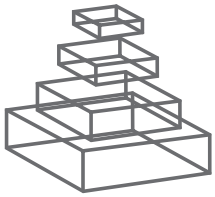
frontiers
RESEARCH TOPICS

ROLE AND PREVALENCE OF ANTIBIOSIS AND THE RELATED RESISTANCE GENES IN THE ENVIRONMENT

Topic Editors
Sylvie Nazaret and Rustam Aminov

frontiers in MICROBIOLOGY



frontiers

Cover image provided by Ibbl sarl, Lausanne CH

ISSN 1664-8714
ISBN 978-2-88919-521-3
DOI 10.3389/978-2-88919-521-3

ABOUT FRONTIERS

Frontiers is more than just an open-access publisher of scholarly articles: it is a pioneering approach to the world of academia, radically improving the way scholarly research is managed. The grand vision of Frontiers is a world where all people have an equal opportunity to seek, share and generate knowledge. Frontiers provides immediate and permanent online open access to all its publications, but this alone is not enough to realize our grand goals.

FRONTIERS JOURNAL SERIES

The Frontiers Journal Series is a multi-tier and interdisciplinary set of open-access, online journals, promising a paradigm shift from the current review, selection and dissemination processes in academic publishing.
All Frontiers journals are driven by researchers for researchers; therefore, they constitute a service to the scholarly community. At the same time, the Frontiers Journal Series operates on a revolutionary invention, the tiered publishing system, initially addressing specific communities of scholars, and gradually climbing up to broader public understanding, thus serving the interests of the lay society, too.

DEDICATION TO QUALITY

Each Frontiers article is a landmark of the highest quality, thanks to genuinely collaborative interactions between authors and review editors, who include some of the world's best academicians. Research must be certified by peers before entering a stream of knowledge that may eventually reach the public - and shape society; therefore, Frontiers only applies the most rigorous and unbiased reviews.
Frontiers revolutionizes research publishing by freely delivering the most outstanding research, evaluated with no bias from both the academic and social point of view.
By applying the most advanced information technologies, Frontiers is catapulting scholarly publishing into a new generation.

WHAT ARE FRONTIERS RESEARCH TOPICS?

Frontiers Research Topics are very popular trademarks of the Frontiers Journals Series: they are collections of at least ten articles, all centered on a particular subject. With their unique mix of varied contributions from Original Research to Review Articles, Frontiers Research Topics unify the most influential researchers, the latest key findings and historical advances in a hot research area!
Find out more on how to host your own Frontiers Research Topic or contribute to one as an author by contacting the Frontiers Editorial Office: researchtopics@frontiersin.org

ROLE AND PREVALENCE OF ANTIBIOSIS AND THE RELATED RESISTANCE GENES IN THE ENVIRONMENT

Topic Editors:
Sylvie Nazaret, CNRS, Ecole Nationale Vétérinaire de Lyon, and Université Lyon 1, Université de Lyon, France.
Rustam Aminov, Technical University of Denmark, National Veterinary Institute, Denmark.

It becomes increasingly clear that the basis of antibiotic resistance problem among bacterial pathogens is not confined to the borders of clinical microbiology but has broader ecological and evolutionary associations. This Research Topic "Role and prevalence of antibiosis and the related resistance genes in the environment" in Frontiers in Microbiology, section Antimicrobials, Resistance and Chemotherapy, presents the examples of occurrence and diversity of antibiotic resistance genes in the wide range of environments, from the grasslands of the Colombian Andes, to the dairy farms and small animal veterinary hospitals in the United Stated, and to the various environments of Continental Europe and Indochina. Besides, various genetic mechanisms and selection/co-selection factors contributing to the dissemination and maintenance of antibiotic resistance genes are presented. The topic is finalized by the mathematical modeling approach to access the probability of rare horizontal gene transfer events in bacterial populations.

Table of Contents

frontiers in
MICROBIOLOGY

EDITORIAL
published: 02 October 2014
doi: 10.3389/fmicb.2014.00520

Role and prevalence of antibiosis and the related resistance genes in the environment

Sylvie Nazaret*[1] *and Rustam Aminov*[2]*

[1] *UMR 5557 Ecologie Microbienne, CNRS, Ecole Nationale Vétérinaire de Lyon, and Université Lyon 1, Université de Lyon, Villeurbanne, France*
[2] *Section for Bacteriology, Pathology and Parasitology, National Veterinary Institute, Technical University of Denmark, Frederiksberg C, Denmark*
**Correspondence: rusam@vet.dtu.dk*

Edited by:
David W. Graham, Newcastle University, UK

Reviewed by:
Timothy LaPara, University of Minnesota, USA

Keywords: antibiotic resistance, mobile genetic elements, environment, model

It becomes increasingly clear that the basis of antibiotic resistance problem among bacterial pathogens is not confined to the borders of clinical microbiology but has broader ecological and evolutionary associations. This Research Topic "Role and prevalence of antibiosis and the related resistance genes in the environment" in *Frontiers in Microbiology: Antimicrobials, Resistance, and Chemotherapy* presents the examples of occurrence and diversity of antibiotic resistance genes (ARGs) in the wide range of environments, from the grasslands of the Colombian Andes, to the dairy farms and small animal veterinary hospitals in the United Stated, and to the various environments of Continental Europe and Indochina. Besides, various genetic mechanisms and selection/co-selection factors contributing to the dissemination and maintenance of ARGs are presented. The topic is finalized by the mathematical modeling approach to access the probability of rare horizontal gene transfer (HGT) events in bacterial populations.

The opinion article by Martínez (2012) summarizes our present understanding of the cycle of ARGs acquisition by bacterial pathogens. The environmental microbiota harbors a vast diversity of genes, which we usually classify as conferring resistance to antibiotics. In natural ecosystems, however, their role may be different and not necessarily associated with this function. Yet, if the certain metabolic genes are acquired by commensal/pathogenic microbiota and appeared to be conferring selective advantage under the pressure of antibiotics, their primary function under these new ecological circumstances becomes resistance to antibiotics. Moreover, upon the amplification under the antibiotic selective pressure, these ARGs are released into the environment thus contributing to the rise of antibiotic resistance in other ecological compartments.

Evidence for the environmental contamination by ARGs can be seen in several articles of this Research Topic. For example, despite the low antibiotic usage in the grassland farms located in the Colombian Andes, there is a significant diversity of tetracycline resistance genes in the microbiota of the animal gut and the environment (Santamaría et al., 2011). But the diversity of the *tet* genes in the former ecosystem is higher thus suggesting the gene flow from the animals into the environment. Another study involved the isolation and characterization of the CTX-M [a major type of extended-spectrum beta-lactamase (ESBL)] producing *Escherichia coli* strains from soils, cattle, and the farm environment in the Burgundy region of France (Hartmann et al., 2012). Environmental and animal strains appeared to be clonally related. The study also suggests a long-term survival of the CTX-M-producing *E. coli* strains in soil since the last manure application has been done 1 year before the actual sampling. Czekalski et al. (2012) demonstrated the increased levels of multidrug-resistant bacteria and ARGs in Lake Geneva, Switzerland due to the discharge from the local wastewater treatment plant. Counterintuitively, wastewater treatment resulted in selection of extremely multidrug-resistant bacteria and accumulation of ARGs although the total bacterial load was substantially decreased. A less favorable situation with the treatment of wastewater is in Indochina, which includes Vietnam, Thailand, Cambodia, Lao PDR, and Myanmar. Suzuki and Hoa (2012) summarized the current knowledge regarding the presence of quinolones, sulfonamides, and tetracyclines as well as the corresponding ARGs in this region. They concluded that: (1) no correlation exists between the quinolone contamination and quinolone resistance; (2) occurrence of the *sul* sulfonamide resistance gene varies geographically; and (3) microbial diversity relates to the oxytetracycline resistance level.

Thames et al. (2012) used qPCR to investigate the effect of feeding milk replacers with various antibiotic doses on the excretion of ARGs by dairy calves. Interestingly, no significant differences have been found in the absolute numbers of ARGs excreted. After the normalization to the 16S rRNA genes the relative *tet*(O) concentration appeared to be higher in animals fed the highest therapeutic doses of antibiotic. Besides, antibiotic feeding provided no obvious health benefits. The authors concluded that the greater than conventional nutritional intake in the study outweighs the previously reported health benefits of antibiotics. Ghosh et al. (2012) reported an interesting observation regarding the carriage of multi-drug resistant enterococci by resident cats in small animal veterinary hospitals. Genotypically identical strains were isolated from cats and surfaces of cage door, thermometer, and stethoscope suggesting that the animals may be involved in cross-contamination of the hospital environment.

What are the factors supporting the dissemination of ARGs? Among the genetic mechanisms, Heuer et al. (2012) identified IncP-1ε plasmids as important vectors for horizontal transfer of antibiotic resistance in agricultural systems. These plasmids are transferable to a wide range of *Beta-* and *Gammaproteobacteria*, with the concurrent transfer of ARGs. Stalder et al. (2012) extensively reviewed the role of integrons in the environmental spread of antibiotic resistance. The main conclusion is that many stress factors including but not limited to antibiotics, quaternary ammonium compounds or high concentrations of heavy metals result in selection of class 1 mobile integron-harboring bacteria. Consistent with the findings of Czekalski et al. (2012), wastewater treatment plants may serve as hot spots for class 1 mobile integron dissemination, with the concurrent dissemination of ARGs. Seiler and Berendonk (2012) reviewed the role of heavy metals in co-selection of antibiotic resistance in soil and water bodies impacted by agriculture and aquaculture. The metals that are extensively used in agriculture such as copper and zinc have the potential to co-select for antibiotic resistance in the environment.

Conventional experimental design for identification of HGT events usually relies on the detection of initial transfer occurrences in fairly small bacterial populations. This approach, however, may fail to detect the rare transfer events thus leading to an inappropriate conclusion regarding the long-term HGT effects. Townsend et al. (2012) addressed this problem using the models that take into consideration various degrees of natural selection, growth dynamics of bacteria with differing fitness, genetic drift, and other variables to build a probabilistic framework for detection of HGT within a given sampling design. This approach will assist to a better design of experiments aimed at detection and analysis of HGT in natural setting.

In summary, the papers in this Research Topic contribute to a better understanding of the dynamic of ARGs within and among different ecological compartments. They emphasize the need for a careful monitoring of the release of pre-selected ARGs into the environment from which they may enter the human food chain. Especially worrying are the findings that the current wastewater treatment systems may serve as hot spots for the amplification of multidrug-resistant bacteria, ARGs, and mobile genetic elements. The topic calls for more research that is needed for identification of crucial checkpoints to limit the circulation of ARGs among different ecological compartments.

REFERENCES

Czekalski, N., Berthold, T., Caucci, S., Egli, A., and Bürgmann, H. (2012). Increased levels of multiresistant bacteria and resistance genes after wastewater treatment and their dissemination into Lake Geneva, Switzerland. *Front. Microbiol.* 3:106. doi: 10.3389/fmicb.2012.00106

Ghosh, A., KuKanich, K., Brown, C. E., and Zurek, L. (2012). Resident cats in small animal veterinary hospitals carry multi-drug resistant enterococci and are likely involved in cross-contamination of the hospital environment. *Front. Microbiol.* 3:62. doi: 10.3389/fmicb.2012.00062

Hartmann, A., Locatelli, A., Amoureux, L., Depret, G., Jolivet, C., Gueneau, E., et al. (2012). Occurrence of CTX-M producing Escherichia coli in soils, cattle, and farm environment in France (Burgundy region). *Front. Microbiol.* 3:83. doi: 10.3389/fmicb.2012.00083

Heuer, H., Binh, C. T. T., Jechalke, S., Kopmann, C., Zimmerling, U., Krögerrecklenfort, E., et al. (2012). IncP-1ε plasmids are important vectors of antibiotic resistance genes in agricultural systems: diversification driven by class 1 integron gene cassettes. *Front. Microbiol.* 3:2. doi: 10.3389/fmicb.2012.00002

Martínez, J. L. (2012). Natural antibiotic resistance and contamination by antibiotic resistance determinants: the two ages in the evolution of resistance to antimicrobials. *Front. Microbiol.* 3:1. doi: 10.3389/fmicb.2012.00001

Santamaría, J., López, L., and Soto, C. Y. (2011). Detection and diversity evaluation of tetracycline resistance genes in grassland-based production systems in Colombia, South America. *Front. Microbiol.* 2:252. doi: 10.3389/fmicb.2011.00252

Seiler, C., and Berendonk, T. U. (2012). Heavy metal driven co-selection of antibiotic resistance in soil and water bodies impacted by agriculture and aquaculture. *Front. Microbiol.* 3:399. doi: 10.3389/fmicb.2012.00399

Stalder, T., Barraud, O., Casellas, M., Dagot, C., and Ploy, M.-C. (2012). Integron involvement in environmental spread of antibiotic resistance. *Front. Microbiol.* 3:119. doi: 10.3389/fmicb.2012.00119

Suzuki, S., and Hoa, P. T. P. (2012). Distribution of quinolones, sulfonamides, tetracyclines in aquatic environment and antibiotic resistance in Indochina. *Front. Microbiol.* 3:67. doi: 10.3389/fmicb.2012.00067

Thames, C. H., Pruden, A., James, R. E., Ray, P. P., and Knowlton, K. F. (2012). Excretion of antibiotic resistance genes by dairy calves fed milk replacers with varying doses of antibiotics. *Front. Microbiol.* 3:139. doi: 10.3389/fmicb.2012.00139

Townsend, J. P., Bøhn, T., and Nielsen, K. M. (2012). Assessing the probability of detection of horizontal gene transfer events in bacterial populations. *Front. Microbiol.* 3:27. doi: 10.3389/fmicb.2012.00027

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 14 August 2014; accepted: 17 September 2014; published online: 02 October 2014.

Citation: Nazaret S and Aminov R (2014) Role and prevalence of antibiosis and the related resistance genes in the environment. Front. Microbiol. ***5****:520. doi: 10.3389/fmicb.2014.00520*

This article was submitted to Antimicrobials, Resistance and Chemotherapy, a section of the journal Frontiers in Microbiology.

frontiers in
MICROBIOLOGY

OPINION ARTICLE
published: 13 January 2012
doi: 10.3389/fmicb.2012.00001

Natural antibiotic resistance and contamination by antibiotic resistance determinants: the two ages in the evolution of resistance to antimicrobials

José L. Martínez*

Departamento de Biotecnología Microbiana, Centro Nacional de Biotecnología-Consejo Superior de Investigaciones Científicas, Madrid, Spain
**Correspondence: jlmtnez@cnb.csic.es*

The study of antibiotic resistance has been historically concentrated on the analysis of bacterial pathogens and on the consequences of acquiring resistance for human health. The development of antibiotic resistance is of course extremely relevant from the clinical point of view, because it can compromise the treatment of infectious diseases as well as other advanced therapeutic procedures as transplantation or anticancer therapy that involve immunosuppression and thus require robust anti-infective preventive therapies. Nevertheless, the studies on antibiotic resistance should not be confined to clinical-associated ecosystems. It was evident soon after introducing antibiotics for human therapy, that bacteria were able to develop resistance, not just as the consequence of mutations in the targets of antibiotics, but by acquiring genes conferring resistance to antimicrobials (Abraham and Chain, 1940). Since those genes were not present before in the human bacterial pathogens, the only suitable source for them was the environmental microbiota, and indeed the presence of *R*-factors (resistance plasmids) in pristine environments without any record of contact with antibiotics was described in the first studies of antibiotic resistance in the field (Gardner et al., 1969).

Given that the origin of antibiotic resistance is the environmental microbiota, it would be necessary to study resistance in natural, non-clinical habitats in order to fully understand the cycle of acquisition of resistance by human pathogens. However, until recently the studies on antibiotic resistance in natural ecosystems have been fragmentary. The availability of metagenomic tools as well as high-throughput sequencing techniques is allowing describing in depth the presence of resistance genes in different ecosystems. Indeed, the use of functional genomic and metagenomic techniques has served to show that natural ecosystems, including not just soils but human gut as well, contain a large number of elements that, upon transfer to a new host, can confer resistance to any type of antimicrobial (D'Costa et al., 2006; Sommer et al., 2009). These include natural antibiotics, which are produced by the environmental microbiota, and synthetic antimicrobials, as quinolones.

One important question from an evolutionary point of view is the function of these resistance genes in their natural environmental hosts (Davies and Davies, 2010). Whereas for naturally produced antibiotics a protective role for resistance genes in the producers organisms (or those coexisting with producers Laskaris et al., 2010) might be foreseen (Benveniste and Davies, 1973), this explanation is not suitable for synthetic antibiotics as quinolones. Indeed, it has been described that the origin of the quinolone resistance gene QnrA, which is now widespread in plasmids present in human pathogens is the environmental non-antibiotic producer *Shewanella algae* (Poirel et al., 2005). This means that a gene that confers resistance in a human pathogen does not necessary play the same role in its original host (Martinez et al., 2009a). The finding that several proteins, involved in basic processes of the bacterial physiology, contribute to intrinsic resistance to antibiotics (Fajardo et al., 2008; Laskaris et al., 2010; Linares et al., 2010), further supports the concept that resistance genes, acquired through horizontal gene transfer by human pathogens, might have evolved in their original host to play a different role than resisting the activity of antimicrobials in natural ecosystems.

We can thus distinguish two ages in the evolution of antibiotic resistance genes. For billions of years (until the use of antibiotics by humans), these genes have been usually chromosomally encoded and had evolved for different purposes. Some of them, as those found in antibiotic producers, likely evolved for detoxifying the original host from the antibiotic it produces, although a role in the biosynthesis of the antibiotic itself has been proposed as well for some of them (Benveniste and Davies, 1973; Doyle et al., 1991). Others, as beta-lactamases might be involved in the biosynthesis of the cell wall (Jacobs et al., 1994; Massova and Mobashery, 1998), whereas others as multidrug efflux pumps might serve for different purposes including the trafficking of signaling molecules, detoxification of metabolic intermediates, or extrusion of plant-produced compounds among others (Martinez et al., 2009b). Like in the case of antibiotics, which do not necessarily have an inhibitory function at the concentrations in which they are present in natural ecosystems (Linares et al., 2006; Yim et al., 2007; Fajardo and Martinez, 2008), the fact that a plasmid-encoded gene produces resistance to antibiotics upon its expression in a new host, is not an unequivocal prove that it confers resistance as well in its original host. This reflection serves to show the relevance of the second age in the evolution of antibiotic resistance determinants. Once a gene is introduced in a new host in which it lacks its original biochemical and genetic context, its function is limited to antibiotic resistance (Baquero et al., 2009). This change of function without changing the sequence of the gene itself, has been named as exaptation (Gould and Vrba, 1982), and is the consequence of the strong selective pressure exerted by antibiotics in the last decades from the time they were introduced for therapy.

Two important aspects are emerging from the studies of natural resistome. First, the environmental microbiota contains a much larger number of resistance genes than those seen to be acquired by bacterial pathogens (Wright, 2007; Davies and Davies, 2010). Furthermore, different ecosystems contain different resistance genes,

which means that we are still far away to have a consistent estimation on the number of potential resistance genes present in natural ecosystems. Finally, the origin of most resistance genes currently found in transferrable elements is still ignored, despite genes (and genetic structures) belonging to the same families are regularly found in different ecosystems, including deep terrestrial subsurface (Brown and Balkwill, 2009), ice (Miteva et al., 2004), and even the permafrost (D'Costa et al., 2011), which have not been in contact with human contaminants. Second, those genes present in mobile elements in human bacterial pathogens can be found nearly everywhere, including pristine ecosystems or wild animals not supposed to be in contact with antibiotics (Martinez, 2009). This indicates that pollution with antibiotic resistance genes is widely spread and that resistance genes can persist even in the absence of a positive selection pressure. The analysis of historical soil archives has shown a consistent increase on the presence of antibiotic resistance genes since 1940 (Knapp et al., 2010), which is a clear prove of the contamination by antibiotic resistance elements of natural ecosystems and the resilience of those elements for their elimination.

In this situation, which type of studies are needed to analyze in depth the role that natural ecosystems may have on the development of resistance in human bacterial pathogens? In my opinion, these studies have two faces (Martinez, 2008). One consists on the analysis of the genes already present in bacterial pathogens. In other words, we will study mainly contamination by antibiotic resistance determinants and how this contamination might increase the risks for the dissemination of those elements (Martinez, 2009). These studies might serve to define reservoirs, elements for enrichment and dissemination of resistance (as wild birds Simoes et al., 2010) or hotspots for the transfer of resistance as waste-water treatment plants (Baquero et al., 2008). For instance, a recent study has shown that soil composition and in particular the presence of heavy metals might enrich for the presence of antibiotic resistance genes in natural ecosystems (Knapp et al., 2011). The other type of studies consists on the analysis, using functional assays, of novel resistance genes in different ecosystems (D'Costa et al., 2006, 2011; Sommer et al., 2009). These studies are useful for defining novel mechanisms of resistance, but making risks assessments on whether those novel antibiotic resistance genes will be transferred to new hosts is likely unsuitable (Martinez et al., 2007). On the other hand tracking the source of currently known resistance gene has demonstrated to be a very difficult task. We have to be extremely careful for assigning the origin of resistance determinants. Only when the genes are nearly identical (as QnrA) and the gene is present in several strains of the original host, with the same synteny and without any sign of a recent acquisition event, we can firmly establish this host being the origin. The report of genes that are highly similar (even above 90%) to antibiotic resistance genes demonstrate their belonging to the same phylogenetic group, not that one is the origin of the other. Does it mean that we will be unable of tracking the source of resistance genes and to propose from this information valuable strategies for reducing antibiotic resistance? I do not believe that. It has been already determined that QnrA was originated in *S. algae* (Poirel et al., 2005) and that chromosomally encoded *qnr* genes are mainly present in water-dwelling bacteria (Sanchez et al., 2008). This suggests that the source of transferrable quinolone resistance is the water microbiota and puts a focus on the effect that the use of quinolones in aquaculture might have had for the emergence and dissemination of these resistance elements (Cabello, 2006).

The study on antibiotic resistance in natural ecosystems and its role on the maintenance and spread of clinically relevant resistance determinants is still in its infancy. It is surprising that large efforts have been used to study the risks for the dissemination of resistance that may have the release of genetic modified organisms containing resistance genes in their chromosomes, whereas the study of the effect of the discharge of human wastes, which contain bacterial pathogens harboring the resistance genes that have demonstrated to be really relevant, in the elements that are important for their dissemination has received few attention if any. Studies in this new field are needed in order to understand the mechanisms involved in the emergence, spread, maintenance, and evolution of antibiotic resistance.

ACKNOWLEDGMENTS

Work in our laboratory is supported by grants BIO2008-00090 from the Spanish Ministry of Science and Innovation and KBBE-227258 (BIOHYPO), HEALTH-F3-2011-282004 (EVOTAR), and HEALTH-F3-2010-241476 (PAR) from European Union.

REFERENCES

Abraham, E. P., and Chain, E. (1940). An enzyme from bacteria able to destroy penicillin. *Nature* 146, 837.

Baquero, F., Alvarez-Ortega, C., and Martinez, J. L. (2009). Ecology and evolution of antibiotic resistance. *Environ. Microbiol. Rep.* 1, 469–476.

Baquero, F., Martinez, J. L., and Canton, R. (2008). Antibiotics and antibiotic resistance in water environments. *Curr. Opin. Biotechnol.* 19, 260–265.

Benveniste, R., and Davies, J. (1973). Aminoglycoside antibiotic-inactivating enzymes in actinomycetes similar to those present in clinical isolates of antibiotic-resistant bacteria. *Proc. Natl. Acad. Sci. U.S.A.* 70, 2276–2280.

Brown, M. G., and Balkwill, D. L. (2009). Antibiotic resistance in bacteria isolated from the deep terrestrial subsurface. *Microb. Ecol.* 57, 484–493.

Cabello, F. C. (2006). Heavy use of prophylactic antibiotics in aquaculture: a growing problem for human and animal health and for the environment. *Environ. Microbiol.* 8, 1137–1144.

Davies, J., and Davies, D. (2010). Origins and evolution of antibiotic resistance. *Microbiol. Mol. Biol. Rev.* 74, 417–433.

D'Costa, V. M., King, C. E., Kalan, L., Morar, M., Sung, W. W., Schwarz, C., Froese, D., Zazula, G., Calmels, F., Debruyne, R., Golding, G. B., Poinar, H. N., and Wright, G. D. (2011). Antibiotic resistance is ancient. *Nature* 477, 457–461.

D'Costa, V. M., Mcgrann, K. M., Hughes, D. W., and Wright, G. D. (2006). Sampling the antibiotic resistome. *Science* 311, 374–377.

Doyle, D., Mcdowall, K. J., Butler, M. J., and Hunter, I. S. (1991). Characterization of an oxytetracycline-resistance gene, otrA, of *Streptomyces rimosus*. *Mol. Microbiol.* 5, 2923–2933.

Fajardo, A., and Martinez, J. L. (2008). Antibiotics as signals that trigger specific bacterial responses. *Curr. Opin. Microbiol.* 11, 161–167.

Fajardo, A., Martinez-Martin, N., Mercadillo, M., Galan, J. C., Ghysels, B., Matthijs, S., Cornelis, P., Wiehlmann, L., Tummler, B., Baquero, F., and Martinez, J. L. (2008). The neglected intrinsic resistome of bacterial pathogens. *PLoS ONE* 3, e1619. doi: 10.1371/journal.pone.0001619

Gardner, P., Smith, D. H., Beer, H., and Moellering, R. C. Jr. (1969). Recovery of resistance (R) factors from a drug-free community. *Lancet* 2, 774–776.

Gould, S. J., and Vrba, S. (1982). Exaptation: a missing term in the science of form. *Paleobiology* 8, 4–15.

Jacobs, C., Huang, L. J., Bartowsky, E., Normark, S., and Park, J. T. (1994). Bacterial cell wall recycling provides cytosolic muropeptides as effectors for beta-lactamase induction. *EMBO J.* 13, 4684–4694.

Knapp, C. W., Dolfing, J., Ehlert, P. A., and Graham, D. W. (2010). Evidence of increasing antibiotic resistance gene abundances in archived soils since 1940. *Environ. Sci. Technol.* 44, 580–587.

Knapp, C. W., Mccluskey, S. M., Singh, B. K., Campbell, C. D., Hudson, G., and Graham, D. W. (2011). Antibiotic resistance gene abundances correlate with metal and geochemical conditions in archived Scottish soils. *PLoS ONE* 6, e27300. doi: 10.1371/journal.pone.0027300

Laskaris, P., Tolba, S., Calvo-Bado, L., and Wellington, L. (2010). Coevolution of antibiotic production and counter-resistance in soil bacteria. *Environ. Microbiol.* 12, 783–796.

Linares, J. F., Gustafsson, I., Baquero, F., and Martinez, J. L. (2006). Antibiotics as intermicrobial signaling agents instead of weapons. *Proc. Natl. Acad. Sci. U.S.A.* 103, 19484–19489.

Linares, J. F., Moreno, R., Fajardo, A., Martinez-Solano, L., Escalante, R., Rojo, F., and Martinez, J. L. (2010). The global regulator Crc modulates metabolism, susceptibility to antibiotics and virulence in *Pseudomonas aeruginosa*. *Environ. Microbiol.* 12, 3196–3212.

Martinez, J. L. (2008). Antibiotics and antibiotic resistance genes in natural environments. *Science* 321, 365–367.

Martinez, J. L. (2009). Environmental pollution by antibiotics and by antibiotic resistance determinants. *Environ. Pollut.* 157, 2893–2902.

Martinez, J. L., Baquero, F., and Andersson, D. I. (2007). Predicting antibiotic resistance. *Nat. Rev. Microbiol.* 5, 958–965.

Martinez, J. L., Fajardo, A., Garmendia, L., Hernandez, A., Linares, J. F., Martinez-Solano, L., and Sanchez, M. B. (2009a). A global view of antibiotic resistance. *FEMS Microbiol. Rev.* 33, 44–65.

Martinez, J. L., Sanchez, M. B., Martinez-Solano, L., Hernandez, A., Garmendia, L., Fajardo, A., and Alvarez-Ortega, C. (2009b). Functional role of bacterial multidrug efflux pumps in microbial natural ecosystems. *FEMS Microbiol. Rev.* 33, 430–449.

Massova, I., and Mobashery, S. (1998). Kinship and diversification of bacterial penicillin-binding proteins and beta-lactamases. *Antimicrob. Agents Chemother.* 42, 1–17.

Miteva, V. I., Sheridan, P. P., and Brenchley, J. E. (2004). Phylogenetic and physiological diversity of microorganisms isolated from a deep greenland glacier ice core. *Appl. Environ. Microbiol.* 70, 202–213.

Poirel, L., Rodriguez-Martinez, J. M., Mammeri, H., Liard, A., and Nordmann, P. (2005). Origin of plasmid-mediated quinolone resistance determinant QnrA. *Antimicrob. Agents Chemother.* 49, 3523–3525.

Sanchez, M. B., Hernandez, A., Rodriguez-Martinez, J. M., Martinez-Martinez, L., and Martinez, J. L. (2008). Predictive analysis of transmissible quinolone resistance indicates *Stenotrophomonas maltophilia* as a potential source of a novel family of Qnr determinants. *BMC Microbiol.* 8, 148. doi: 10.1186/1471-2180-8-148

Simoes, R. R., Poirel, L., Da Costa, P. M., and Nordmann, P. (2010). Seagulls and beaches as reservoirs for multidrug-resistant *Escherichia coli*. *Emerging Infect. Dis.* 16, 110–112.

Sommer, M. O., Dantas, G., and Church, G. M. (2009). Functional characterization of the antibiotic resistance reservoir in the human microflora. *Science* 325, 1128–1131.

Wright, G. D. (2007). The antibiotic resistome: the nexus of chemical and genetic diversity. *Nat. Rev. Microbiol.* 5, 175–186.

Yim, G., Wang, H. H., and Davies, J. (2007). Antibiotics as signalling molecules. *Philos. Trans. R. Soc. B Biol. Sci.* 362, 1195–1200.

Received: 15 November 2011; accepted: 02 January 2012; published online: 13 January 2012.

Citation: Martínez JL (2012) Natural antibiotic resistance and contamination by antibiotic resistance determinants: the two ages in the evolution of resistance to antimicrobials. Front. Microbiol. ***3****:1. doi: 10.3389/fmicb.2012.00001*

This article was submitted to Frontiers in Antimicrobials, Resistance and Chemotherapy, a specialty of Frontiers in Microbiology.

frontiers in
MICROBIOLOGY

ORIGINAL RESEARCH ARTICLE
published: 14 December 2011
doi: 10.3389/fmicb.2011.00252

Detection and diversity evaluation of tetracycline resistance genes in grassland-based production systems in Colombia, South America

Johanna Santamaría[1]*, Liliana López[1] and Carlos Yesid Soto[2]

[1] *Environmental Microbiology Laboratory, Department of Biology and Environmental Sciences, University Jorge Tadeo Lozano, Bogotá, Colombia*
[2] *Department of Chemistry, National University of Colombia, Bogotá, Colombia*

Edited by:
Rustam I. Aminov, University of Aberdeen, UK

Reviewed by:
Henning Sørum, Norwegian School of Veterinary Science, Norway
Ludek Zurek, Kansas State University, USA
Carlos F. Amábile-Cuevas, Fundación Lusara, Mexico

****Correspondence:***
Johanna Santamaría, Laboratorio de Microbiología Ambiental, Departamento de Biología y Ciencias Ambientales, Universidad Jorge Tadeo Lozano, Carrera 4 No. 22-61, Módulo 7ª, piso cuarto, Bogotá, Colombia.
e-mail: johanna.santamaria@utadeo.edu.co

Grassland-based production systems use ∼26% of land surface on earth. However, there are no evaluations of these systems as a source of antibiotic pollution. This study was conducted to evaluate the presence, diversity, and distribution of tetracycline resistance genes in the grasslands of the Colombian Andes, where administration of antibiotics to animals is limited to treat disease and growth promoters are not included in animals' diet. Animal (ruminal fluid and feces) and environmental (soil and water) samples were collected from different dairy cattle farms and evaluated by PCR for the genes *tet*(M), *tet*(O), *tet*B(P), *tet*(Q), *tet*(W), *tet*(S), *tet*(T), *otr*(A), which encode ribosomal protection proteins (RPPs), and the genes *tet*(A), *tet*(B), *tet*(D), *tet*(H), *tet*(J), and *tet*(Z), encoding efflux pumps. A wide distribution and high frequency for genes *tet*(W) and *tet*(Q) were found in both sample types. Genes *tet*(O) and *tet*B(P), detected in high frequencies in feces, were detected in low frequencies or not detected at all in the environment. Other genes encoding RPPs, such as *tet*(M), *tet*(S), and *tet*(T), were detected at very low frequencies and restricted distributions. Genes encoding efflux pumps were not common in this region, and only two of them, *tet*(B) and *tet*(Z), were detected. DGGE–PCR followed by comparative sequence analysis of *tet*(W) and *tet*(Q) showed that the sequences detected in animals did not differ from those coming from soil and water. Finally, the farms sampled in this study showed more than 50% similarity in relation to the *tet* genes detected. In conclusion, there was a remarkable presence of *tet* genes in these production systems and, although not all genes detected in animal reservoirs were detected in the environment, there is a predominant distribution of *tet*(W) and *tet*(Q) in both animal and environmental reservoirs. Sequence similarity analysis suggests the transmission of these genes from animals to the environment.

Keywords: antibiotic resistance, cattle farms, Colombian Andes, environmental pollution, grasslands, tetracycline

INTRODUCTION

Antibiotics are broadly used around the world in concentrated animal feeding operations (CAFOs), not only to prevent and control diseases but also to promote faster growth by mixing antibiotics with livestock feed. These practices help reduce production costs and retail prices to consumers. Nonetheless, the formation of antibiotic resistant microbial pools in animal guts as a result of antibiotic administration (Aminov et al., 2001; Aarestrup, 2005; Sawant et al., 2007) is well documented. These pools have been identified as the source of many antibiotic resistant pathogenic bacteria transmitted to humans (van den Bogaard and Stobberingh, 2000), and the consumption of meat, meat-derived products, eggs, and food contaminated with antibiotic resistant zoonotic bacterial pathogens, is considered the most common transmission route of antibiotic resistance from animal farms to humans (Aarestrup, 2006).

An emergent concern is that antibiotic resistance could be transmitted from environmental resistance reservoirs to humans and animal pathogens by consuming polluted water or agricultural food products. Environmental reservoirs of resistance may be the result of three different process: the selective pressure exerted by the antibiotics released in animal waste (Winckler and Grafe, 2001; Campagnolo et al., 2002), the horizontal gene transfer between the resistant bacteria released in feces and indigenous bacteria (Götz and Smalla, 1997; Witte, 2000; Sengelov et al., 2003; Schwarz et al., 2006; Heuer and Smalla, 2007; Binh et al., 2008), and finally, natural selective pressures on the microbial community (Aminov, 2009; Martinez, 2009). Previous studies conducted in United States and Europe have shown the presence of tetracycline resistance pools in surface and ground water of industrial livestock facilities proving that CAFOs and land application of manure are important sources of environmental antibiotic pollution that promote environmental reservoirs of resistance (Aminov et al., 2001; Chee-Sanford et al., 2001; Koike et al., 2007; Patterson et al., 2007; Peak et al., 2007; Knapp et al., 2010; Heuer et al., 2011).

Unlike industrial facilities, grassland-based production systems rely upon natural vegetation growing in the field for feeding animals that live off the land, with low numbers of livestock units per area. The administration of antibiotics is restricted to disease control, and no food concentrates containing antibiotics are used. However, even though the amount of antibiotics administered to animals in grasslands is significantly lower to the amount administered in CAFOs, the possibility of animal and environmental reservoirs of resistance, as a result of the livestock activity in these extensive production systems, cannot be ruled out. It has been demonstrated the presence of resistant bacteria in animals when no growth promoters have been administered (Blake et al., 2003; Bryan et al., 2004; Alexander et al., 2008). Despite the fact that grasslands represent ∼26% of the land surface on earth (Steinfeld et al., 2006), there are no evaluations of grassland-based systems as a source of antibiotic pollution.

The first objective of the present study was to evaluate the presence, distribution, and diversity of microbial genes encoding resistance to the antibiotic tetracycline in animal (ruminal fluid and feces) and environmental (soil and water) samples from grassland-based systems located in the tropical highlands of Colombia. The presence of resistance in the total microbial community, including viable but non-culturable bacteria, was evaluated by PCR detection of the genes *tet*(M), *tet*(O), *tet*B(P), *tet*(Q), *tet*(W), *tet*(S), *tet*(T), *otr*(A), which encode ribosomal protection proteins (RPPs), and the genes *tet*(A), *tet*(B), *tet*(D), *tet*(H), *tet*(J), and *tet*(Z), encoding tetracycline efflux pumps (Roberts, 2005). Tetracycline resistance was chosen because this antibiotic is frequently used in Colombia and other regions of the world for veterinary purposes. In addition, no antibiotic resistance evaluations of the total microbial community at the animal and environmental levels have been previously conducted in Latin America and the Caribbean Region, where grassland animal production is one of the most important economic activities (FAO, 2008). The second objective of this study was to establish whether the *tet* genes found in the environment of the highlands were part of an intrinsic resistance in the indigenous bacteria community or originating from the animal reservoirs in the region.

MATERIALS AND METHODS

STUDY SITE

This study evaluated six different grassland-based cattle farms, mainly used for milk production: Manitas, Puente Luna, Granada, Lindaraja, Alisos, and Corpoica. These sites are located on the Eastern Cordillera of the Colombian Andes in a high plateau known as Altiplano Cundiboyacense (**Figure 1**). The administration of antibiotics to animals on these farms is limited to the treatment of health problems. This limitation is part of a strict management because the milk produced in the highlands of Colombia is purchased by the dairy industry, and when traces of antibiotics are

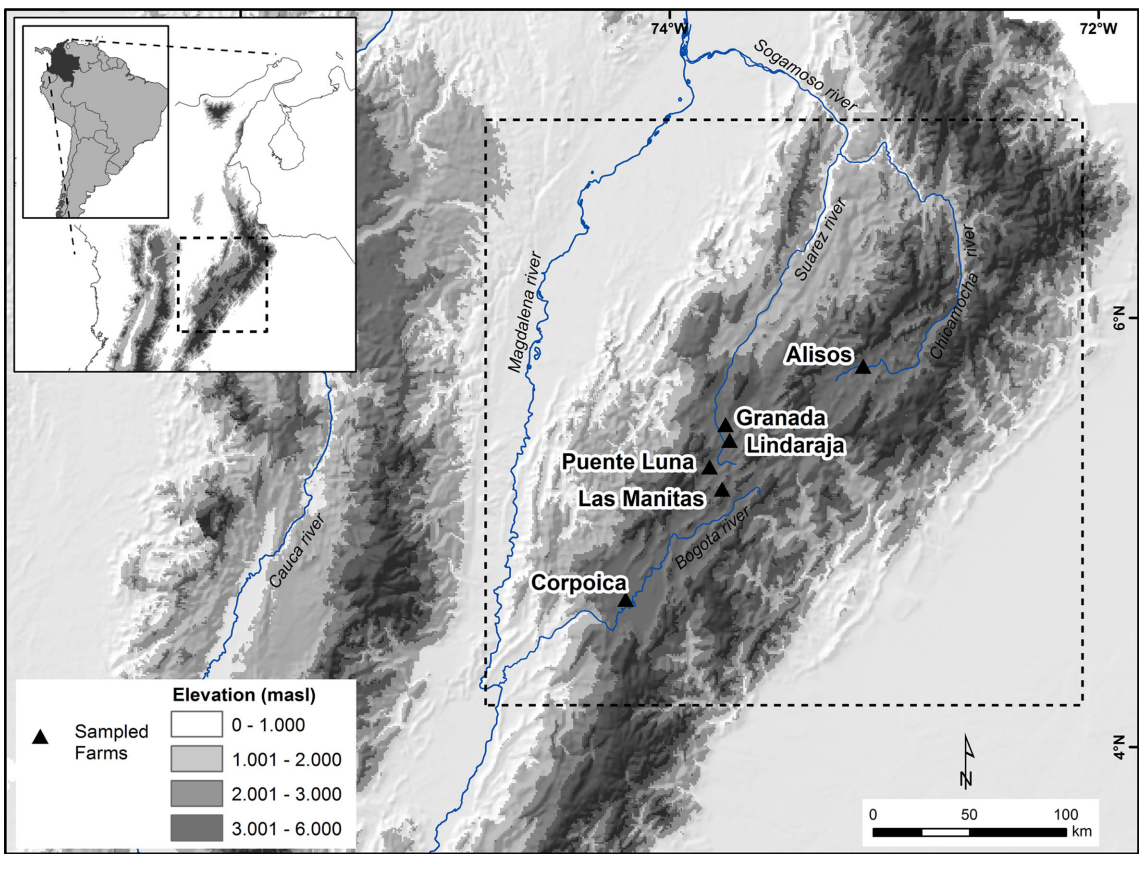


FIGURE 1 | Locations of the sampling sites at the Altiplano Cundiboyacense, Colombian Andes.

found, the milk is rejected. In addition, many farms belong to small producers who cannot pay the high cost of the drugs used in this region.

The most often administered antibiotic in these farms is the oxytetracycline dehydrate, followed by the ampicillin. Unfortunately, farms managers do not keep record of dose and frequency of use. Nonetheless, based on data supplied by veterinarians working for these extensive production systems, it was estimated that the evaluated farms, with a number of animals between 60 and 100, use approximately between 13 and 130 g of oxytetracycline dihydrate in a week. These values are well below the estimated 9 kg of chlortetracycline used per week in a medium size intensive Colombian pig farm with 3000 animals.

SAMPLE COLLECTION

One sampling event was conducted at each farm where soil, water, feces, and ruminal fluid samples were collected.

Soil samples were collected from two different plots under the influence of cattle waste. At each plot, four composite samples were obtained, each resulting from four pooled soil cores. The soil cores were randomly taken from the surface (0–5 cm) along four transects ∼5 m apart and 10 m long.

Four composite feces samples were collected at one of the plots, according to the sampling design described for soils. For this sampling, 50 g of feces were taken from the inner center of a fresh cow pie at each sampling point.

Runoff, ground, and animal drinking water samples were collected depending on their availability at the sites. In all farms, except Manitas, 50 ml samples of runoff water were collected from a ditch draining system. In Manitas, runoff water samples were collected from a pond where waters collect and remain stagnant. To obtain the samples, a 50-ml sterile container was submerged about 50 cm below the water surface at different locations around the waterbody. Animal drinking water samples had different sources depending on the farm. In Puente Luna and Corpoica, animal drinking water is the same potable water consumed by the human population and is supplied by a municipal water processing plant. In Granada, Lindaraja, and Alisos, animal drinking water is *in situ* chemically treated runoff water. Approximately 100 ml of this water was collected from cattle troughs in a sterile container. The groundwater samples from active farming wells were collected from the valve system installed at the head of each well. Water ran for 30 min before filling a sterile 150 ml glass bottle.

Approximately 100 ml of ruminal fluid was collected from each animal using a bovine esophageal probe.

All samples were stored in a refrigerated cooler, transported to the laboratory, and processed within 24 h. The number of collected samples per farm and the number of samples analyzed are listed in **Table 1**.

DNA EXTRACTION

Total DNA was extracted in duplicate from 0.5 g of each soil and feces composite samples and from 0.5 ml of each ruminal fluid sample. DNA isolation was performed using the Ultra Clean DNA kit from Mo Bio Laboratories Inc., according to manufacturer's instructions with modifications. Briefly, samples in the bead solution tubes containing SDS were boiled in water for 10 min before proceeding with the protocol. Subsequently, an equal volume of phenol was added to remove excess organic matter present in the samples previously treated with the protein precipitation reagent. After separating the phenol/aqueous phases by centrifugation, the aqueous phase was mixed with the DNA binding salt solution and passed through the silica membrane of the spin filter. Samples were resuspended in 50 μl of the elution buffer (10 mM Tris Buffer, pH 8.5) and stored at −20˚C.

For DNA extraction, 50, 100, and 150 ml samples of runoff, animal drinking, and ground water, respectively, were filtered through a 25-mm polyester sulfone membrane with a 0.22-μm pore size (Supor 200, Pall Corporation). Subsequently, the membranes were cut into small squares of approximately 5 mm × 5 mm, and the DNA was extracted with the Mo Bio kit as described previously, except the phenol step was omitted for the groundwater samples.

POLYMERASE CHAIN REACTION

Amplification reactions were performed with a Labnet Thermal Cycler in a final reaction volume of 50 μl. The reaction mixtures contained 1X PCR reaction buffer, 2 mM $MgCl_2$, 200 nM of each dNTP, 200 nM of forward and reverse primers, 1U Taq polymerase (Promega), 400 ng/μl BSA (Bioline), and 1 μl of DNA solution. The following basic thermocycling program was used for all the PCR reactions: 94˚C for 4 min (1 cycle); 94˚C for 50 s, X˚C for 40 s, and 72˚C for 30 s (30 cycles); 72˚C for 4 min. The annealing temperature (X) was specific for each primer pair used to amplify the genes encoding for RPPs and tetracycline efflux pumps. Primers details and annealing temperatures are described in Aminov et al. (2001) and Aminov et al. (2002). DNA fragments of 250 bp, containing annealing sequences for *tet* genes, were synthesized by DNA Technologies, Inc., San Diego, CA, USA and used as positive controls. The control sequences were obtained from Aminov et al. (2001, 2002). Negative controls were also included in each PCR reaction. Before testing isolated DNA with *tet* primers, the DNA quality was assessed by performing an initial amplification with the 16S rDNA universal *Bacteria* primers 8F and 1541R using PCR conditions described by Loffler et al. (2000).

To assess the similarity among sampled farms in terms of presence and frequency of the detected *tet* genes, a cluster analysis was conducted based on the percentage similarity index and the UPGMA method of hierarchical agglomeration using the XLSTAT 6.0 software (Addinsoft, NY, USA, 20011).

DENATURING GEL ELECTROPHORESIS ANALYSIS AND SEQUENCING OF AMPLIFIED REGIONS

Animal and environmental DNA samples that were positive for two of the most frequent genes were selected for PCR–Denaturing gel electrophoresis analysis (DGGE). Positive *tet* samples were newly amplified by PCR using a forward primer with a GC tail (Kobayashi et al., 2007). PCR conditions were the same as described before. The Bio-Rad D-Code Universal Mutation System was used to analyze the polymorphisms found in these PCR products by loading 30 μl on an 8% polyacrylamide/bis acrylamide 37.5:1 gel in 1X TAE. The denaturing gradient was 30–50%, and the electrophoresis was performed for 18 h at 40 V and 60˚C. To visualize the DNA, gels were stained in ethidium bromide solution

Table 1 | PCR evaluation results for the presence of *tet* genes in the samples collected from each farm sampled.

Farm	Type of sample	Total number of samples analyzed by PCR	No. of samples positive for *tet* genes							
			tet Genes encoding for RPPs						*tet* Genes encoding for membrane efflux proteins	
			tet(M)	*tet*(O)	*tet*B(P)	*tet*(Q)	*tet*(W)	*tet*(S)	*tet*(T)	*tet*(B)	*tet*(Z)
Manitas	S	16	–	–	–	–	3	–	–	–	2
	RW	10	–	10	–	10	10	–	–	–	4
	ADW	n/a									
	GW	1	–	–	–	–	–	–	–	–	–
	RF	n/a									
	F	8	–	8	–	8	8	–	–	–	–
Puente Luna	S	16	–	–	–	6	2	–	–	–	–
	RW	5	–	–	–	–	–	–	–	–	–
	ADW	2	–	–	–	–	–	–	–	–	–
	GW	n/a									
	RF	2	–	–	–	2	2	–	–	–	–
	F	8	–	8	7	8	7	–	–	–	–
Granada	S	16	–	–	–	–	11	–	–	–	–
	RW	5	–	–	–	1	5	–	–	–	–
	ADW	1	–	–	–	1	1	–	–	–	–
	GW	n/a									
	RF	2	–	–	–	2	2	–	–	–	–
	F	8	1	8	4	8	8	–	–	–	–
Linadaraja	S	16	–	–	–	2	8	–	–	–	–
	RW	5	–	–	–	4	–	–	–	–	–
	ADW	1	–	–	–	1	–	–	–	–	–
	GW	n/a									
	RF	2	–	–	–	2	2	–	–	–	–
	F	8	–	8	8	8	8	–	–	–	–
Alisos	S	16	–	4	–	16	16	–	–	7	–
	RW	6	–	–	–	6	5	–	–	–	–
	ADW	2	–	–	–	2	1	–	–	–	–
	GW	1	–	–	–	1	–	–	–	–	–
	RF	2	–	–	–	2	2	–	–	–	–
	F	8	–	8	8	8	8	–	–	4	–
Corpoica	S	16	–	–	–	–	16	–	3	–	–
	RW	5	–	5	–	–	4	5	–	3	–
	ADW	1	–	1	–	–	1	1	–	–	–
	GW	1	–	–	–	–	–	1	–	–	–
	RF	2	–	2	–	–	2	–	–	–	–
	F	8	–	8	8	–	8	–	8	6	–
	Total		1	70	35	98	140	7	11	20	6

S, soil; RW, runoff water; ADW, animal drinking water; GW, ground water; RF, ruminal fluid; F, feces; –, there were no positive samples for the gene; n/a, not available sample.

(5 μg/ml), and digital images were taken using a UVP Gel Doc-It™ system.

Denaturing gel electrophoresis analysis bands were sequenced to confirm the identity of the amplified products and to compare which PCR products originated from animal and environmental samples. For the analysis, bands were excised from the gel and stored in distilled water for 12 h. Water containing the diffused template was used for PCR, and the reamplification products were sequenced using a DYEnamic ET Dye terminator kit (MegaBACE) on a MegBACE 1000 (GE Amersham) sequencer.

The identity of PCR amplicons was confirmed by comparing nucleotide sequences with the Gene Bank Database at National Center for Biotechnology (NCBI) using the basic logical alignment search tool (BLAST). Sequence alignment and cluster analysis,

including the *tet* genes from animal and environmental origin detected in the present study and other studies, were performed with Sequencher 4.1 and Clustal W.

RESULTS

DETECTION OF TETRACYCLINE RESISTANCE GENES

All of the farms evaluated were positive for *tet* genes in both animal and environmental samples (**Table 1**). The detected genes include *tet*(W), *tet*(Q), *tet*B(P), *tet*(O), *tet*(M), *tet*(S), *tet*(T), *tet*(B), and *tet*(Z). The genes *otr*(A), *tet*(A), *tet*(D), *tet*(H), and *tet*(J) were not detected in any sample. The highest detection frequencies were for those genes encoding RPPs with 355 out of 388 positive PCR reactions. Among this group, the most frequent were *tet*(W) with 36% (140 out of 388), *tet*(Q) with 25% (98 out of 388), and *tet*(O) with 18% (70 out of 388; **Table 2**).

Considering the frequency of detection per sample type, feces and ruminal fluid showed the highest frequencies of PCR positives for *tet* genes. Specifically, *tet*(O), *tet*(W), *tet*(Q), and *tet*B(P) were present in 100, 98, 83, and 70% of the feces samples, respectively (**Table 2**), while *tet*(W) and *tet*(Q) with frequencies of 100 and 80%, respectively, were the most common genes detected in the ruminal fluid. Genes *tet*(T) and *tet*(B) were also detected in feces, although with low frequencies.

Generally, *tet*(W) and *tet*(Q) were detected simultaneously in feces and runoff water samples from the farms, except in Lindaraja and Puente Luna. In Lindaraja, *tet*(W) was detected only in feces, while in Puente Luna both genes were found only in the feces samples (**Table 1**). Of all the genes that were detected simultaneously in feces and runoff water, only *tet*(W) and *tet*(Q) were present in soil samples at relatively high frequencies (**Table 2**). Treated runoff water, used as animal drinking water in Granada, Lindaraja, and Alisos, tested positive for the most common genes *tet*(W) and *tet*(Q). Resistance was not detected in animal drinking water from the municipal processing plant in Puente Luna; however, *tet*(W), *tet*(S), and *tet*(O) were detected in the samples from Corpoica (**Table 1**). Of the three groundwater samples analyzed, the samples collected from Granada and Corpoica were positive only for genes *tet*(Q) and *tet*(S), respectively.

An analysis of the gene patterns detected per reservoir type (**Table 3**) revealed that in four of the six sampled farms, the *tet* gene diversity was higher in animal samples as compared with the environmental samples. We also observed that the *tet*B(P) gene was only found in animal samples, and in the contrast, the genes *tet*(S) and *tet*(Z) were only found in environmental samples, although at low frequencies (**Table 2**).

Cluster analysis shows that the geographically closest sites, Puente Luna, Lindaraja, and Granada, are significantly more similar in relation to the genes detected and their frequencies (**Figures 1** and **2**). Manitas is more distant from this group because the gene *tet*(Z) was detected at this site but not *tet*(B/P). Corpoica is less similar with the other sites due to the absence of gene *tet*(Q) and the presence of genes *tet*(S). The *tet*(B) gene was not detected in the other sampling sites.

DGGE AND SEQUENCE ANALYSIS

Denaturing gel electrophoresis analysis results did not show genetic polymorphisms for *tet*(W) amplicons from any farm sampled. Sequences originating from both, animals and environment, exhibited a single band at the same gel level (data not shown). Blast analysis confirmed that the sequences of the bands corresponded to gene *tet*(W), and the alignment analysis showed no difference among the sequences from the samples, including the positive control, *B. fibrisolvens* (Nikolich et al., 1992). In addition, Clustal W analysis confirmed that the *tet*(W) sequences reported in this study had 100% identity with more than 83% of counterpart *tet*(W) sequences reported in databank.

In contrast, DGGE patterns for gene *tet*(Q) amplicons showed several bands per sample (data not shown). Blast analysis confirmed that sequences of all the bands observed corresponded to gene *tet*(Q), and the alignment analysis showed that most of

Table 2 | PCR evaluation results for the presence of *tet* genes in animal and environmental samples.

Sample type	No. of samples evaluated by PCR	No. of samples positive for *tet* genes									Total PCR reactions
		tet Genes encoding RPPs							*tet* Genes encoding efflux pumps		
		tet(M)	*tet*(O)	*tet*B(P)	*tet*(Q)	*tet*(W)	*tet*(S)	*tet*(T)	*tet*(B)	*tet*(Z)	
ENVIRONMENTAL											
S	96	–	4 (4)	–	24 (25)	56 (58)	–	–	7 (7)	2 (2)	93
RW	36	–	15 (42)	–	21 (58)	24 (67)	5 (14)	3 (38)	3 (8)	4 (11)	75
ADW	7	–	1 (14)	–	4 (57)	3 (43)	1 (14)	–	–	–	9
GW	3	–	–	–	1 (33)	–	1 (33)	–	–	–	2
ANIMAL											
RF	10	–	2 (20)	–	8 (80)	10 (100)	–	–	–	–	20
F	48	1 (2)	48 (100)	35 (73)	40 (83)	47 (98)	–	8 (17)	10 (21)	–	189
	Total	1	70	35	98	140	7	11	20	6	388

( ), Frequency of detection = No. of samples positive for a given gen/total number of evaluated samples∗100.

–, There were no positive samples for the gene; S, soil; RW, runoff water; ADW, animal drinking water; GW, ground water; RF, ruminal fluid; F, feces.

the bands in a sample shared the same sequence. We observed a maximum of two bands with different *tet*(Q) genotypes within a sample. The multiple bands for the same sequence in the DGGE patterns of *tet*(Q) probably resulted from the presence of single-stranded DNA and the formation of a PCR product with multiple denaturation domains (Kocherginskaya et al., 2001; Kobayashi et al., 2007; Calabria de Araujo and Schneider, 2008). Cluster analysis classified 70 of the *tet*(Q) band sequences observed in the DGGE analysis into five different groups based on nucleotide variations, rather than the sample type (**Figure 3**). Only five different genotypes were found, which differed at position 32, where a thymine had been replaced by cytosine and also at position 65 where a thymine was replaced by adenine or guanine as compared with the positive control *B. thetaiotaomicron* (Aminov et al., 2001). There were no particular *tet*(Q) genotypes associated exclusively with the environmental or animal samples for groups two, four, and five. However, group one only presented sequences detected in environmental samples, although these sequences grouped together with the control, which is a sequence of clinical origin (**Figure 3**). Notably, most sequences were included in Group 5, which was characterized by nucleotide changes at positions 32 and 65, where the thymine was replaced by an adenine. These changes were also observed in *tet*(Q) nucleotide sequences previously reported (Aminov and Mackie, 2007). According to Clustal and Generunner analyses, these mutations do not produce changes in the protein sequence when they are compared with the Tet(Q) protein of the reference strain *B. thetaiotaomicron*; in both positions, the amino acid glycine is conserved.

Table 3 | Gene patterns detected in the animal and environmental samples from each sampled farm.

Farm	Type of sample	
	Animal	**Environmental**
Manitas	*tet* (O), (Q), (W)	*tet*(O), (Q), (W), (Z)
Puente Luna	*tet* (O), (Q), (W), B(P)	*tet* (Q), (W)
Granada	*tet* (O), (Q), (W), B(P), (M)	*tet* (Q), (W)
Lindaraja	*tet* (O), (Q), (W), B(P)	*tet* (Q), (W)
Alisos	*tet* (O), (Q), (W), B(P), (B)	*tet* (O), (Q), (W), (B)
Corpoica	*tet* (O), (W), B(P), (B),(T)	*tet* (O), (W), (B), (T), (S)

DISCUSSION

Assessing the presence of resistance genes to the antibiotic tetracycline in the highlands of Colombia has revealed not only resistance reservoirs in dairy cattle raised in this region but also a wide distribution of *tet* genes in the environment of grassland-based production systems. The detection frequency and spatial distribution of resistance genes in the runoff water and soils was remarkable, especially for genes *tet*(W) and *tet*(Q). It is known that resistant bacteria occur in nature as a result of natural selective pressures (Aminov, 2009; Martinez, 2009), as it was shown by some studies reporting tetracycline resistance in a national park and in fecal bacteria of wild animals in areas which apparently are not under the exposure of urban or agricultural antibiotic contamination (Gilliver et al., 1999; Yang et al., 2010; Rahman et al., 2008).

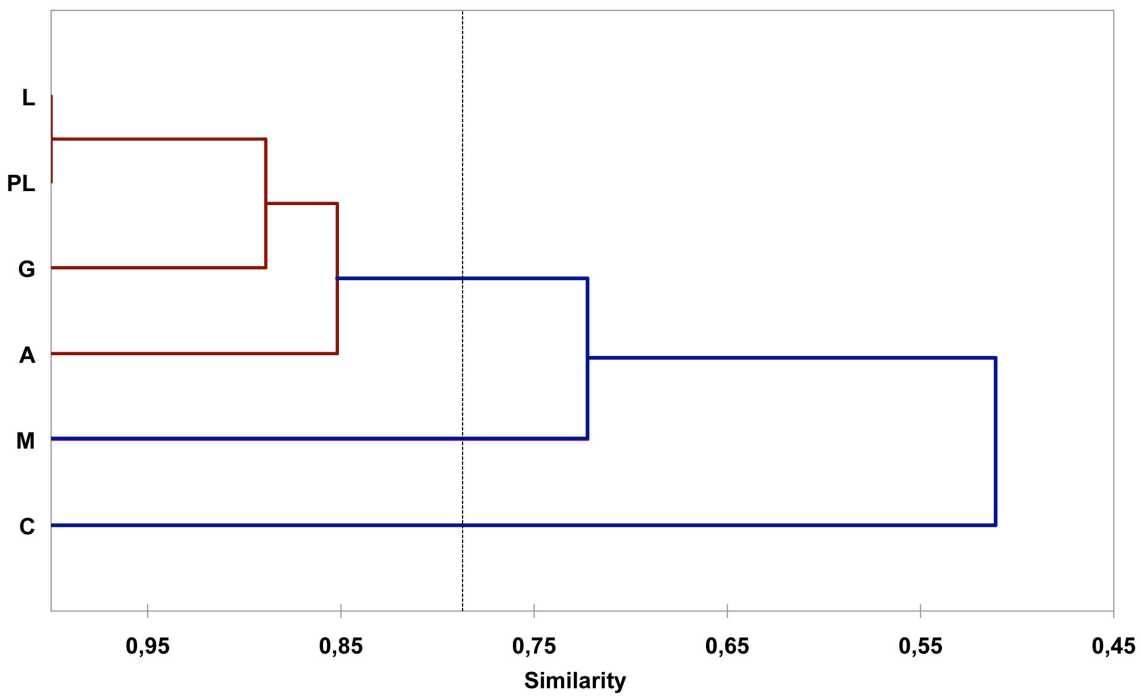


FIGURE 2 | Cluster analysis (UPGMA, percentage similarity index) for farms sampled, based on the presence and frequency of *tet* genes. M, Manitas; PL, Puente Luna; G, Granada; L, Lindaraja; A, Alisos; C, Corpoica.

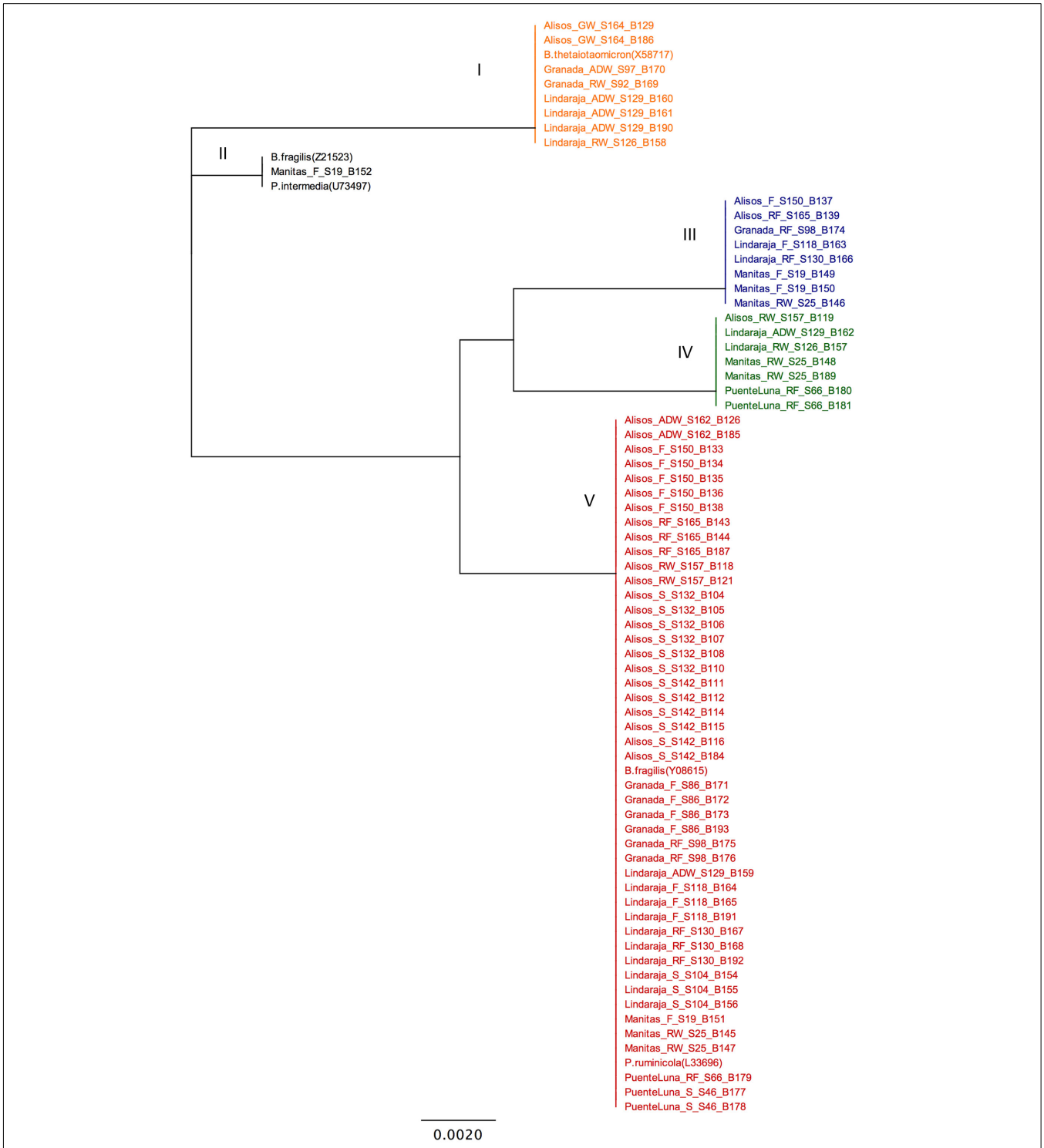


FIGURE 3 | Unrooted phylogram showing the genetic distances among gene *tet*(Q) sequences (neighbor-joining). S_, soil; RW_, runoff water; ADW_, animal drinking water; GW_, ground water; RF_, ruminal fluid; F_, feces, S, Sample ID; B, DGGE band sequence ID.

However, *tet* genes detected in the evaluated grasslands-based production systems in Colombia, do not seem to be the result of natural selective pressures. Instead, they are probably the result of horizontal gene transfer because, the same *tet*(W) and *tet*(Q) sequences were detected in animal, soil, and water samples. Additionally, it was not found any novel sequence for these genes at the Altiplano region. Sequences detected for these genes did not differ from most of the *tet*(Q) and *tet*(W) sequences reported in

the literature. It was not feasible to include in this study plots of grazing land free areas in order to compare samples of natural environments with those samples collected in the active grazing plots. The vast majority of the land in the Altiplano Cundiboyacense is (or had been during the last decades) under the influence of cattle waste given the predominant extensive cattle production system. Therefore, it was not possible to determine the presence and frequency of the *tet* genes in an environment of the Altiplano that is not under the exposure of antibiotic contamination.

Cattle raised in the grassland-based production systems at the highlands of Colombia, seem to be a source of tetracycline resistance genes into the environment, especially those coding for RPPs. This transmission was concluded on the basis that detection frequencies for genes *tet*(Q) and *tet*(W) in the digestive tract of the animals sampled in this region was greater than 80%, and these genes were also common in the environmental samples, especially in water. Moreover, the sequences of the genes *tet*(W) and *tet*(Q) detected in soil and water did not differ from those found in samples of animal origin, and no *otr*(A) genes were detected from antibiotic-producing bacteria. Finally, Lopez et al. (Submitted manuscript) examined the presence of *tet* genes and their sequences in 150 heterotrophic tetracycline resistant bacteria isolated by culture techniques from the animal and environmental samples analyzed in the present study. These data showed that a high percentage of the isolates were positive for genes *tet*(W) (53)% and *tet*(Q) (35%). Gene *tet*(W) did not show any genetic polymorphism as it was observed for *tet*(W) sequences detected in the total DNA samples. Sequences of gene *tet*(Q) detected in the isolates, showed three different genotypes which were identical to those in group one, two, and five in **Figure 3**. These groups included sequences detected in bacteria isolated from both, animal and environmental samples. 16S rRNA sequences analysis of the bacteria isolates, revealed that genera where genes *tet*(W) and *tet*(Q) were detected included *Enterococcus*, *Staphylococcus*, *Escherichia*, *Klebsiella* and *Shigella*, and also non-strict pathogenic, non-clinical bacteria commonly found in the environment such as *Burkholderia*, *Chryseobacterium*, *Variovorax*, *Acinetobacter*, *Pseudomonas,* and *Dyella.* Therefore, based on the foregoing, it is likely that the resistance genes detected at the Altiplano Cundiboyacense originated from animal reservoirs and are neither associated with native bacteria genes that produce antibiotics nor the result of selective pressure exerted by the tetracycline released into the environment on microbial populations.

A comparison of gene patterns detected in animal and environmental samples from the Altiplano (**Table 3**) revealed that the detected genes are not equally distributed in animal and environmental reservoirs. Despite having a 100% frequency of detection in feces from all the sampling sites, gene *tet*(O) was not detected in environmental samples from Puente Luna, Granada, and Lindaraja. Gene *tet*B(P), also with high frequencies of detection in feces, was not detected in water or soil samples from any site. These results are consistent with the mobility reported for *tet* genes in the literature. Genes *tet*(W) and *tet*(Q), found in 18 and 15 different bacteria genera, respectively, have high transferability rates because they are associated with conjugative chromosomal elements encoding their own transfer (Roberts, 2005). However, genes *tet*(O) and *tet*B(P), found in 10 and 1 bacteria genera, respectively, are not normally associated with conjugative transposons or other types of mobile genetic elements (Chopra and Roberts, 2001; Roberts, 2005), which could limit the flow of these genes into soil and water bacteria.

Runoff water might be playing an important role in the transmission of genes with genetic mobility to the regions of Altiplano and the north of Colombia. This transmission is possible because runoff water in the ditch systems, where samples were collected, flows into the Bogotá and Sogamoso watersheds, which in turn drain into the Magdalena River Basin (**Figure 1**). Considering the high detection frequencies of *tet*(Q) and *tet*(W) genes in runoff waters and the high rainfall precipitation throughout most of the year in this region (IDEAM, 2010) resistance genes might be dispersed by the waters of Magdalena because this river crosses the country from north to south emptying into the great slope of the Caribbean. Although this study did not analyze river water impacted by runoff, Kobayashi et al. (2007) and Tao et al. (2010) demonstrated the presence of resistance genes in waters and sediments from rivers impacted by waste coming from developed areas in southern Vietnam and China, where significant amounts of antibiotics are administrated to humans and animals.

There seems to be a geographical distribution of some *tet* genes in the Altiplano region. Sampled farms showed more than 50% similarity in relation to the detected *tet* genes and their frequencies (**Figure 2**), and this similarity increases with decreasing geographical distances. For instance, Puente Luna, Granada, and Lindaraja, close and under the influence of a dense hydrographic network that flows into the Sogamoso River, are significantly more similar to each other than to Corpoica, located further apart and under the influence of a different hydrographic network that flows into the Bogota River. Moreover, the presence of genes *tet*(S) and *tet*(T) and the absence of *tet*(Q) make Corpoica different from the other sites. Manitas, a farm surrounded by fewer streams per square kilometer was the only site positive for gene *tet*(Z) and the only site where *tet*(B/P) was not detected. The differences in the geographical distribution of some *tet* genes might result from differences in the management practices for the administration of antibiotics. Alexander et al. (2011), showed that cattle fed with different subtherapeutic antibiotics presented different abundances of certain resistance genes. Future studies are needed to clarify how the geographical location, presence of hydrological networks, and management practices for the administration of antibiotics determines the distribution of *tet* genes at the highland plateau of the Altiplano Cundiboyacense.

Ruminal fluid samples did not reveal the variety of *tet* genes occurring in animals reservoirs as feces did. Genes *tet*B(P), *tet*(T), and *tet*(B) were found in feces but not in the forestomach, may be as a result of differences in the microbial populations along the ruminant digestive tract. It has been demonstrated that rumen and feces have different microbial communities as a result of differences in the environmental conditions between rumen and the end of the digestive tract, such as pH, degree of anaerobiosis, and available substrates (Michelland et al., 2009; Callaway et al., 2011). Therefore, genes *tet*(W) and *tet*(Q), reported in a wide range of genera (Roberts, 2005), are detected in bacteria associated with both rumen and the end of the intestinal tract. However, genes with narrow distribution ranges, such as *tet*B(P), *tet*(T),

and *tet*(B) (Roberts, 2005), were associated only with bacteria at the end of the intestinal tract. In fact gene *tet*B(P) has been only reported in *Clostridium*, one of the predominant genera in feces (Callaway et al., 2011). Surprisingly, *tet*(M), although having a wide distribution range (Roberts, 2005), was only present in feces. This gene was not common on any sampled farm at the Altiplano region; it was only detected in one feces sample from Granada. Nevertheless, we cannot rule out the presence of *tet*(M) in the animal and environmental microbial community, where it might be present at a low frequency, making it difficult to detect by PCR.

Genes *tet*(W), *tet*(Q), and *tet*(O), detected in higher frequencies at the Altiplano Cundiboyacense, were also frequently detected in studies conducted in the United States by Aminov et al. (2001) and Peak et al. (2007) for ruminal content and waste water lagoons near cattle feedlots. The most particular results found in this present study were related to the frequencies detected for genes *tet*(M) and *tet*B(P). Specifically, *tet*(M), often associated with conjugative transposons and identified in 35 genera from variety of ecosystems, including humans, animals, and soils (Roberts, 2005), was not common at the Altiplano Cundiboyacense. By contrast, in other studies, it is reported as a common gene (Peak et al., 2007; Alexander et al., 2011). Gene *tet*B(P), which is commonly found in the intestinal tract of cows from most sampled farms in this study, is not reported in previous studies of ruminal content, cow feces, soil amended with cow manure, or waste water lagoons near cattle feedlots conducted in Europe and the United States. Finally, the most frequent genes found in the highlands of Colombia are those encoding RPPs. Genes encoding membrane efflux pumps are neither diverse nor frequent. Conversely, Sawant et al. (2007) in the United States showed that genes *tet*(B) and *tet*(A) encoding for efflux pumps, were the *tet* genes most frequently found in feces of dairy cattle. Additionally, a study conducted by Kobashi et al. (2007) in various agricultural environments of Japan, showed that *tet* genes encoding efflux pumps were more frequent than those encoding for RPPs. As far as we know, only Yang et al. (2010), have reported in the USA that gene pools of *tet*(O) and *tet*(W), were bigger than those encoding efflux pumps in fecal and water samples of cattle operations.

In conclusion, grassland production systems at the highlands plateau of the Altiplano Cundiboyacense present animal and environmental reservoirs of tetracycline resistance genes, especially for genes *tet*(W) and *tet*(Q) encoding RPPs. The high frequency of detection of these genes in soil and water is probably the result of horizontal gene transfer from fecal bacteria to environmental bacteria. The range of diversity of the *tet* genes found in animal reservoirs and particularly at the end of the animal digestive tract is wider than for those found in soil and water, indicating that genes encoding resistance do not have the same probability of transmission to the environment. Runoff water might be an important dissemination agent for the most frequent *tet* genes in this region; however, the impact of infiltrated wastewaters on groundwater appears to be lower, although it is necessary to include more ground water sampling sites to reach a conclusion.

ACKNOWLEDGMENTS

This work was supported by the Administrative Department of Science, Technology and Innovation of the Republic of Colombia (COLCIENCIAS), Project No. 460-2008, and the University Jorge Tadeo Lozano. We thank the Colombian Corporation for Agricultural Research (Corpoica), Ernesto Guerra, Fabio Reina, Salvador Rincon, Rafael Reina, and Mauricio Sandoval for allowing us to collect samples on their dairy cattle farms. The authors also like to thank Andrés León for helping in sample collection and PCR experiments. We declare that this project has The Permit for Scientific Research in Biodiversity (No. 9, December 5, 2008) and an Agreement for Access to Genetic Resources from the Scientific Interest Free of Trade Interest (No. 24) issued by the Ministry of Environment, Housing, and Territorial Development of the Republic of Colombia.

REFERENCES

Aarestrup, F. M. (2005). Veterinary drug usage and antimicrobial resistance in bacteria of animal origin. *Basic Clin. Pharmacol. Toxicol.* 96, 271–281.

Aarestrup, F. M. (2006). "Origin, evolution and local and global dissemination of antimicrobial resistance," in *Antimicrobial Resistance in Bacteria of Animal Origin*, ed. F. M. Aarestrup (Washington, DC: ASM Press), 339–359.

Alexander, T. W., Yanke, J. L., Reuter, T., Topp, E., Read, R. R., Selinger, B. L., and McAllister, T. A. (2011). Longitudinal characterization of antimicrobial resistance genes in feces shed from cattle fed different subtherapeutic antibiotics. *BMC Microbiol.* 11, 19–30. doi:10.1186/1471-2180-11-19

Alexander, T. W., Yanke, L. J., Topp, E., Olson, M. E., Read, R. R., Mork, D. W., and McAllister, T. A. (2008). Effect of subtherapeutic administration of antibiotics on the prevalence of antibiotic resistant *Escherichia coli* bacteria in feedlot cattle. *Appl. Environ. Microbiol.* 74, 4405–4416.

Aminov, R. I. (2009). The role of antibiotics and antibiotic resistance in nature. *Environ. Microbiol.* 11, 2970–2988.

Aminov, R. I., Chee-Sanford, J. C., Garrides, N., Teferedegne, B., Krapac, I. J., White, B. A., and Mackie, R. I. (2002). Development, validation and application of PCR primers for detection of tetracycline efflux genes of gram negative bacteria. *Appl. Environ. Microbiol.* 68, 1786–1793.

Aminov, R. I., Garrigues-Jean-Jean, M., and Mackie, R. I. (2001). Molecular ecology of tetracycline resistance: development and validation of primers for detection of tetracycline resistance genes encoding ribosomal protection proteins. *Appl. Environ. Microbiol.* 67, 22–32.

Aminov, R. I., and Mackie, R. I. (2007). Evolution and ecology of antibiotic resistance genes. *FEMS Microbiol. Lett.* 271, 147–161.

Binh, C. T. T., Heuer, H., Kaupenjohann, M., and Smalla, K. (2008). Piggery manure used for soil fertilization is a reservoir for transferable antibiotic resistance plasmids. *FEMS Microbiol. Ecol.* 66, 25–37.

Blake, D. P., Humphry, R. W., Scott, K. P., Hillman, K., Fenlon, D. R., and Low, J. C. (2003). Influence of tetracycline exposure on tetracycline resistance and carriage of tetracycline resistance genes within commensal populations. *J. Appl. Microbiol.* 94, 1087–1097.

Bryan, A., Shapir, N., and Sadowsky, M. J. (2004). Frequency and distribution of tetracycline resistance genes in genetically diverse, nonselected, and nonclinical *Escherichia coli* strains isolated from diverse human and animal sources. *Appl. Environ. Microbiol.* 70, 2503–2507.

Calabria de Araujo, J., and Schneider, R. P. (2008). DGGE with genomic DNA: suitable for detection of numerically important organisms but not for identification of the most abundant organisms. *Water Res.* 42, 5002–5010.

Callaway, T. R., Dowd, S. E., Edrington, T. S., Anderson, R. C., Krueger, N., Bauer, N., Kononoff, P. J., and Nisbet, D. J. (2011). Evaluation of bacterial diversity in the rumen and feces of cattle fed different levels of dried distillers grains plus solubles using bacterial tag-encoded FLX amplicon pyrosequencing. *J. Anim. Sci.* 88, 3977–3983.

Campagnolo, L., Jonhson, K., Karpati, A., Rubin, C., Kolpin, D., Meyer, M., Esteban, E., Currier, R., Smith, K., Thu, K., and McGeehin, M. (2002). Antimicrobial residues in animal waste and water resources proximal to large scale swine and poultry feeding operations. *Sci. Total Environ.* 299, 89–95.

Chee-Sanford, J. C., Aminov, R. I., Krapac, I. J., Garrigues-Jean-Jean, N., and Mackie, R. J. (2001). Occurrence and diversity of tetracycline resistance genes in lagoons and groundwater underlying two swine production facilities. *Appl. Environ. Microbiol.* 67, 1494.

Chopra, I., and Roberts, M. (2001). Tetracycline antibiotics: mode of action, applications, molecular biology, and epidemiology of bacterial resistance. *Microbiol. Mol. Biol. Rev.* 65, 232–260.

Gilliver, M., Bennett, M., Begon, M., Hazel, S., and Hart, C. (1999). Antibiotic resistance found in wild rodents. *Nature* 401, 233–234.

Götz, A., and Smalla, K. (1997). Manure enhances plasmid mobilization and survival of *Pseudomonas putida* introduced into field soil. *Appl. Environ. Microbiol.* 63, 1980–1986.

Heuer, H., Schmitt, H., and Smalla, K. (2011). Antibiotic resistance gene spread due to manure applications on agricultural fields. *Curr. Opin. Microbiol.* 14, 1–8.

Heuer, H., and Smalla, K. (2007). Manure and sulfadiazine synergistically increased bacterial antibiotic resistance in soil over at least two months. *Environ. Microbiol.* 9, 657–666.

IDEAM. (2010). Informe Anual sobre el Estado del Medio Ambiente y los Recursos Naturales Renovables en Colombia – Bosques. Bogotá, DC: Instituto de Hidrología, Meteorología y Estudios Ambientales de Colombia.

Knapp, C. W., Schmitt, H., and Smalla, K. (2010). Evidence of increasing antibiotic resistance gene abundances in archived soils since 1940. *Environ. Sci. Technol.* 44, 580–587.

Kobashi, Y., Hasebe, A., Nishi, M., and Uchiyama, H. (2007). Diversity of tetracycline resistance genes in bacteria isolated from various agricultural environments. *Microbes Environ.* 22, 44–51.

Kobayashi, T., Suehiro, F., Tuyen, B. C., and Suzuki, S. (2007). Distribution and diversity of tetracycline resistance genes encoding ribosomal protection proteins in Mekong River sediments in Vietnam. *FEMS Microbiol. Ecol.* 59, 729–737.

Kocherginskaya, S., Aminov, R. I., and White, B. (2001). Analysis of the rumen bacterial diversity under two different diet conditions using denaturing gradient gel electrophoresis, random sequencing, and statistical ecology approaches. *Anaerobe* 7, 119–134.

Koike, S., Krapad, I. G., Oliver, H. D., Yanarel, A. C., Chee-Sanford, J. C., Aminov, R. I., and Mackie, R. I. (2007). Monitoring and source tracking of tetracycline resistance genes in lagoons and groundwater adjacent to swine production facilities over a 3 year period. *Appl. Environ. Microbiol.* 73, 4813–4823.

Loffler, F. E., Sung, Q. J., Li, J., and Tiedje, J. M. (2000). 16S rRNA gene based detection of tetrachloroethene dechlorinating *Desulfuromonas* and *Dehalococcoides* species. *Appl. Environ. Microbiol.* 66, 1369–1374.

Martinez, J. L. (2009). The role of natural environments in the evolution of resistance traits in pathogenic bacteria. *Proc. Biol. Sci.* 276, 2521–2530.

Michelland, R. J., Monteils, V., Zened, A., Combes, S., Cauquil, L., Gidenne, T., Hamelin, J., and Fortun-Lamothe, L. (2009). Spatial and temporal variations of the bacterial community in the bovine digestive tract. *J. Appl. Microbiol.* 107, 1642–1650.

Nikolich, M. P., Shoemaker, N. B., and Salyers, A. A. (1992). A bacteroides tetracycline resistance gene represents a new class of ribosome protection tetracycline resistance. *Antimicrob. Agents Chemother.* 36, 1005–1012.

Oficina Regional de la FAO para América Latina y el Caribe (FAO). (2008). *Ayudando a desarrollar una ganadería sustentable en América Latina y el Caribe. Lecciones a Partir de Casos Exitosos.* Santiago: Organización de las Naciones Unidas Para la Agricultura y la Alimentación.

Patterson, J. A., Colangelli, R., Spigaglia, P., and Scott, K. P. (2007). Distribution of specific tetracycline and erythromycin resistance genes in environmental samples assessed by microarray detection. *Environ. Microbiol.* 9, 703–715.

Peak, N., Knapp, C. W., Yang, R. K., Hanfelt, M. M., Smith, M. S., Aga, D. S., and Graham, D. W. (2007). Abundance of six tetracycline resistance genes in wastewater lagoons at cattle feedlots with different antibiotic use strategies. *Environ. Microbiol.* 9, 143–151.

Rahman, H. M., Sakamoto, K. Q., Nonaka, L., and Suzuki, S. (2008). Occurrence and diversity of tetracycline resistance gene tet(M) in enteric bacteria of Antarctic Adélie penguin. *J. Antimicrob. Chemother.* 62, 627–628.

Roberts, M. C. (2005). "Tetracycline resistance due to ribosomal protections proteins," in *Frontiers In Antimicrobial Resistance*, eds D. G. White, M. N. Alekshun, and P. F. McDermott (Washington, DC: ASM Press), 19–28.

Sawant, A. A., Narasimha, V. H., Straley, B. A., Donaldson, S. C., Love, B., Knabel, S. J., and Jayarao, B. M. (2007). Antimicrobial resistant enteric bacteria from dairy cattle. *Appl. Environ. Microbiol.* 73, 156–163.

Schwarz, S., Cloeckaert, A., and Roberts, C. (2006). "Mechanisms and spread of bacterial resistance to antimicrobial agents," in *Antimicrobial Resistance in Bacteria of Animal Origin*, ed. F. M. Aareestrup (Washington, DC: ASM Press), 73–98.

Sengelov, G., Agerso, Y., Halling-Sorencen, B., Beloda, S. B., Anderdsen, J. S., and Jensen, L. B. (2003). Bacterial antibiotic resistance levels in Danish farmland as a result of treatment with pig manure slurry. *Environ. Int.* 28, 587–595.

Steinfeld, H., Gerber, P., Wassernaar, T., Castel, V., Rosales, M., and deb Hann, C. (2006). *Livestock's Long Shadow-Environmental Issues and Options.* Rome: LEAD/FAO.

Tao, R., Ying, G., Su, H. C., Zou, H. W., and Sidhu, J. P. S. (2010). Detection of antibiotic resistance and tetracycline resistance genes in *Enterobacteriaceae* isolated from Pearl rivers in South China. *Environ. Pollut.* 158, 2101–2109.

van den Bogaard, A. E., and Stobberingh, E. E. (2000). Epidemiology of resistance to antibiotics links between animal and humans. *Int. J. Antimicrob. Agents* 14, 327–335.

Winckler, C., and Grafe, A. (2001). Use of veterinary drugs in intensive animal production facilities evidence of persistence of tetracycline in pig slurry. *J. Soils Sediments* 1, 66–70.

Witte, W. (2000). Ecological impact of antibiotic use in animals on different complex microflora environment. *Int. J. Antimicrob. Agents* 14, 321–325.

Yang, H., Byelashov, O. A., Geornaras, I., Goodridge, L. D., Nightingale, K. K., Belk, K. E., Smith, G. C., and Sofos, J. N. (2010). Presence of antibiotic resistant commensal bacteria in samples from agricultural, city, and national park environments evaluated by standard culture and real time PCR methods. *Can. J. Microbiol.* 56, 761–770.

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 04 September 2011; accepted: 29 November 2011; published online: 14 December 2011.

Citation: Santamaría J, López L and Soto CY (2011) Detection and diversity evaluation of tetracycline resistance genes in grassland-based production systems in Colombia, South America. Front. Microbio. ***2****:252. doi: 10.3389/fmicb.2011.00252*

This article was submitted to Frontiers in Antimicrobials, Resistance and Chemotherapy, a specialty of Frontiers in Microbiology.

frontiers in
MICROBIOLOGY

ORIGINAL RESEARCH ARTICLE
published: 09 March 2012
doi: 10.3389/fmicb.2012.00083

Occurrence of CTX-M producing *Escherichia coli* in soils, cattle, and farm environment in France (Burgundy region)

Alain Hartmann[1,2]*, Aude Locatelli[1], Lucie Amoureux[3], Géraldine Depret[1,2], Claudy Jolivet[4], Eric Gueneau[5] and Catherine Neuwirth[3]

[1] INRA, UMR Microbiologie des Sols et de l'Environnement (MSE), The Institut National de la Recherche Agronomique, Dijon, France
[2] Université de Bourgogne, Dijon, France
[3] Laboratory of Bacteriology, University Hospital of Dijon, Dijon, France
[4] Unité InfoSol, The Institut National de la Recherche Agronomique, Orléans, France
[5] Laboratoire Départemental de la Côte d'Or, Dijon, France

Edited by:
Rustam I. Aminov, University of Aberdeen, UK

Reviewed by:
Luisa Peixe, Faculdade de Farmácia da Universidade do Porto, Portugal
Timothy LaPara, University of Minnesota, USA
Yoshimi Matsumoto, Osaka University, Japan

***Correspondence:**
Alain Hartmann, UMR Agroécologie 1347, INRA, The Institut National de la Recherche Agronomique Centre, 17 rue Sully, BP 86510, Dijon 21065, France.
e-mail: alain.hartmann@dijon.inra.fr

CTX-M [a major type of extended-spectrum beta-lactamase (ESBL)] producing *Escherichia coli* are increasingly involved in human infections worldwide. The aim of this study was to investigate potential reservoirs for such strains: soils, cattle, and farm environment. The prevalence of $bla_{\text{CTX-M}}$ genes was determined directly from soil DNA extracts obtained from 120 sites in Burgundy (France) using real-time PCR. $bla_{\text{CTX-M}}$ targets were found in 20% of the DNA extracts tested. Samples of cattle feces ($n = 271$) were collected from 182 farms in Burgundy. Thirteen ESBL-producing isolates were obtained from 12 farms and further characterized for the presence of *bla* genes. Of the 13 strains, five and eight strains carried $bla_{\text{TEM-71}}$ genes and $bla_{\text{CTX-M-1}}$ genes respectively. Ten strains of CTX-M-1 producing *E. coli* were isolated from cultivated and pasture soils as well as from composted manure within two of these farms. The genotypic analysis revealed that environmental and animal strains were clonally related. Our study confirms the occurrence of CTX-M producing *E. coli* in cattle and reports for the first time the occurrence of such strains in cultivated soils. The environmental competence of such strains has to be determined and might explain their long term survival since CTX-M isolates were recovered from a soil that was last amended with manure 1 year before sampling.

Keywords: extended-spectrum beta-lactamase, CTX-M, cattle, soil, Burgundy, farm environment

INTRODUCTION

The production of extended-spectrum beta-lactamases (ESBLs) is one of the most significant mechanisms of resistance to oxyiminocephalosporin antibiotics in *E. coli* (Pitout and Laupland, 2008). Among these enzymes, the CTX-M type ESBLs have emerged worldwide, they have progressively replaced the TEM and SHV families (Bonnet, 2004). To date, 123 $bla_{\text{CTX-M}}$ genes have been reported[1], the corresponding CTX-M enzymes are clustered in five groups, group 1, 9, and 2 being predominant. The producing organisms are sometimes involved in nosocomial infections but are widely encountered in community settings (Arpin et al., 2009; Woerther et al., 2010). Their rate of dissemination might suggest the occurrence of environmental reservoirs potentially leading to human contamination through water, food consumption or direct contact with animals (Leverstein-Van Hall et al., 2011). There are many descriptions of fecal carriage of such organisms among food-producing animals especially poultry (broilers) and pigs (Costa et al., 2009; Bortolaia et al., 2010; Cortes et al., 2010). The reports concerning livestock cattle are much less abundant (Horton et al., 2011), and there is no published data about the prevalence of CTX-M producing *E. coli* in soils. Nevertheless cultivated soils are frequently fertilized with agricultural or urban organic residues that may contain antibiotic resistant microorganisms (Moodley and Guardabassi, 2009; Reinthaler et al., 2010) and thus might act as environmental reservoirs.

The aim of this study was to develop an integrated approach encompassing soils, livestock, and farm environment in a whole region (Burgundy, France). A systematic large scale study was conducted using a molecular detection approach to detect $bla_{\text{CTX-M}}$ directly from soil DNA extracts. Conventional bacteriological methods were used to isolate ESBL-producing *E. coli* from cattle feces from 182 farms. When positive animals were detected, the corresponding farm environment, i.e., cultivated and pasture soils as well as cattle manure was sampled and analyzed by bacteriological methods. All environmental and animal isolates recovered during the study have been subjected to genotypic characterization and $bla_{\text{CTX-M}}$ genes have been sequenced.

MATERIALS AND METHODS

SOIL SAMPLING

Soils were sampled in the "Réseau de Mesures de la Qualité des Sols" (RMQS = French Soil Quality Monitoring Network) which is a network based on the sampling of soil with a 16 × 16 km systematic grid covering the whole French territory (Arrouays et al., 2002). The RMQS consisted in 2200 monitoring sites, which are located close to the center of each 16 × 16 km cell. Corresponding

[1] http://www.lahey.org/studies/

land covers were recorded and categorized as: large scale crops, pastures, orchards, vineyards, natural vegetation such as forests or meadows. Each site is geo-positioned with a precision <0.5 m. Twenty five individual core samples were collected from the top-soil (0–30 cm) using a stratified random sampling design within a 20 m × 20 m area. Core samples were bulked to obtain a composite sample for each site. Soil samples were air-dried and sieved to 2 mm before analysis. For this study 120 soil samples corresponding to the Burgundy region (four departments Côte d'Or, Saône et Loire, Yonne, and Nièvre) were analysed.

BOVINE FECES SAMPLING

From April 2009 to June 2009, a total of 271 fecal swabs of cattle were collected from 182 farms located in three departments of the Burgundy region, namely Côte d'Or, Nièvre, and Saône et Loire. Three groups of animals were sampled: (i) healthy adults (218), (ii) enteritic diseased calves (35), and healthy adults linked with sick calves (18). Swabs (COPAN, CML, France) were immediately transferred into tubes containing Amies agar gel transport medium. Bacteriological analyses of the fecal swabs were performed within 3 days after sampling. In farm 2 where positive animals had been detected in 2009, feces samples of the whole cow herd were analyzed (90 animals) in July 2010 as described above.

FARM ENVIRONMENT SAMPLING

For three farms (farms 1, 2 and 3), where CTX-M producing *E. coli* occurred in animals, we conducted further analysis of several environmental samples during the autumn 2009. These farms were chosen on the basis of the willingness of the farmers to cooperate to the study. In each farm one cropped and one pasture soils were sampled by taking three individual cores in the top soil (using a 7 cm width disinfected auger) that were pooled to obtain a composite sample. Manure samples were collected in the three farms using the same equipment (disinfected auger). All samples were kept moist in single use plastic bags at room temperature. Bacteriological analyses were performed on these samples within 3 days after sampling.

SOIL MICROBIAL COMMUNITY DNA EXTRACTION

Microbial DNA was extracted from bulk RMQS soil samples according to the method described by Ranjard et al. (2003). Briefly, 1.5 g of each soil was mixed with 5 ml of a solution containing 100 mM Tris (pH 8.0), 100 mM EDTA (pH 8.0), 100 mM NaCl, and 2% (w/v) sodium dodecyl sulfate. Two grams of 106 μm diameter glass beads and eight glass beads of 2-mm diameter were added in a bead-beater tube. The samples were then homogenized for 30 s at 1,600 rpm in a mini bead-beater cell disruptor (Mikrodismembrator; S.B. Braun Biotech International) and centrifuged at 7,000 × g for 5 min at 4°C after 30 min incubation at 70°C. The collected supernatants were incubated for 10 min on ice after adding 1/10 volume of 3 M potassium acetate (pH 5.5) and centrifuged at 14,000 × g for 5 min. After precipitation with one volume of ice-cold isopropanol, the nucleic acids were washed with 70% ethanol. DNA extracts were purified on polyvinyl polypyrrolydone minicolumns (Bio-Rad, France) by centrifugation at 1,000 × *g* for 2 min at 10°C. Residual impurities from DNA extracts were finally removed by using a Geneclean Turbo kit as recommended by the manufacturer (Q Biogene®, Illkirch, France). DNA's were quantified by agarose gel electrophoresis (1% agarose in TBE buffer) using calf thymus DNA dilutions as standards and the ImageQuant software (Applied Biosytems). Five nanograms of DNA were used per PCR reaction.

DEVELOPMENT AND USE OF A REAL-TIME PCR ASSAY FOR *bla*$_{\text{CTX-M}}$ DETECTION FROM SOIL EXTRACTED DNA

Twenty seven *bla*$_{\text{CTX-M}}$ genes sequences were aligned using the ClustalW program. Sequences were chosen to be representative from the five distinct groups of CTX-M enzymes described by Bonnet (2004). Seven sequences from CTX-M-1 group were used (GenBank accession numbers: DQ658221, X92506, EU921825, NC004464, Y10278, NC005327, and AY238472) corresponding to CTX-M-12, CTX-M-1, CTX-M-32, CTX-M-3, CTX-M-15, CTX-M-33, respectively. Four sequences from CTX-M-9 group (GenBank accession numbers: AF174129, EU916273, EU921824, and EU921826) corresponding to CTX-M-9, CTX-M-27, CTX-M-24, and CTX-M-65, respectively. Three sequences from CTX-M-2 group were used (GenBank accession numbers: X92504, EF374097, and AM982521) corresponding to CTX-M-2, CTX-M-56, and CTX-M-77 respectively. Three sequences from CTX-M-25 group were used (GenBank accession numbers: AF518567, DQ023162, and AM982522) corresponding to CTX-M-25, CTX-M-41, and CTX-M-78 respectively. Two sequences from CTX-M-8 group were used (GenBank accession number: AF189721 and AY750914) corresponding to CTX-M-8 and CTX-M-40. From these alignments, primers and Taqman type probes specific for either *bla*$_{\text{CTX-M}}$ genes encoding CTX-M-1 or CTX-M-9 groups were selected using Primer Express software (Applied Biosystems). For CTX-M-1 group, primers were F469 (5′-CAGCTGGGAGACGAAACGTT-3′) and R532 (5′-CCGGAATGGCGGTGTTTA-3′) and the probe was S490 (5′ -6FAM-CGTCTCGACCGTACCGAGCCGAC-TAMRA-3′). For the CTX-M-9 group, primers were F446 (5′-GAGGCGTGACGGCTTTTG-3′) and R513 (5′-CGTAGGTTCAG TGCGATCCA-3′), and the probe was S470 (5′-6FAM-CGATCGGCGATGAGACGTTTCGT-TAMRA-3′). Duplicate real-time PCRs were run with the ABI Prism 7900 sequence detection system (Applied Biosystems, France) and Absolute QPCR ROX master mix (Thermo Scientific, France) in 25-μl reaction mixtures and under reaction conditions of 95°C for 15 min (enzyme activation), and 40 two-step cycles consisting of 95°C for 15 s and 60°C for 1 min. Primers concentrations were 400 nM and Taqman type probe concentration was 200 nM, 1-μl of T4 GP32 (MP Biomedicals, France) was added per reaction. Five microliters of template DNA was added per reaction. PCR targets were cloned in pCR II-TOPO plasmid and recombinant linearized plasmids were used as standards. Gene copy numbers were calculated by amplifying six serial dilutions of the standard (10^1–10^5 copies per reaction mixture) in parallel with the samples. The specificity of the CTX-M detection systems has been checked *in silico* using the BLASTN algorithm across the GenBank nucleotide database (NCBI website). The specificity of the CTX-M detection systems has also been validated by

Table 1 | Human clinical strains used to validate the CTX-M-1 group and CTX-M-9 group detection systems (clinical strains were collected between 2006 and 2007 at CHU Dijon, France).

Strain	Strain origin	*bla* gene (CTX-M Group)	Detection by CTX-M-1 group PCR	Detection by CTX-M-9 group PCR
E. coli 913SE	CHU Dijon, bacteriology	CTX-M-1 (1)	+++	–
E. coli 882SE	CHU Dijon, bacteriology	CTX-M-2 (2)	–	–
E. coli 886SE	CHU Dijon, bacteriology	CTX-M-3 (1)	+++	–
E. coli 803SE	CHU Dijon, bacteriology	CTX-M-9 (9)	–	+++
E. coli 912SE	CHU Dijon, bacteriology	CTX-M-14 (9)		+++
E. coli 902SE	CHU Dijon, bacteriology	CTX-M-15 (1)	+++	–
K. ascorbata 268SL	CHU Dijon, bacteriology	KLUA (2)	–	–
K. cryocrescens 254SL	CHU Dijon, bacteriology	KLUG-1 (8)	–	–

E. coli, Escherichia col; K. ascorbata, Kluyvera ascorbata; K. cryocrescens, Kluyvera cryocrescens.

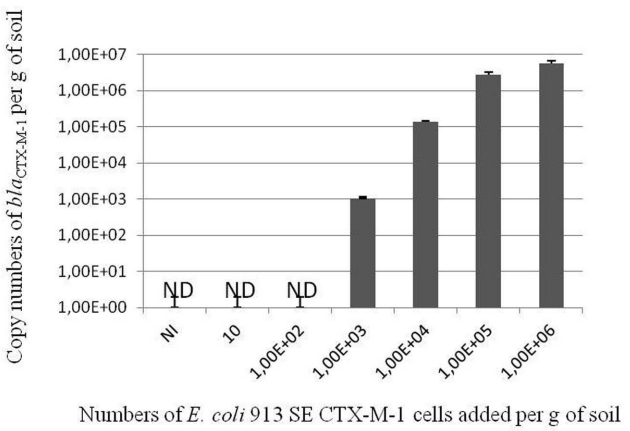


FIGURE 1 | Detection levels of $bla_{CTX-M-1}$ genes by real-time PCR (CTX-M-1 group detection system) in soil microcosms spiked with increasing amounts of *E. coli* strain 913SE CTX-M-1. ND, not detected. Results are the means of two replicates and bars represent standard deviations.

using human clinical isolates harboring known bla_{CTX-M} genes (**Table 1**). In order to determine the efficiency of these detection systems in soil, soil samples (2 *g*) were spiked with known titers of *E. coli* strain EC 913 SE carrying $bla_{CTX-M-1}$, soil DNA was extracted immediately as described above and subjected to real-time PCR using the primers and probe specific for CTX-M-1 group (**Figure 1**).

BACTERIOLOGICAL METHODS AND ANTIBIOTIC RESISTANCE TESTING

All fecal samples from the cows and all environmental samples have been inoculated on ESBL screening agar plates containing Drigalsky medium supplemented with either cefotaxime (4 mg/l) or ceftazidime (4 mg/l). The antibiotic susceptibility testings have been performed by the disk diffusion method. A range of antibiotics including penicillins, cephalosporins, carbapenem (imipenem), aminoglycosides (kanamycin, tobramycin, gentamycin, streptomycin, amikacin, netilmicin), chloramphenicol, quinolones (ciprofloxacin, ofloxacin), doxycycline, cotrimoxazol, and colistin was used to determine antibiotic susceptibility patterns of the isolates and the production of ESBL was assessed by the double-disk synergy test (Jarlier et al., 1988). Guidelines for the interpretation of antibiotic susceptibility testing were from the Clinical and Laboratory Standards Institute (CLSI, 2010).

IDENTIFICATION OF ESBL TYPES

The characterization of the *bla* gene was performed by PCR on isolates with a positive double-disk synergy test by using primers specific for the genes encoding ESBL from TEM, SHV, and CTX-M families (Chanal et al., 1992; Neuwirth et al., 1995; Sabate et al., 2002). PCR products were sequenced on both strands using ABI PRISM 3100 (Applied Biosystems, France).

GENOTYPING BY THE DIVERSILAB SYSTEM (BIOMÉRIEUX)

The Diversilab system is a typing technique which is based on the repetitive-sequence-based PCR (rep-PCR) and proved to be a useful method for genotyping of *E. coli* (Fluit et al., 2010). All reagents, automates and software used for this study were provided by bioMérieux, France. DNA was extracted from *E. coli* colonies using an UltraClean Microbial DNA isolation kit and following the manufacturer's instructions. The extracted DNA was amplified using a DiversiLab *Escherichia* DNA fingerprinting kit. Electrophoresis of the amplified fragments using a microfluidics LabChip with an Agilent 2100 Bioanalyzer and analysis were performed according to the protocol of the manufacturer. Isolates with a similarity of <95% were considered different, and isolates with a similarity of >98% were considered indistinguishable.

MLST TYPING OF *E. COLI* STRAINS

MLST typing of *E. coli* strains was done according to recommendations found at http://mlst.ucc.ie/mlst/dbs/Ecoli/documents/primersColi_html. The *E. coli* MLST scheme uses internal fragments of the following seven house-keeping genes: *adk* (adenylate kinase), *fum*C (fumarate hydratase), *gyr*B (DNA gyrase), *icd* (isocitrate/isopropylmalate dehydrogenase), *mdh* (malate dehydrogenase), *pur*A (adenylosuccinate dehydrogenase), *rec*A (ATP/GTP binding motif). MLST typing was done at the Institute of Microbiology and Epizootics, Freie Universität Berlin, Berlin, Germany, by Dr. Sebastian Guenther.

RESULTS

bla_{CTX-M} DETECTION IN SOILS

The method of real-time PCR detection of bla_{CTX-M} has been validated on the basis of amplification of DNA from pure strains or from inoculated soil DNA. The two detection systems proved to be sensitive and specific of bla_{CTX-M} genes encoding group 1 and group 9 enzymes. Detection threshold was estimated to be one copy per PCR reaction. Detection limit in soil samples was estimated to be 10^3 copies per gram of soil (**Figure 1**). Out of the 120 soil extracted DNA's from Burgundy, 22 were found to be positive using one or two detection systems (**Figure 2**). The proportion of positive soil for occurrence of $bla_{CTX-M\ 1\ or\ 9}$ was of 3.3% in Yonne, 6.2% in Côte d'Or, 16.0% in Nièvre, and 24.2% in Saône et Loire. These results demonstrate the wide distribution of some bla_{CTX-M} genes in soils from Burgundy. No obvious correlation was found between soil physical and chemical properties and the occurrence of bla_{CTX-M} genes [analysis by principal component analysis (PCA), data not shown]. The causes of the higher prevalence of bla_{CTX-M} genes in soils from Saône et Loire remain to be deciphered.

DETECTION OF ESBL-PRODUCING *E. COLI* FROM LIVESTOCK

Out of the 271 feces samples analyzed in 2009, 13 proved to contain ESBL-producing *E. coli* (**Table 2**). Five isolates harbored the TEM-71-ESBL. Interestingly all these isolates were originated from "Nièvre." Four isolates were genotypically indistinguishable. Among them isolates 32 and 101 were originated from two farms located in the same village. The others (92 and 107) were recovered in animals from farms located 16 km apart. The eight remaining isolates harbored CTX-M-1. The CTX-M-1 producing isolates have been isolated in three departments (Nièvre, Saône et Loire, and Côte d'Or). The genotypical analysis performed by rep-PCR with Diversilab revealed a wide diversity among the strains isolated from different farms. However, the strains 234 and 235 originated from two animals sampled in a same farm (farm 2) were clonally related (not distinguishable).

One year later (July 2010), feces samples of the whole cow herd from farm 2 were analyzed (90 animals), and CTX-M-1 producing *E.coli* isolates were detected from two animals. Strains V71 and V9 were genotyped and were different from the strains 234 and 235 isolated in June 2009 (**Table 2**).

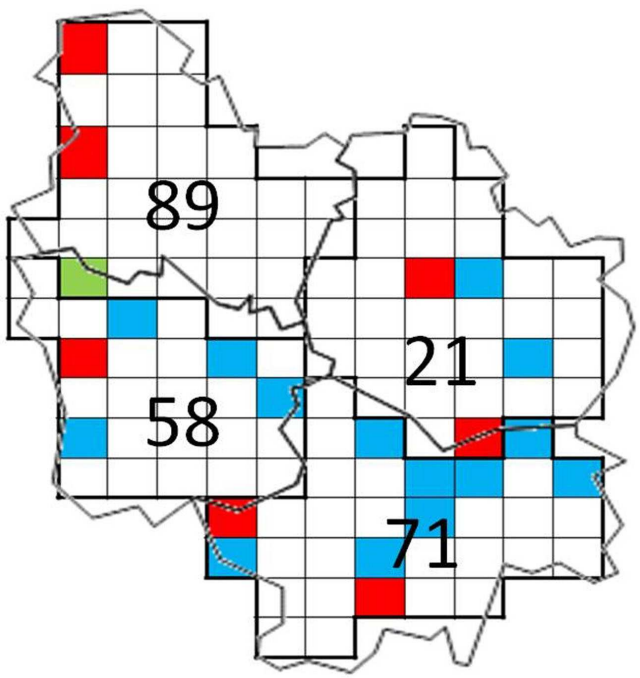


FIGURE 2 | Distribution of RMQS soils where bla_{CTX-M} genes were detected (colored squares) in the four departments of Burgundy (21: Côte d'Or, 58: Nièvre, 89: Yonne, and 71: Saône et Loire). Red square: detection of $bla_{CTX-M-1}$, blue square: detection of $bla_{CTX-M-9}$, green square: detection of both gene types.

DETECTION OF ESBL *E. COLI* ISOLATES IN ENVIRONMENTAL SAMPLES

The environments of three farms where positive animals have been detected were chosen for further investigations. In the farm 1, no ESBL-producing *E. coli* were found in the farm environment. On the contrary, 4 and 6 CTX-M-1 producing *E. coli* were detected in farms 2 and 3 respectively (**Table 2**). In farm 2, a cultivated soil amended one year before with liquid cow manure from the farm was found to contain CTX-M harboring *E. coli* strains. These isolates were genotypically indistinguishable from the animal strains F2/CO/234 and F2/CO/235. Interestingly the soil sampling site was located 3 km away from the cattle barn. In farm 3 the positive samples were: pasture soil, and composted manure. In that case, composted manure isolate was identical to the animal isolate, whereas one soil isolate had a different genotype.

DISCUSSION

The worldwide emergence of CTX-M producing *E. coli* in human clinical samples is a public health concern (Pitout and Laupland, 2008) and raises several interrogations regarding their high dissemination rate. These strains are also widely described in animals (pets, farm animals; Costa et al., 2009; Bortolaia et al., 2010; Cortes et al., 2010). It can be hypothesized that there is a cross-transmission between the human being and the animals (Leverstein-Van Hall et al., 2011), or that there are common environmental sources leading to human and animal contaminations. The treatment regimes for eradication of infections caused by such strains are sometimes very limited, i.e., when the CTX-M production is associated with the production of aminoglycosides modifying enzymes and gyrase mutations (fluoroquinolones resistance). We report in this study our findings concerning the prevalence of fecal carriage of *E. coli* CTX-M producing in cattle as well as the contamination of the farm environment.

DETECTION OF ESBL-PRODUCING *E. COLI* IN LIVESTOCK

We have detected ESBL-producing *E. coli* in livestock (5% of the animals tested). In Europe such strains have been only sporadically described in cattle: in Germany (Guerra et al., 2007), England (Horton et al., 2011), and Spain (Brinas et al., 2005). To our best knowledge this is the second report of ESBL-producing *E. coli* in cattle in France where Meunier et al. (2006) reported three isolates (carrying either CTX-M-1 or CTX-M-15 ESBL) responsible for infections in cow. Our results demonstrate that ESBL-producing *E.*

Table 2 | Characteristics of the *E. coli* strains isolated from animals, manure and soils.

Strain designation[a], farm number/department/ isolate number	Origin[b]	Type of ESBL	Rep-PCR genotype (Diversilab)	Associated non-beta-lactam antibiotic resistance[c]	ST[d]
F1/SL/250	Animal EDC	CTX-M-1	E	CIP-G	
F2/CO/234	Animal H	CTX-M-1	A	SXT	
F2/CO/235	Animal H	CTX-M-1	A	SXT	2497
F2/CO/RET12	Cultivated soil	CTX-M-1	A	SXT	155
F2/CO/RET15	Cultivated soil	CTX-M-1	A	SXT	
F2/CO/RET20	Cultivated soil	CTX-M-1	A	SXT	155
F2/CO/RET21	Cultivated soil	CTX-M-1	A	SXT	155
F2/CO/V9 (2010)	Animal H	CTX-M-1	F	SXT	
F2/CO/V71 (2010)	Animal H	CTX-M-1	G	SXT	
F3/CO/241	Animal H	CTX-M-1	B	SXT	2498
F3/CO/RET23	Pasture soil	CTX-M-1	H	CIP-Cm-SXT	58
F3/CO/RET24	Pasture soil	CTX-M-1	B	SXT	58
F3/CO/RET25	Pasture soil	CTX-M-1	B	SXT	58
F3/CO/RET26	Pasture soil	CTX-M-1	B	SXT	58
F3/CO/RET27	Composted manure	CTX-M-1	B	SXT	2498
F3/CO/RET28	Composted manure	CTX-M-1	B	SXT	
F3/CO/RET29	Pasture soil	CTX-M-1	B	Cm-SXT	2499
F4/N/32	Animal HLEDC	TEM-71	C	CIP-G-Cm-SXT	
F5/N/87	Animal H	CTX-M-1	I	CIP-G-Cm-SXT	
F6/N/92	Animal EDC	TEM-71	C	CIP-G-Cm-SXT	
F7/N/101	Animal HLEDC	TEM-71	C	CIP-G-Cm-SXT	178
F8/N/105	Animal H	TEM-71	J	CIP-G-Cm-SXT	
F9/N/107	Animal H	TEM-71	C	CIP-G-Cm-SXT	
F10/SL/190	Animal EDC	CTX-M-1	K	CIP-Cm-SXT	
F11/CO/240	Animal H	CTX-M-1	D	SXT	
F12/CO/245	Animal H	CTX-M-1	D	SXT	

[a] *SL, department of Saône et Loire; CO, department of Côte d'Or; N, department of Nièvre. For farms 2 and 3, isolates from cattle and from environment were listed together to allow strain comparison. In farm 2, the two isolates isolated in 2010 are indicated.*

[b] *Strains were isolated from animal feces, manure or soil. H, healthy animal; EDC, enteritic disease calf; HLEDC, healthy animal linked with an enteritic disease calf.*

[c] *Associated antibiotic resistances: CIP, ciprofloxacin; G, gentamicin; Cm, chloramphenicol; SXT, Cotrimoxazol.*

[d] *ST, sequence type from MLST analysis as defined at University College Cork (UCC, Ireland) http://mlst.ucc.ie/mlst/dbs/Ecoli/GetTableInfo_html*

coli strain are carried indifferently by sick and healthy animals. Our results are in agreement with previously reported data: CTX-M-1 is the ESBL which is the most frequently encountered in animals (Girlich et al., 2007; Bonnedahl et al., 2009; Bortolaia et al., 2010).

Nevertheless we have detected for the first time *E. coli* TEM-71-producing in five animals whereas they have been exceptionally reported in human infection (Wong-Beringer et al., 2001; Rasheed et al., 2002; De Champs et al., 2004). This finding is interesting from the epidemiological point of view because four isolates are genotypically related whereas they were originated from different farms (F4, F6, F7, and F9) This indicates that some clonal isolates are widespread or might circulate (through animal transfer) at a regional scale or that there is a common reservoir in the region. At the farm level, this might suggest a potential cross-contamination among cattle or the presence of a reservoir within the farm. Finally, this is the first description of TEM-71 producing *E. coli* in animals. It is noteworthy that the TEM-71 producing isolates are multiresistant to ciprofloxacin, gentamicin, chloramphenicol, and cotrimoxazol. On the opposite, most of the CTX-M-1 producing isolates harbor a single associated resistance to cotrimoxazol (**Table 2**). Therefore, cefotaxim resistant *E. coli* recovered from animals originated from different farms show significantly different antibiotic resistance patterns. This might reflect different exposition of the animals to antimicrobial agents or different sources of contamination.

In farm 2, strains isolated from cattle in 2010 have a different genotype compared to those isolated in 2009. Several hypothesis might explain this result: (i) horizontal gene transfer may occur between *E. coli* genotypes carried by cattle, since $bla_{\text{CTX-M}}$ genes are carried by plasmids, (ii) new exogenous *E. coli* strains may have been disseminated in the farm environment.

DETECTION OF ESBL-PRODUCING *E. COLI* IN FARM ENVIRONMENT

Among the 12 farms where ESBL-producing *E. coli* were detected from animals, we chose three farms to further investigate the dissemination of these strains in the farm environment. Farms were chosen on the basis of which farmers will decide to cooperate to our study.

In two of the three farms where positive animals were reported, ESBL *E. coli* isolates were detected in environmental samples.

Interestingly, in farm 2 one of the soil sampling sites was a crop field that has been amended 1 year before (autumn 2008) with liquid manure collected in the barn. This crop field was located 3 km away from the cattle barn. In autumn 2009, we were able to isolate several CTX-M producing *E. coli* strains from this soil, indicating that such strains have the ability to survive at least 1 year after soil amendment under environmental conditions. In farm 3, the positive samples were: pasture soil, and composted manure. In that case, composted manure isolate was identical to the animal isolate, whereas one soil isolate had a different genotype.

FURTHER GENOTYPIC COMPARISON OF *E. COLI* STRAINS

MLST typing of *E. coli* strains isolated from this study has been partially done. Preliminary results indicate that some *E. coli* strains isolated from animal and soil belong to previously described ST58, ST155, and ST178 as defined in the MLST database hosted at UCC[2]. Four strains appear to belong to new genotypes namely ST2497, ST2498, and ST2499. $bla_{\text{CTX-M-1}}$ genes were found in *E. coli* isolates with variable ST. ST8 and ST155 grouped strains from animal and human origin, some strains belonging to these ST are pathogenic for human or animals. Plasmids of four strains were sequenced (454 pyrosequencing), $bla_{\text{CTX-M}}$ genes were found on plasmids and located close to one copy of ISEcp1 in each strain (data not shown).

MOLECULAR DETECTION OF $bla_{\text{CTX-M}}$ GENES IN SOILS FROM BURGUNDY

The origin of the $bla_{\text{CTX-M}}$ genes detected in soils remains to be elucidated, several hypotheses might be investigated. *E. coli* strains harboring $bla_{\text{CTX-M}}$ genes might have been disseminated in soils through manure application on cultivated soils (animal origin), sewage sludge application (human origin), or through irrigation with treated or untreated waste-water. As the progenitor $bla_{\text{CTX-M}}$ gene is the chromosomal *bla* genes of different species of *Kluyvera* (Decousser et al., 2001; Poirel et al., 2002) we can also hypothesize that *bla* genes detected in the studied soils might be harbored by bacterial species other than *E. coli*. These bacteria might thus act as potential environmental *bla* genes reservoirs. To our best knowledge, this is the first report about occurrence of $bla_{\text{CTX-M}}$ genes in soil.

The detection of CTX-M producing *E. coli* in soil was the major finding of this study. Our work demonstrated the survival of CTX-M producing *E. coli* in soil at least for one year. Future work will aim at linking $bla_{\text{CTX-M}}$ occurrence with the capacity of *E. coli* strains to survive under environmental conditions. Finally, potential risks for public health through water or vegetables contamination will have to be determined.

[2]http://mlst.ucc.ie/mlst/dbs/Ecoli/GetTableInfo_html

ACKNOWLEDGMENTS

This work was co-funded by the Conseil Régional de Bourgogne (Regional Council of Burgundy), and by MERIAL SA. We wish to thank the Groupement Technique Vétérinaire (GTV) of Saône et Loire for animal feces sampling. Finally, we wish to thank Dr. Sebastian Guenther (Institute of Microbiology and Epizootics, Freie Universität Berlin, Berlin, Germany) for his invaluable help in MLST typing of the *E. coli* strains. The authors wish to thank the GenoSol Platform (particularly M. Lelièvre and S. Dequiedt) of UMR Agroecology, for supplying soil DNA extracts from Burgundy and for data-mining.

REFERENCES

Arpin, C., Quentin, C., Grobost, F., Cambau, E., Robert, J., Dubois, V., Coulange, L., and Andre, C. (2009). Nationwide survey of extended-spectrum beta-lactamase-producing Enterobacteriaceae in the French community setting. *J. Antimicrob. Chemother.* 63, 1205–1214.

Arrouays, D., Jolivet, C., Boulonne, L., Bodineau, G., Saby, N., and Grolleau, E. (2002). A new initiative in France: a multi-institutional soil quality monitoring network. Une initiative nouvelle en France: la mise en place d'un reseau multi-institutionnel de mesure de la qualite des sols (RMQS). *C. R. Acad. Agric. Fr.* 88, 93–103.

Bonnedahl, J., Drobni, M., Gauthier-Clerc, M., Hernandez, J., Granholm, S., Kayser, Y., Melhus, A., Kahlmeter, G., Waldenstrom, J., Johansson, A., and Olsen, B. (2009). Dissemination of Escherichia coli with CTX-M type ESBL between humans and yellow-legged gulls in the south of France. *PLoS One* 4, e5958. doi:10.1371/journal.pone.0005958

Bonnet, R. (2004). Growing group of extended-spectrum beta-lactamases: the CTX-M enzymes. *Antimicrob. Agents Chemother.* 48, 1–14.

Bortolaia, V., Guardabassi, L., Trevisani, M., Bisgaard, M., Venturi, L., and Bojesen, A. M. (2010). High diversity of extended-spectrum beta-lactamases in *Escherichia coli* isolates from Italian broiler flocks. *Antimicrob. Agents Chemother.* 54, 1623–1626.

Brinas, L., Moreno, M. A., Teshager, T., Saenz, Y., Porrero, M. C., Dominguez, L., and Torres, C. (2005). Monitoring and characterization of extended-spectrum beta-lactamases in *Escherichia coli* strains from healthy and sick animals in Spain in 2003. *Antimicrob. Agents Chemother.* 49, 1262–1264.

Chanal, C., Poupart, M. C., Sirot, D., Labia, R., Sirot, J., and Cluzel, R. (1992). Nucleotide sequences of CAZ-2, CAZ-6, and CAZ-7 beta-lactamase genes. *Antimicrob. Agents Chemother.* 36, 1817–1820.

Clinical and Laboratory Standards Institute (CLSI). (2010). "Performance standards for antimicrobial susceptibility testing," in *Twentieth Informational Supplement M100-S20* (Wayne, PA: CLSI).

Cortes, P., Blanc, V., Mora, A., Dahbi, G., Blanco, J. E., Blanco, M., Lopez, C., Andreu, A., Navarro, F., Alonso, M. P., Bou, G., Blanco, J., and Llagostera, M. (2010). Isolation and characterization of potentially pathogenic antimicrobial-resistant *Escherichia coli* strains from chicken and pig farms in Spain. *Appl. Environ. Microbiol.* 76, 2799–2805.

Costa, D., Vinue, L., Poeta, P., Coelho, A. C., Matos, M., Saenz, Y., Somalo, S., Zarazaga, M., Rodrigues, J., and Torres, C. (2009). Prevalence of extended-spectrum beta-lactamase-producing *Escherichia coli* isolates in faecal samples of broilers. *Vet. Microbiol.* 138, 339–344.

De Champs, C., Chanal, C., Sirot, D., Baraduc, R., Romaszko, J. P., Bonnet, R., Plaidy, A., Boyer, M., Carroy, E., Gbadamassi, M. C., Laluque, S., Oules, O., Poupart, M. C., Villemain, M., and Sirot, J. (2004). Frequency and diversity of Class A extended-spectrum beta-lactamases in hospitals of the Auvergne, France: a 2 year prospective study. *J. Antimicrob. Chemother.* 54, 634–639.

Decousser, J. W., Poirel, L., and Nordmann, P. (2001). Characterization of a chromosomally encoded extended-spectrum class A beta-lactamase from *Kluyvera cryocrescens*. *Antimicrob. Agents Chemother.* 45, 3595–3598.

Fluit, A. C., Terlingen, A. M., Andriessen, L., Ikawaty, R., Van Mansfeld, R., Top, J., Stuart, J. W. C., Leverstein-Van Hall, M. A., and Boel, C. H. E. (2010). Evaluation of the DiversiLab system for detection of hospital outbreaks of infections by different bacterial species. *J. Clin. Microbiol.* 48, 3979–3989.

Girlich, D., Poirel, L., Carattoli, A., Kempf, I., Lartigue, M. F., Bertini, A., and Nordmann, P. (2007). Extended-spectrum beta-Lactamase CTX-M-1 in *Escherichia coli* isolates from healthy poultry in France. *Appl. Environ. Microbiol.* 73, 4681–4685.

Guerra, B., Avsaroglu, M. D., Junker, E., Schroeter, A., Beutin, L., and Helmuth, R. (2007). Detection and characterisation of ESBLs in German *Escherichia coli*, isolated from animal, foods, and human origin between 2001-2006. *Int. J. Antimicrob. Agents* 29, S270–S271.

Horton, R. A., Randall, L. P., Snary, E. L., Cockrem, H., Lotz, S., Wearing, H., Duncan, D., Rabie, A., Mclaren, I., Watson, E., La Ragione, R. M., and Coldham, N. G. (2011). Fecal carriage and shedding density of CTX-M extended-spectrum beta-lactamase-producing *Escherichia coli* in cattle, chickens, and pigs: Implications for environmental contamination and food production. *Appl. Environ. Microbiol.* 77, 3715–3719.

Jarlier, V., Nicolas, M. H., Fournier, G., and Philippon, A. (1988). Extended broad-spectrum beta-lactamases conferring transferable resistance to newer beta-lactam agents in Enterobacteriaceae – hospital prevalence and susceptibility patterns. *Rev. Infect. Dis.* 10, 867–878.

Leverstein-Van Hall, M. A., Dierikx, C. M., Stuart, J. C., Voets, G. M., Van Den Munckhof, M. P., Van Essen-Zandbergen, A., Platteel, T., Fluit, A. C., Van De Sande-Bruinsma, N., Scharinga, J., Bonten, M. J. M., and Mevius, D. J. (2011). Dutch patients, retail chicken meat and poultry share the same ESBL genes, plasmids and strains. *Clin. Microbiol. Infect.* 17, 873–880.

Meunier, D., Jouy, E., Lazizzera, C., Kobisch, M., and Madec, J. Y. (2006). CTX-M-1- and CTX-M-15-type beta-lactamases in clinical *Escherichia coli* isolates recovered from food-producing animals in France. *Int. J. Antimicrob. Agents* 28, 402–407.

Moodley, A., and Guardabassi, L. (2009). Transmission of IncN plasmids carrying bla(CTX-M-1) between commensal *Escherichia coli* in pigs and farm workers. *Antimicrob. Agents Chemother.* 53, 1709–1711.

Neuwirth, C., Siebor, E., Duez, J. M., Pechinot, A., and Kazmierczak, A. (1995). Imipenem resistance in clinical isolates of *Proteus mirabilis* associated with alterations in penicillin binding proteins. *J. Antimicrob. Chemother.* 36, 335–342.

Pitout, J. D. D., and Laupland, K. B. (2008). Extended-spectrum beta-lactamase-producing enterobacteriaceae: an emerging public-health concern. *Lancet Infect. Dis.* 8, 159–166.

Poirel, L., Kampfer, P., and Nordmann, P. (2002). Chromosome-encoded ambler class A beta-lactamase of *Kluyvera georgiana*, a probable progenitor of a subgroup of CTX-M extended-spectrum beta-lactamases. *Antimicrob. Agents Chemother.* 46, 4038–4040.

Ranjard, L., Lejon, D. P. H., Mougel, C., Schehrer, L., Merdinoglu, D., and Chaussod, R. (2003). Sampling strategy in molecular microbial ecology: influence of soil sample size on DNA fingerprinting analysis of fungal and bacterial communities. *Environ. Microbiol.* 5, 1111–1120.

Rasheed, J. K., Anderson, G. J., Queenan, A. M., Biddle, J. W., Oliver, A., Jacoby, G. A., Bush, K., and Tenover, F. C. (2002). TEM-71, a novel plasmid-encoded, extended-spectrum beta-lactamase produced by a clinical isolate of *Klebsiella pneumoniae*. *Antimicrob. Agents Chemother.* 46, 2000–2003.

Reinthaler, F. F., Feierl, G., Galler, H., Haas, D., Leitner, E., Mascher, F., Melkes, A., Posch, J., Winter, I., Zarfel, G., and Marth, E. (2010). ESBL-producing *E. coli* in Austrian sewage sludge. *Water Res.* 44, 1981–1985.

Sabate, M., Miro, E., Navarro, F., Verges, C., Aliaga, R., Mirelis, B., and Prats, G. (2002). beta-Lactamases involved in resistance to broad-spectrum cephalosporins in *Escherichia coli* and *Klebsiella* spp. clinical isolates collected between 1994 and 1996, in Barcelona (Spain). *J. Antimicrob. Chemother.* 49, 989–997.

Woerther, P. L., Angebault, C., Lescat, M., Ruppe, E., Skurnik, D., El Mniai, A., Clermont, O., Jacquier, H., Da Costa, A., Renard, M., Bettinger, R. M., Epelboin, L., Dupont, C., Guillemot, D., Rousset, F., Arlet, G., Denamur, E., Djossou, F., and Andremont, A. (2010). Emergence and dissemination of extended-spectrum beta-lactamase-producing *Escherichia coli* in the community: lessons from the study of a remote and controlled population. *J. Infect. Dis.* 202, 515–523.

Wong-Beringer, A., Hindler, J., Loeloff, M., Queenan, A. M., Lee, N., Pegues, D. A., Quinn, J. P., and Bush, K. (2001). Molecular correlation for the treatment outcomes in bloodstream infections caused by *Escherichia coli* and *Klebsiella pneumoniae* with reduced susceptibility to ceftazidime. *Clin. Infect. Dis.* 34, 135–146.

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 15 November 2011; accepted: 19 February 2012; published online: 09 March 2012.

Citation: Hartmann A, Locatelli A, Amoureux L, Depret G, Jolivet C, Gueneau E and Neuwirth C (2012) Occurrence of CTX-M producing Escherichia coli in soils, cattle, and farm environment in France (Burgundy region). Front. Microbio. ***3****:83. doi: 10.3389/fmicb.2012.00083*

This article was submitted to Frontiers in Antimicrobials, Resistance and Chemotherapy, a specialty of Frontiers in Microbiology.

frontiers in
MICROBIOLOGY

ORIGINAL RESEARCH ARTICLE
published: 22 March 2012
doi: 10.3389/fmicb.2012.00106

Increased levels of multiresistant bacteria and resistance genes after wastewater treatment and their dissemination into Lake Geneva, Switzerland

Nadine Czekalski[1]*, Tom Berthold[2], Serena Caucci[2], Andrea Egli[1] and Helmut Bürgmann[1]

[1] *Department of Surface Waters – Research and Management, Eawag: Swiss Federal Institute of Aquatic Science and Technology, Kastanienbaum, Switzerland*
[2] *Department of Environmental Microbiology, UFZ, Helmholtz Centre for Environmental Research, Leipzig, Germany*

Edited by:
Sylvie Nazaret, Centre National de la Recherche Scientifique, France

Reviewed by:
Chris Rensing, RTI International, USA
Julie Zilles, University of Illinois Urbana-Champaign, USA

****Correspondence:***
Nadine Czekalski, Department of Surface Waters – Research and Management, Eawag: Swiss Federal Institute of Aquatic Science and Technology, Seestrasse 79, 6047 Kastanienbaum, Switzerland.
e-mail: nadine.czekalski@eawag.ch

At present, very little is known about the fate and persistence of multiresistant bacteria (MRB) and their resistance genes in natural aquatic environments. Treated, but partly also untreated sewage of the city of Lausanne, Switzerland is discharged into Vidy Bay (Lake Geneva) resulting in high levels of contamination in this part of the lake. In the present work we have studied the prevalence of MRB and resistance genes in the wastewater stream of Lausanne. Samples from hospital and municipal raw sewage, treated effluent from Lausanne's wastewater treatment plant (WTP) as well as lake water and sediment samples obtained close to the WTP outlet pipe and a remote site close to a drinking water pump were evaluated for the prevalence of MRB. Selected isolates were identified (16S rRNA gene fragment sequencing) and characterized with regards to further resistances, resistance genes, and plasmids. Mostly, studies investigating this issue have relied on cultivation-based approaches. However, the limitations of these tools are well known, in particular for environmental microbial communities, and cultivation-independent molecular tools should be applied in parallel in order to take non-culturable organisms into account. Here we directly quantified the sulfonamide resistance genes *sul1* and *sul2* from environmental DNA extracts using TaqMan real-time quantitative PCR. Hospital sewage contained the highest load of MRB and antibiotic resistance genes (ARGs). Wastewater treatment reduced the total bacterial load up to 78% but evidence for selection of extremely multiresistant strains and accumulation of resistance genes was observed. Our data clearly indicated pollution of sediments with ARGs in the vicinity of the WTP outlet. The potential of lakes as reservoirs of MRB and potential risks are discussed.

Keywords: aquatic, environment, sewage, pollution, sediment, qPCR, antibiotics

INTRODUCTION

Antibiotic resistance has been the focus of research primarily in clinical settings (human and veterinary medicine), whereas the impact of environmental microbes as reservoirs of resistance factors and the impact of releasing antibiotic resistant bacteria (ARB) into the environment was not considered until quite recently. However, it is now known, that ARB and antibiotic resistance genes (ARGs) are ubiquitous in nature (Alonso et al., 2001; Martinez, 2008; Aminov, 2009) and can occur in high concentrations in clinical, industrial, and communal wastewater (Schwartz et al., 2003; Kümmerer, 2004; Volkmann et al., 2004) as well as in animal husbandry (Lanz et al., 2003; Perreten and Boerlin, 2003; Heuer et al., 2008). These environments frequently also contain elevated levels of antibiotics and other pharmaceuticals. Consequently they are considered to select for antibiotic resistance and to be important hot spots for horizontal gene transfer (HGT) of resistance genes, and thus sites of resistance evolution (Baquero et al., 2008; Kemper, 2008; Kümmerer, 2008). In addition, liquid and solids discharged from these environments have been described as major sources of ARB in surface waters (Young, 1993; Kümmerer, 2004; Blasco et al., 2009; Martinez, 2009) and soils (Sengelov et al., 2003; Heuer and Smalla, 2007; Heuer et al., 2008). There are two key concerns related to this continuous introduction of ARB and their resistance genes into the environment. The possible persistence and further dissemination of ARB in natural aquatic environments may contribute to the increase in infections with resistant pathogens. Secondly the dissemination of ARB and ARG may lead to an increase of the ARG pool in environmental bacteria – thus facilitating transfer of resistance into current and emerging pathogens.

Multiresistant bacteria (MRB) are assumed to emerge primarily from hospitals and other environments where high amounts of antibiotics are frequently used. Thus, an elevated occurrence of MRB in the environment is assumed to be a more appropriate incidence for anthropogenic pollution compared to strains carrying only a single resistance. In the culture-based part of the present study we directly selected for multiresistant strains by applying combinations of clinically relevant antibiotics representing seven different antibiotic classes. The antibiotics incorporated in our approach were chosen in order to include representatives of different modes of action (e.g., inhibition of folic acid synthesis as for sulfamethoxazole and trimethoprim, protein biosynthesis as

for clarithromycin, tetracycline, and streptomycin, cell wall synthesis as for ceftazidime, and DNA replication as for norfloxacin), different time spans of clinical deployment (e.g., sulfamethoxazole representing an "old" and norfloxacin, ceftazidime representing "newer" antibiotic classes) and different origins (either natural, e.g., streptomycin, semi- or fully synthetic, e.g., clarithromycin and norfloxacin, respectively). Further criteria were the global but also local clinical relevance of the substances as well as their recent detection as micropollutants at our study sites (Blanc, 2010; Bonvin et al., 2011). Preference was given to broad-spectrum antibiotics. The three antibiotic combinations applied in the present study represent "old" (sulfamethoxazole, trimethoprim, and streptomycin), "mixed-old and new" (clarithromycin, tetracycline), and "new" (norfloxacin, ceftazidime) classes. Furthermore, sulfamethoxazole and trimethoprim are mostly applied together in clinics, which led us to apply them in combination also in our study.

Switzerland is a country with comparatively low antibiotic consumption (lowest defined daily dose per 1000 inhabitants among European countries; Filippini et al., 2006). Monitoring programs for antibiotic resistance in clinical settings have been established only recently (NRP49, 2007). Several studies on the release of antibiotics and other micropollutants and their occurrence in sewage and natural environments have been conducted in Switzerland (Alder et al., 2001; Giger et al., 2003; McArdell et al., 2003; Escher et al., 2011). However, the exposure of Swiss lakes to ARB and ARG has so far not received much attention, which is also the case for lakes in general. Most of the studies conducted on antibiotic resistance in surface waters focused on rivers (Iwane et al., 2001; Pei et al., 2006; Li et al., 2009). However, the significance of lakes as environments providing drinking water, food, and recreational activities marks them as highly relevant study objects. Lakes might not only be exposed to pollution with ARB and ARG but might also function as natural resistance reservoirs: Jones et al. (1986) found elevated levels of ARB in Lake Windermere and even higher resistance prevalence in two upland tarns. Picao et al. (2008) detected plasmid-mediated quinolone resistance genes in *Aeromonas* species isolated from Lake Lugano, Switzerland. However, Auerbach et al. (2007) found only 1 out of 10 tetracycline resistance genes in lake samples. The low number of studies and somewhat conflicting information they provide shows that there is still a considerable knowledge gap on the function of lakes as resistance reservoirs, which needs to be addressed.

Lake Geneva is the largest fresh water reservoir of Western Europe but at the same time receives wastewaters from the surrounding cities. The largest wastewater treatment plant (WTP) belongs to the city of Lausanne. The discharge of sewage from this plant has led to heavy pollution of its receiving water, the Vidy Bay, which is considered the most contaminated part of the lake. Several studies have reported on pollution of the sediment in the bay with heavy metals (Loizeau et al., 2004), micropollutants (Bonvin et al., 2011), and fecal indicator bacteria (Haller et al., 2009b; Poté et al., 2009a) as well as changes to the sediment conditions such as increased organic matter content (Poté et al., 2008; Bravo et al., 2011). To the best of our knowledge no previous studies have been conducted on the input of ARB and ARG into Vidy Bay/Lake Geneva.

In the present study, we aimed to evaluate the occurrence of ARB and ARGs in the wastewater stream of Lausanne and the role of Lausanne's WTP. In particular, we wanted to evaluate whether the WTP acts as a barrier for MRB in the wastewater stream, or conversely, provides an environment for selection of MRB and horizontal transfer of resistance factors. Finally, the role of the lake as a potential reservoir of ARB and ARG is discussed. We combined application of culture-based and culture-independent methods to allow both: the identification of important key species that might carry and further disseminate antibiotic resistance into the aquatic environment, as well as a culture-independent description of the dynamics of ARGs in the various compartments of the Lausanne wastewater system and Lake Geneva.

MATERIALS AND METHODS

SAMPLING CAMPAIGNS AND STUDY SITE

Lake Geneva is located in the South Western part of Switzerland (**Figure 1**) and has a volume of 89 km^3, a surface area of 580 km^2 and a maximum depth of 310 m. The Vidy Bay, which accounts for 0.3% of the lake's total volume (Chèvre et al., 2011), is located at the northern shore of the lake, next to the city of Lausanne. Lausanne's WTP treats sewage from 214000 inhabitants, including wastewater from several health care centers. The largest one is the Centre Hospitalier Universitaire Vaudois (CHUV). The most important building in terms of capacity and antibiotic consumption is the main building which accounts for 71% of the CHUV sewage output. On average 410 $m^3\ day^{-1}$ of raw sewage are released from this building to the Lausanne municipal sewer system (Blanc, 2010). There is no pharmaceutical industry located in Lausanne, and intensive animal production is not prevalent in the vicinity. Hence it is assumed that the healthcare facilities are likely the main source of ARB. The WTP receives on average 107734 $m^3\ day^{-1}$ of wastewater and discharges 86631 $m^3\ day^{-1}$ of treated sewage (usually 1–3 $m^3\ s^{-1}$ up to 5–6 $m^3\ s^{-1}$) directly into Vidy Bay (Vioget et al., 2011). The discharge point is situated 700 m offshore at 30 m depth. During heavy rain events the capacity of the WTP is exceeded and untreated wastewaters are then discharged into the Bay. This has led to heavy pollution (Loizeau et al., 2004; Poté et al., 2009b). Only 3.2 km southwest from the WTP discharge point, Lausanne pumps lake water for its drinking water supply (St. Sulpice). Situated 1 km offshore and at 50 m depth the pump withdraws 100000 m^3 of fresh water per day and drinking water is prepared via sand filtration and chlorination.

Three sampling campaigns were conducted between February and April 2010 in order to sample all of the following sites once. (a) Wastewater samples were taken from the sewage pipe exiting the main building of the CHUV (March 8, ∼09:00 a.m., sample code: HOS) and (b) at the in- and outlet of the WTP of Lausanne (Feb 22, ∼12:00 a.m., WTPin/WTPout). Additionally, (c) lake water and sediment cores were sampled at two points in the Vidy Bay: directly at the outlet of the wastewater discharge pipe (April 7, 12:00 a.m., sample code: STEP, "Station d'épuration") and close to the end of the pipe supplying the drinking water pump (April 7, 04:15 p.m., DP) of St. Sulpice (**Figure 1**). All water samples were filled into ethanol-washed canisters after rinsing twice with sample water. One liter of wastewater was sampled using a repeatedly rinsed scoop and filled into the canisters. Lake water (5 l) and sediment

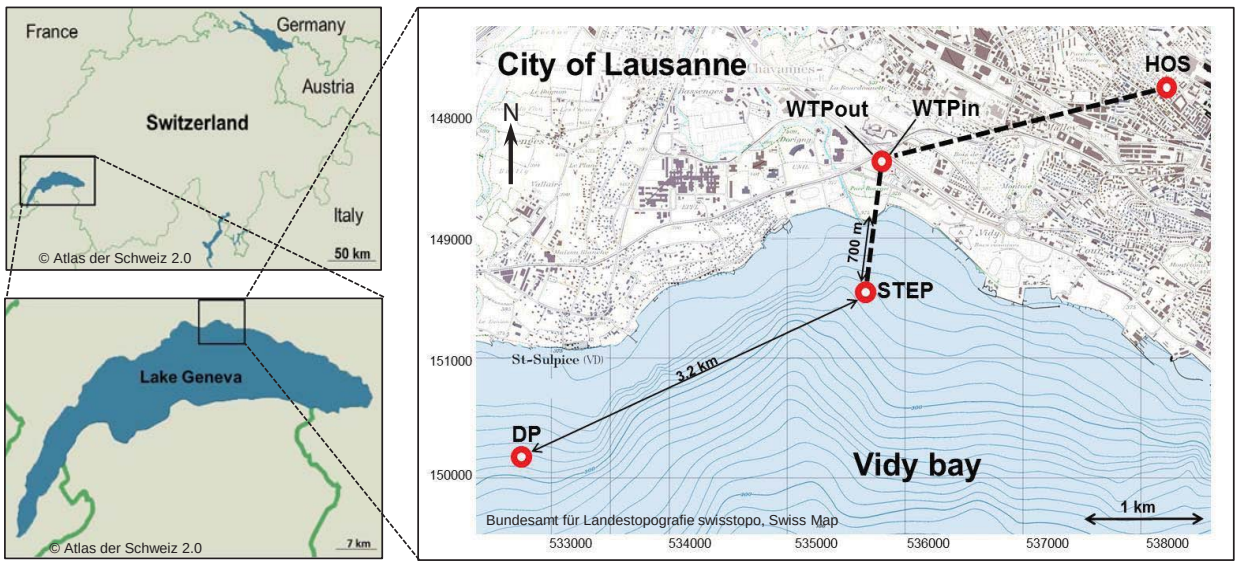


FIGURE 1 | Map of the study area and sampling sites. Effluents of Lausanne's wastewater treatment plant (WTP) are discharged via pipe 700 m off shore at 30 m depth (STEP, Station d'épuration, Swiss coordinates: 534672/ 151540). 3.2 km to the southwest, lake water is withdrawn for Lausanne's drinking water supply (DP, Swiss coordinates: 531748/150195). Water was sampled at all points, sediment was sampled at STEP and DP.

were sampled from the R/V La Licorne (Institute Forel, Geneva), equipped with a crane to which either a sediment corer (Uwitec, Austria) or a rosette sampling device (1018 Rosette Sampling System, General Oceanics Inc., FL, USA), consisting of eleven 1.7-l Niskin bottles, were attached. The rosette was coupled to a CTD device (OCEAN SEVEN 316 Plus CTD, IDRONAUT S.r.l., General Oceanics), which allowed for online monitoring of dissolved oxygen, temperature, pH, and conductivity. Lake water was sampled at 20 m depth (below the thermocline) subsequent to recording a CTD profile (**Figure A1** in Appendix). The maximum water depth at STEP and DP is 30 and 56 m, respectively.

Immediately after sampling, 45 ml aliquots of sample water were transferred to 50-ml polypropylene centrifuge tubes (Becton, Dickinson and Company, Sparks, NJ, USA) containing 5 ml of a 20% paraformaldehyde solution (final concentration 2%) in order to preserve cells for flow-cytometric counts of total bacteria (Troussellier et al., 1995). Samples were transported to the laboratory and stored at 4°C in the dark. Plating and filtration of water samples was carried out within 3–6 h of sampling. Sediment cores were stored for up to 1 week at 4°C in the dark before processing. Fixed samples for flow-cytometric counts were stored at −80°C and flow-cytometric analysis was carried out within 2–7 days.

ISOLATION AND QUANTIFICATION OF MULTIPLE ANTIBIOTIC RESISTANT BACTERIA

For total and resistant viable cell counts (colony forming units – CFU), samples were processed as follows: (a) *Lake water close to the DP*: 4 l were filtered through sterile 0.2-μm-pore-sized polycarbonate Isopore™membrane filters (147 mm in diameter, Millipore, Billerica, MA, USA). Filters were cut into small pieces and placed into 5–10 ml of sterile 0.05 M pyrophosphate (pp) solution (pH 8.5). Cells were suspended in pp by vigorous vortexing and saline solution was added to give a final volume of 40 ml and a 100-fold concentrated microbial suspension relative to the raw sample. (b) *Sediment cores*: In order to detach bacteria from particles of the sediment surface layer (0–3 cm depth) 3 g sediment were added to 30 ml pp and vortexed for 2 h (Lindahl, 1996; Amalfitano and Fazi, 2008). Particles were then allowed to settle for 18 min.

The concentrated DP microbial suspension from procedure (a) was applied directly as well as diluted 1–10. The suspensions of procedure (b) as well as raw HOS, WTPin, WTPout, and STEP samples were diluted from 1:10 up to $1:10^5$ in sterile 0.9% saline solution. Hundred microliters of each dilution were plated in triplicate on the following agar media cast in 9 cm diameter Petri dishes: nutrient agar (NAg) for copiotrophic bacterial counts, R2A for heterotrophs, phenylethyl alcohol (PEA) agar to select for Gram-positive bacteria, *Pseudomonas* isolation (PIA) agar to select for pseudomonads, and eosin methylene blue (EMB) agar to select for Gram-negative enterobacteria. All media (Difco™, Becton, Dickinson and Company, Sparks, NJ, USA) were supplemented with three combinations of antibiotics (Sigma-Aldrich, MO, USA) at inhibitory concentrations: (a) *"old" Sul/Trm/Str*: sulfamethoxazole/trimethoprim (Sul/Trm, 64 $\mu g\,ml^{-1}$, 8:1) and streptomycin (Str, 32 $\mu g\,ml^{-1}$), (b) *"new" Nor/Cef*: norfloxacin (Nor, 2 $\mu g\,ml^{-1}$)/ceftazidime (Cef, 32 $\mu g\,ml^{-1}$), and (c) *"mixed" Cla/Tet*: clarithromycin (Cla, 4 $\mu g\,ml^{-1}$)/tetracycline (Tet, 8 $\mu g\,ml^{-1}$). Combination (c) was not tested with EMB and PIA media that enrich for Gram-negative organisms, as clarithromycin mainly selects against Gram-positive bacteria. The concentration of each substance was applied according to DIN norms [German Institute for Standardization (DIN) which develops official standards for susceptibility testing of antimicrobials in Germany and Europe in close collaboration with European Committee on Antimicrobial Susceptibility Testing (EUCAST) similar to CLSI guidelines in the US; Deutsches Institut für Normung e. V. [DIN], 2004]. Three plates of each medium

without antibiotics served as positive controls and for determining total viable counts and one plate of each medium not receiving sample was incubated as a sterile control. In order to favor growth of bacteria which are adapted to lower temperatures, NAg and R2A plates were incubated at 25°C and evaluated for formation of colonies after 24, 48, and 72 h. PEA, PIA, and EMB plates were incubated at 37°C and evaluated after 24 and 48 h. All plates were incubated under aerobic conditions.

TOTAL BACTERIAL COUNTS FROM LAKE AND WASTEWATER SAMPLES

Total bacterial counts of all water samples were determined once per sample by flow-cytometry (FC) as follows: 1 ml of the fixed sample was diluted 100–1000 times (to give 1000–10000 events per second) and stained with 10 µl of diluted SYBR® Green I solution (Invitrogen, Basel, Switzerland, 1:100 dilution in anhydrous dimethyl sulfoxide). After 15 min of incubation at 20°C in the dark, samples were analyzed on an PARTEC-PAS-III CyFlow Space flow cytometer (Partec GmbH, Münster, Germany) according to Berney et al. (2008).

IDENTIFICATION OF MULTIPLE RESISTANT BACTERIAL ISOLATES FROM WASTEWATER BY 16S rDNA GENE FRAGMENT SEQUENCING

From R2A and EMB plates supplemented with Sul/Trm/Str approximately 25 colonies were picked from each of the following environments: HOS, WTPin, and WTPout. Antibiotic R2A and EMB plates inoculated with lake samples contained less than 25 colonies. For this reason, colonies from all media supplemented with Sul/Trm/Str were picked. For identification, we performed colony PCR amplification of bacterial 16S rDNA gene fragments. The final reaction volume of 100 µl contained 20 µl of 5× Colorless GoTaq® Flexi buffer (Promega, Madison, WI, USA), 3 mM $MgCl_2$, 0.2 µM of each of the general bacterial primers 27f and 1492r (**Table 1**), 0.2 µM dNTPs, 1 mg ml^{-1} bovine serum albumin, 0.025 u µl^{-1} of GoTaq® Flexi DNA polymerase (Promega) and material from a single colony picked with sterile inoculation needles. 10 ng of DNA from *E. coli* served as a positive control and 1 µl of nuclease-free water (Qiagen, Germany) served as negative control in all PCRs carried out in this study. PCR reactions were run on a Touchgene Gradient Thermal Cycler (Techne, Cambridge, UK) with the following temperature program: initial denaturation for 5 min at 94°C followed by 35 cycles consisting of 30 s at 94°C, 1 min at 55°C and 1.5 min at 72°C, final extension for 5 min at 72°C.

PCR products were digested overnight at 37°C with *Hha*I (Promega) for analysis of restriction fragment length polymorphism (RFLP) typing. The final reaction volume of 20 µl contained 2 µl of 10× multicore buffer, 0.2 µl of enzyme (2 u), 7.8 µl water and 10 µl of PCR product. Lambda phage DNA (Promega) served as positive control. RFLP digests were visualized by gel electrophoresis on 3% NuSieve Agarose gels (Cambrex Bio Science Rockland, Inc., ME, USA) and stained with GelRed™(Biotium, Inc., Hayward, CA, USA). Isolates were grouped into ribo-types according to identical restriction patterns and one representative

Table 1 | Primers and probes used in the present study.

Target name	Primer/probe name	Primer/probe sequence (5′–3′)	Size (bp)	*T* (°C)	Reference
Sul1	SulF1	CTGAACGATATCCAAGGATTYCC	245	50	Heuer and Smalla (2007)
	SulR1	AAAAATCCCATCCCCGGRTC	245	50	
Sul2	SulF2	CTCAATGATATTCGCGGTTTYCC	245	50	
	SulR2	AAAAACCCCATGCCGGGRTC	245	50	
Sul3	SulF3	ATTAATGATATTCAAGGTTTYCC	245	50	
	SulR3	AAGAAGCCCATACCCGGRTC	245	50	
Sul1	qSUL653f	CCGTTGGCCTTCCTGTAAAG			
	qSUL719r	TTGCCGATCGCGTGAAGT	67	60	
	tpSUL1	*FAM*-CAGCGAGCCTTGCGGCGG-*TAMRA*			
	qSUL2_595f	CGGCTGCGCTTCGATT			Modified from Heuer et al. (2008)
Sul2	qSUL2_654r	CGCGCGCAGAAAGGATT	60	60	
	tpSUL2_614	*FAM*-CGGTGCTTCTGTCTGTTTCGCGC-*TAMRA*			
16S rRNA	27f	AGAGTTTGATCMTGGCTCAG	1465	55	Lane (1991)
	1492r	GGCTACCTTGTTACGACTT			
16S rRNA	1369F	CGGTGAATACGTTCYCGG	124	56/60	Suzuki et al. (2000), Newcombe et al. (2005)
	1492R	GGWTACCTTGTTACGACT			
	TM1389F	CTTGTACACACCGCCCGTC			
IncP1$_{\alpha,\beta}$	trfA2 1	CGAAATTCRTRTGGGAGAAGTA	241	57	Gotz et al. (1996)
	trfA2 2	CGYTTGCAATGCACCAGGTC			
IncP7	rep1	CCCTATCTCACGATGCTGTA	524	52	Levchuk et al. (2006)
	rep2	GCACAAACGGTCGTCAG			
IncQ	oriT1	TTCGCGCTCGTTGTTCTTCGAGC	191	57	Gotz et al. (1996)
	oriT2	GCCGTTAGGCCAGTTTCTCG			
IncW	IncW_f	CCTAAGAACAACAAAGCCCCCG	242	64	Carattoli et al. (2005)
	IncW_r	GGTGCGCGGCATAGAACCGT			
IncN	IncN-f	GTCTAACGAGCTTACCGAAG	559	55	
	incN_r	GTTTCAACTCTGCCAAGTTC			

of each group was selected for sequencing. The respective PCR products were cleaned using the Wizard® SV Gel and PCR Clean-Up System (Promega) and quantified using a NanoDrop 1000 Spectrophotometer (Thermo Fisher Scientific Inc., Wilmington, USA). Unidirectional sequencing was carried out by Microsynth AG (Balgach, Switzerland) using the 27f primer. Sequences were analyzed via the BLASTn program (Altschul et al., 1990) and the Ribosomal Database Project (release 10) Classifier tool (Wang et al., 2007). All identified bacterial isolates from the wastewater compartments underwent analysis of minimal inhibition concentrations (MICs; provided subculturing succeeded).

DETERMINATION OF MINIMAL INHIBITION CONCENTRATIONS

Identified wastewater bacteria (HOS, WTPin, WTPout) isolated on R2A and EMB plates in the presence of Sul/Trm/Str were investigated for the MICs of various antibiotics using the Sensititre® broth dilution technique. As for the lake samples less than 25 colonies had formed on antibiotic supplemented plates, these were not included neither in MIC testing nor in plasmid and resistance gene detection (see below). Strains were grown on R2A and EMB agar plates. However, for several bacterial isolates subculturing on these media resulted in poor or even no growth for which reason also other media, including NAg, EMB, Columbia Agar with 5% sheep blood (LaboLife Sàrl, Pully, Switzerland) or Tryptic Soy Agar (Difco™) plates were used, according to the growth requirements of the different identified genera. All agar media were supplemented with Sul/Trm/Str as described above and strains were allowed to grow for 72 h at the temperatures which were used during isolation (25 or 37°C), and at 30°C for identified pseudomonads. One to four colonies per strain were picked (dependent on quantity of biomass formed) and transferred to 1 ml of sterile millipore water. After vortexing, 10 µl of the suspension were transferred to 11 ml of a liquid medium based on the agar medium used for growth [R2A broth, Mueller Hinton broth (MHB), nutrient broth (NB), brain heart infusion broth (BHB), tryptic soy broth (TSB); replacing solid media listed above in this order]. All media were purchased from Dicfo™, except R2A broth which was prepared according to Reasoner and Geldreich (1985).

Subsequently, Sensititre® 96 well susceptibility plates (TREK Diagnostic Systems, West Sussex, UK; plate formats applied in this study: EUMVS for Gram-negatives and NLM4 for Gram-positives) were inoculated with 50 µl culture medium per slot. A negative control was inoculated with sterile medium and a slot without antibiotic served as positive control. Plates were incubated at the appropriate temperature for each strain and evaluated for bacterial growth after 24 and 48 h. Plates were checked for biomass formation in each slot by measuring absorption at 570 nm using an Synergy™HT Multi Detection Microplate Reader (BioTek, Winooski, VT, USA) and by eye. Strains were classified as resistant to the tested substances based on the observed minimum inhibitory concentration according to Deutsches Institut für Normung e. V. [DIN] (2004) norms and European Committee on Antimicrobial Susceptibility Testing [EUCAST] (2011). As for florfenicol no resistance breakpoints are available, strains were classified as resistant according to breakpoints for the closely related chloramphenicol (CHL).

EXTRACTION AND REPLICON TYPING OF PLASMIDS FROM ISOLATED STRAINS

All Gram-negative wastewater bacteria referred to in the previous paragraphs were grown in 5 ml R2A broth, nutrient bouillon, LB, TSB, or BHB supplemented with 64 µg ml^{-1} Sul/Trm (8:1) and 32 µg ml^{-1} Str. The liquid cultures were shaken in 15-ml polypropylene tubes at 180 rpm and 25, 30, or 37°C for 24–48 h, depending on growth-preferences and growth rates of strains. Cells were harvested by centrifugation at 5000 rpm for 10 min at room temperature using an Eppendorf 5804R centrifuge with a swinging bucket rotor for 15-ml centrifuge tubes (Eppendorf, Hamburg, Germany). The supernatant was discarded and the pellets frozen at −20°C until plasmid extraction. Plasmid DNA was purified from frozen cell pellets using the PureYield™Plasmid Miniprep System (Promega) for Gram-negative bacteria according to the manufacturers' protocol. As positive controls we used *E. coli* J53 harboring the 33-kb-sized plasmid R388, which inherits the *sul1* gene for sulfonamide resistance. Liquid cultures of this strain were prepared in LB with 20 µg ml^{-1} trimethoprim. For Gram-positive strains we applied the PureYield™Plasmid Midiprep System (Promega) adapted according to Wegmüller and Schmid (2009) with a few modifications: in brief, cells were grown in 50 ml BHB for 20 h at 37°C and 180 rpm. *Bacillus subtilis* ssp. *subtilis* BD170 (DSMZ, Germany) carrying the 4.5-kb-sized plasmid pUB110 served as a positive control during extraction. Cell pellets were resuspended in 3 ml of the cell suspension solution supplied with the kit and incubated for 10 min at 37°C with 1 mg ml^{-1} lysozyme added (*B. subtilis* cells), or for 30 min at 37°C and 10 mg ml^{-1} of lysozyme and 1 ml of a 75% (w/v) saccharose solution (environmental isolates). Subsequently, cell lysis and neutralization was carried out according to the manufacturers guidelines, taking into account the recommended time to allow the formation of white flocculent before centrifugation of the lysate (Wegmüller and Schmid, 2009). Plasmid DNA was further purified following the Promega protocol and finally eluted in 400 µl of nuclease-free water.

Visualization of plasmid DNA was done by gel electrophoresis in 0.8% agarose gels at 50 V. Plasmid extracts showing several sharp bands were screened for the following Inc groups: IncP$_{1\alpha,\beta}$, IncP$_7$, IncN, IncW, and IncQ, using the primers and PCR conditions as described previously (**Table 1**). PCR products were screened by gel electrophoresis. Plasmid extracts were evaluated as positive for the tested Inc groups if the PCR product exhibited a band of the correct size.

DETECTION OF SULFONAMIDE RESISTANCE GENES IN SELECTED ISOLATES

All plasmid extracts were screened for the presence of the three sulfonamide resistance genes *sul1*, *sul2*, and *sul3* using a multiplex PCR assay as described previously (**Table 1**). The presence of *sul* genes was determined from detection of a PCR amplicon band of the expected size following agarose gel electrophoresis. A post-PCR identification of which *sul* type was present was not performed. As positive controls for *sul1*, *sul2*, and *sul3* served plasmids R388 (*E. coli* J53), RSF1010 (*E. coli* DH5α), and pVP440 (*E. coli* K12), respectively. The formation of PCR products of the correct size was evaluated via gel electrophoresis in 2% agarose gels.

DNA EXTRACTION FROM WATER FILTERS AND SEDIMENTS

Water samples were filtered using 5- and 0.2-μm pore-size polycarbonate membrane filters (Isopore™, Ø 147-mm, Millipore). Filters were cut into quarters, folded, packed into clean self-seal bags, and shock-frozen in liquid nitrogen for subsequent storage at −80°C until DNA extraction (3–5 months). One quarter of the filter from each sampled location was extracted as follows: lysis of bacterial cells was carried out by a two-step procedure: (a) bead-beating in GOS buffer (Hönerlage et al., 1995) for 40 s at 4 ms^{-1} with a FastPrep®-24 Instrument (Biomedicals Europe, Illkirch, France). For this step we used 0.2 g of a 1:1 mixture of 106 μm and 150–212 μm glass beads (Sigma-Aldrich). (b) Freezing in liquid nitrogen for 1 min and thawing at room temperature (rt). Cell debris was removed by centrifugation for 30 min at maximum speed (13000 rpm, Eppendorf Centrifuge 5415R) at rt. The supernatant was treated for 30 min at 37°C with 10 mg ml^{-1} RNase A. DNA was extracted with 1/2 volume of phenol (pH 8, Sigma-Aldrich) and 1/2 volume of chloroform/isoamyl alcohol (24:1, CIA, both Sigma-Aldrich) followed by a second extraction with 1 volume of CIA. DNA was precipitated with 1 volume of isopropanol. After centrifugation at 4°C and maximum speed for 30 min the DNA pellet was rinsed with 70% cold ethanol and dissolved in 20–30 μl of sterile Tris–EDTA buffer.

Out of each sediment core, 0.5 g of surface layer material were transferred to sterile 2 ml screw-cap tubes (BRAND GmbH + Co KG, Wertheim, Germany) and stored at −80°C in 1.5 ml GOS buffer until DNA extraction. For cell lysis, a bead mixture as described above was added and after vortexing two times for 40 s each at 3000 rpm (maximum vortex velocity, Vortex Genie 2, Scientific Industries, Inc., NY, USA) the supernatant was extracted with 500 μl phenol (pH 8, Sigma-Aldrich), followed by extraction with 200 μl CIA and finally with 400 μl chloroform. Precipitation of DNA and subsequent steps were as described for the DNA extraction from filters. For both water filters and sediments one blank extract was prepared, containing only beads and chemicals, respectively one quarter of a clean filter.

QUANTIFICATION OF SULFONAMIDE RESISTANCE GENES AND BACTERIAL 16S rRNA GENE FRAGMENTS IN ENVIRONMENTAL DNA EXTRACTS

The sulfonamide resistance genes *sul1* and *sul2* and bacterial 16S rRNA gene fragments were quantified from environmental DNA extracts using quantitative real-time *Taq*man®-PCR. Reactions were run in volumes of 20 μl (*sul1* and *sul2*) and 30 μl (16S rRNA), containing 1× *Taq*Man® Fast Universal PCR-Master Mix (Applied Biosystems, Foster City, CA, USA) and 5 μl of DNA (dilutions of quantification standards or diluted (1:10 to $1:10^5$) DNA extracts of samples). Extraction blanks (see above) as well as PCR blanks containing 5 μl of nuclease-free water were run as negative controls. To check for the presence of PCR inhibitors, standard PCR amplification of bacterial 16S rRNA gene fragments was carried out as described above, using 1 μl of template at various dilutions. Dilutions revealing successful amplification according to agarose gel electrophoresis were then used for real-time PCR. Primers and probes for *sul1* and *sul2* genes (**Table 1**), as well as their corresponding concentrations for PCR were as previously described (Heuer and Smalla, 2007; Heuer et al., 2008). PCR conditions for *sul1* and *sul2* were as follows: 2 min activation at 50°C, initial denaturation for 10 min at 94°C and 40 cycles of denaturation at 95°C for 15 s and annealing at 60°C for 3 min. For quantification of 16S rRNA genes we used universal primers and probes described by Suzuki et al. (2000). Primer and probe concentrations as well as PCR conditions were applied according to Newcombe et al. (2005). All samples and standards were run in triplicate in 96 well plates on a 7500 Fast Real-Time PCR System (Applied Biosystems) and analyzed using the automated settings of the 7500 Fast System SDS Software (Applied Biosystems). Standard curves were prepared from serial dilutions of the plasmids serving as positive controls for *sul1* (R388) and *sul2* (RSF1010) and a pGEM®-T Easy plasmid containing the target fragment of the 16S rRNA gene of *E. coli*. (position 1369–1492 of the *E. coli* reference genome, 123 bp). Dilution series were prepared as recommended by the Applied Biosystems Tutorial "Creating Standard Curves with Genomic DNA or Plasmid DNA Templates for Use in Quantitative PCR." Efficiency (E) of each qPCR run was calculated from the slope of the standard curves according to the formula $E = 10^{(-1/\text{slope})} - 1$. The limit of quantification (LOQ) was determined by the most diluted standard in the standard curve that exhibited a standard deviation of Ct values of the triplicates smaller than 0.5 (Life Technologies Corporation, 2012).

STATISTICAL ANALYSIS

In order to determine significant differences of measurements in excess of the error of measurement, we performed pairwise t-tests in R, based on the triplicate measurements of either qPCR or plate counts. Correlation analyses for comparison of the outcome of different methods were performed using the data analysis tool in Microsoft Excel.

RESULTS

TOTAL BACTERIAL LOAD ALONG THE WASTEWATER FLOW LINE

We determined the total bacterial load in samples along the wastewater flow of Lausanne into the Vidy Bay, Lake Geneva using a culture-based (plate counts) and two culture-independent methods (total cell counts by FC and direct quantification of 16s rRNA gene fragments using quantitative real-time PCR). For 16S rRNA genes the PCR efficiency was calculated to be 109.94% and the LOQ was determined at 300 copies of the target per 5 μl.

All three methods show a decrease in total bacteria along the wastewater flow path from the urban catchment to the lake (**Figures 2** and **3A**). The two culture-independent methods, agreed very well in all analyzed water samples (correlation coefficient $r^2 = 0.94$, **Figure 3A**). On all of the five tested media, and for all tested samples, viable bacterial numbers determined by plate counts were at least two orders of magnitude lower than the bacterial numbers determined with the culture-independent approaches. The highest numbers were detected on the two general media R2A and NAg.

The highest bacterial load was determined in the HOS sample based on FC and 16S rRNA gene copy numbers ($p \leq 0.01$). Plate counts on the three selective media PIA ($p \leq 0.001$), PEA ($p \leq 0.01$), and EMB also revealed highest viable bacterial numbers in the HOS sample, although for EMB the difference was within the error of measurement. Both the general media R2A

and NAg revealed slightly higher viable bacterial concentrations in the WTPin compared to the HOS sample but the difference was not significant ($p \geq 0.05$).

The discrepancy between untreated and treated wastewater samples (WTPin and WTPout, respectively) was about 0.5–1 orders of magnitude for all three methods but for 16S rRNA gene copy numbers the difference was not significant. According to this data wastewater treatment reduced the total bacterial load is by 73% based on total viable counts (averaged over all five media), 78% according to FC and 42% according to 16S rRNA gene copy numbers, respectively.

Total viable counts in STEP lake water ranged from 10^1 to 10^4 CFU ml^{-1} but they were considerably lower (by 1–3 orders of magnitude) at the DP site (**Figure 2**). The trend of CFU-Total and FC counts along the flow path was mostly coherent, irrespective of the fact that the latter were generally 2–3 orders of magnitude higher. However, we observed an increasing divergence of the two methods in the lake samples: in contrast to the drop of bacterial plate counts from STEP to DP ($p \leq 0.05$ for all media, except for PEA), FC counts revealed a similar bacterial load in both lake water samples of 10^6 events ml^{-1} (compare **Figures 2** and **3A**). 16S rRNA gene copy numbers were even 1 order of magnitude lower for the STEP water sample compared to the DP sample by $p \leq 0.01$.

In sediment samples taken close to STEP total viable counts were approximately 10 times higher compared to DP sediments (**Figure 2**). Note that sediment viable counts per gram dry weight were at least 10 times higher compared to plate counts per milliliter of the overlying water columns at both lake sites. No qPCR quantification results for 16S rRNA genes are presented for sediments as we observed considerable inhibition for the method with these samples.

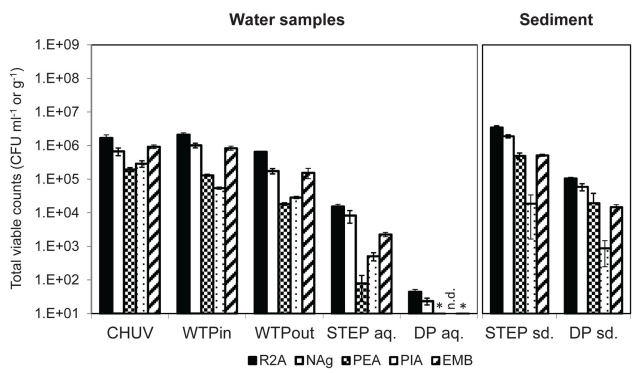


FIGURE 2 | Total viable counts, normalized to sample volume or mass, on agar media without antibiotics. An asterisk (∗) indicates less than 10 CFU ml^{-1}. n.d., no colonies detected. Error bars indicate standard deviation of triplicate platings of the same sample.

MULTIRESISTANT BACTERIA AND RESISTANCE GENES ALONG THE WASTEWATER FLOW LINE

As for the total bacterial load we also detected a decreasing trend for MRB determined by plate counts along the wastewater flow path from the urban catchment to the lake (HOS > WTPin > WTPout > STEP > DP, **Figures 4A–C**). Plate counts for "new" antibiotics (Nor/Cef) were comparatively low along the whole wastewater flow line on four out of five of the tested media. Solely on PEA plates which select for Gram-positive bacteria, Nor/Cef resistance occurred in the same range as the numbers determined for the remaining antibiotics at the respective sampling sites (**Figure 4C**).

The highest concentration of viable potentially MRB along the flow path was found in the HOS sample, regardless of the antibiotic combination or growth medium (**Figures 4A–C**). However, in case of Nag plates supplemented with Sul/Trm/Str and Cla/Tet counts on PEA plates the differences between HOS and WTPin samples were within the error of measurement ($p = 0.08$ for both, respectively).

Relative abundances of potentially MRB (i.e., MRB plate counts normalized to the total viable bacterial counts on the same medium) were mostly, but not exclusively, observed to be highest in HOS as well, ranging from 0.01 to 66% (**Figures 4D–F**). Besides the high values in HOS we also observed high relative abundance of Cla/Tet resistance on PEA plates in the WTPin sample (40%) and NAg plates showed high relative abundances for this antibiotic combination in the WTPout sample (21%). Both values were in the same range as the relative abundances determined for the HOS sample (36 and 21% for PEA and Nag, respectively; **Figure 4E**). For Nor/Cef resistance slightly elevated relative

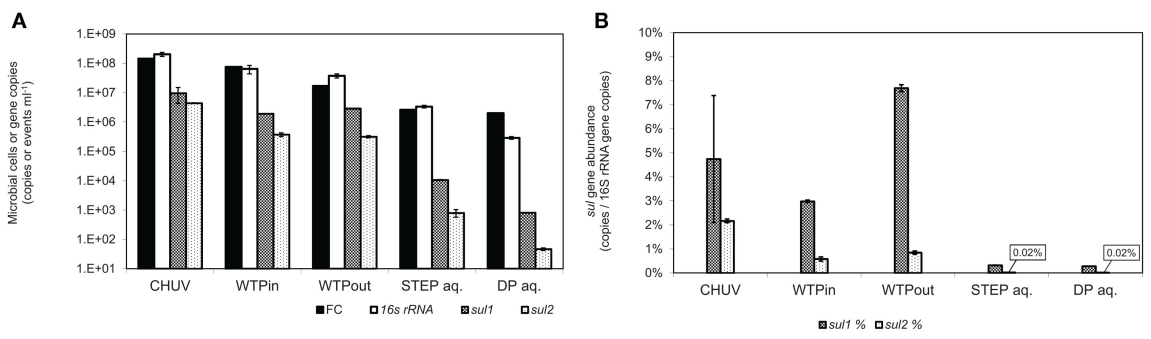


FIGURE 3 | (A) Total gene copy numbers and flow-cytometric cell counts, normalized to sample volume. **(B)** Relative abundance of sulfonamide resistance gene copy numbers normalized to 16S rRNA gene copies. Error bars indicate standard deviation from triplicate real-time PCR amplification of the same sample.

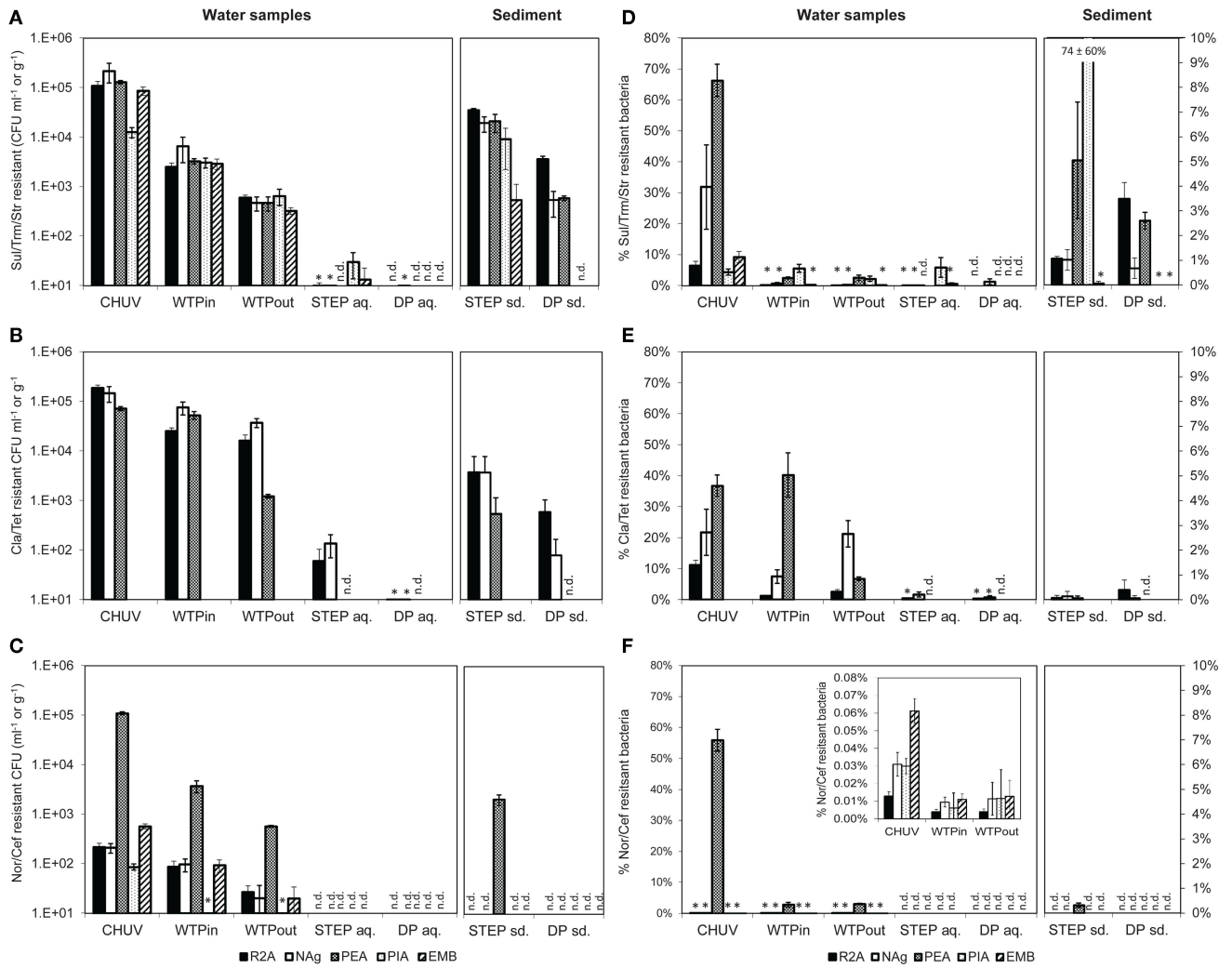


FIGURE 4 | (A–C) Total viable counts, normalized to sample volume or mass, in the presence of **(A)** Sul/Trm/Str, **(B)** Cla/Tet, and **(C)** Nor/Cef. **(D–F)** Abundance, relative to total viable counts without antibiotics, in the presence of **(D)** Sul/Trm/Str, **(E)** Cla/Tet, and **(F)** Nor/Cef. The insert in **(F)** shows abundance of selected samples at an adjusted scale. An asterisk (∗) indicates less than 10 CFU ml^{-1} in **(A–C)** and less than 1% relative abundance in **(D–F)**. n.d., no colonies detected. Error bars indicate standard deviation of triplicate platings of the same sample.

abundances were observed in WTPin and WTPout, but only for Gram-positives (PEA medium, 2.8 and 3.1%, respectively) whereas their abundance was low for the remaining media (less than 1%). Abundances of Sul/Trm/Str MRB were quite homogenous for all media and ranged from 0.01 to 5.6%.

Viable counts of potentially MRB were 0.5–1 orders of magnitude higher in the untreated versus the treated wastewater sample (WTPin and WTPout) which is similar to the trend observed for total viable counts. Calculated removal of MRB during wastewater treatment (averaged over all five media) was 62 ± 26, 78 ± 6, and 84 ± 6% for Cla/Tet, Nor/Cef (disregarding the much lower PIA counts), and Sul/Trm/Str MRB, respectively.

To some extent we noticed variations in removal efficiency among the different media and antibiotics. For instance, Cla/Tet resistant Gram-positives (PEA medium) seem to be more drastically reduced during wastewater treatment compared to other Cla/Tet resistant bacteria growing on R2A and NAg (**Figure 4B**). Nor/Cef resistant Gram-positives (PEA medium) were found at 10-fold higher number compared to viable bacteria on the remaining media, and appeared to retain this elevated number even after passing the WTP (**Figure 4C**). Although relative abundances of potentially MRB usually decreased from WTPin to WTPout, we also observed similar proportions in both samples, e.g., for Nor/Cef resistant Gram-positives (2.8 and 3.1%) and even increasing numbers, e.g., from 7.5 to 22% on NAg plates supplemented with Cla/Tet, respectively (**Figure 4E**).

The lake water generally exhibited low amounts of resistant bacteria. From STEP lake water samples we were able to isolate bacteria in the presence of Sul/Trm/Str (10^0 to 10^2 CFU ml^{-1}) and Cla/Tet (10^1 to 10^2 CFU ml^{-1}) but not in the presence of

Nor/Cef. At the DP site, hardly any bacteria were cultured from lake water in the presence of antibiotics, despite the additional sample concentration step (**Figures 4A–C**).

In sediment samples Nor/Cef resistant colonies were formed solely on PEA plates from STEP, while none were obtained from DP sediments. Generally, STEP sediments contained higher numbers of MRB than DP sediments (**Figure 4C**). Compared to the overlying water column, the concentration of MRB in sediment were 1–3 orders of magnitude higher. Relative abundances of MRB in both sediment and lake samples were mostly below 1% except for Sul/Trm/Str plates which, on different selected media, reached up to 5% of the respective total viable counts (**Figure 4D**).

Real-time PCR quantification of sulfonamide resistance genes *sul1* and *sul2* in wastewater and lake water DNA-extracts revealed the highest load of both traits in the HOS sample and a general decrease along the flow path (**Figure 3A**). This trend is in good agreement with the outcome of the culture-based approach. *sul1* gene copy numbers correlated with Sul/Trm/Str plate counts on R2A and NAg plates ($r^2 = 0.91$, for both media), and the correlation was even closer for *sul2* ($r^2 = 0.99$, for both media). However, copy numbers per milliliter of water or gram sediment (dwt) of both ARGs were higher by 1–4 orders of magnitude compared to the respective viable counts in all analyzed samples (compare **Figures 3A** and **4A**).

In contrast to the culture-based results, qPCR results revealed a twofold increased level of *sul1* ($p \leq 0.001$) and only a 15% lower level of *sul2* ($p = 0.3$) in the WTPout sample compared to the WTPin sample.

Normalized to the 16S rRNA gene, the *sul* genes occurred at abundances of 0.28 to 7.7 and 0.02 to 2.1% for *sul1* and *sul2*, respectively. The highest *sul1* abundance was observed in the WTPout sample followed by HOS (**Figure 3B**).

The abundance of *sul1* was found to be higher than *sul2* in all samples with increasing discrepancy from wastewater to the lake samples. In lake water, the relative abundance of the two resistance genes was considerably lower (<1%) than in wastewater samples.

qPCR efficiencies derived by the slopes of the standard curves for both *sul* genes were 93% for *sul1* and 102% for *sul2*. The LOQ for both standards was at 300 copies per 5 µl. All diluted samples amplified within the range of the standard curve, above the LOQ.

IMPORTANT GENERA IDENTIFIED IN WASTEWATER AND LAKE SAMPLES

In total, 163 isolates from HOS, WTPin, and WTPout obtained on Sul/Trm/Str supplemented R2A and EMB plates were identified to the genus level by RFLP typing and sequencing of the rRNA gene. Sequences that passed quality control and the chimera check (using Bellerophon, version 3; DeSantis et al., 2006) were submitted to GenBank and are available under accession numbers: JQ595461–JQ595555 (the FASTA file provided as supplementary material also includes the lower quality sequences). We identified 27 different genera (**Table 2**), 16 in HOS, 14 in WTPin, and 13 in WTPout. The majority were Gram-negative bacteria, but we also identified a few Gram-positives in HOS ($n = 6$) and WTPin ($n = 5$), mostly *Enterococci*. *Pseudomonads* were found to be most abundant among isolates from all three sites. *Enterococci* and *Brevundimonas* were quite common in HOS, but the latter was also detected in WTPout. *Escherichia/Shigella* and *Acidovorax* species dominated the WTPin isolates. *Escherichia/Shigella* was also isolated frequently from WTPout, as were *Sphingobacteria* (accounting for ∼25% of the WTPout isolates). Furthermore, 17 genera were identified among the lake water bacteria (both STEP and DP) grown in the presence of Sul/Trm/Str (**Table 2**). In sediment from STEP and DP, *Bacillus* and *Solibacillus*, respectively, were the most frequently isolated strains. A further dominant genus in STEP sediment was again *Brevundimonas*.

PREVALENCE OF HIGHLY AND EXTREMELY MULTIRESISTANT BACTERIA ALONG THE WASTEWATER FLOW PATH

Resistance of identified multiresistant wastewater isolates obtained from Sul/Trm/Str plates was confirmed by determining the MICs of 14 antibiotics. The results of this experiment are summarized in **Figure 5**. Resistance toward the antibiotics applied in the isolation plates (Sul/Trm/Str) was confirmed. This resistance pattern usually coincided with a high tolerance or resistance to ampicillin, which was present in 76 and 77% of HOS and WTPin isolates ($n = 42$ and 22, respectively) and 87% of WTPout isolates ($n = 45$). Resistance patterns toward the 11 remaining substances tested varied among the different wastewater environments: the number of antibiotics to which more than 50% of the tested strains were resistant was 8 for the HOS sample. Among WTPin isolates only resistances against CHL and nalidixic acid (NAL) were detected frequently (in 55 and 67% of the isolates, respectively). When comparing the proportions of bacteria resistant against specific antibiotics in untreated (WTPin) and treated (WTPout) wastewater, similar or higher proportions were found in WTPout for 9 out of the 11 antibiotics not used in isolation. In the cases of gentamicin (GEN) and colistin (COL) the ratio was more than twice as high for WTPout. Only the resistances to fluoroquinolones (NAL and ciprofloxacin, CIP) occurred more frequently in WTPin isolates.

We have furthermore evaluated the number of resistances per strain. Strains with resistance against more than six or eight antibiotics were labeled as highly and extremely multiresistant, respectively. When comparing the occurrence of highly and extremely MRB at the three sites (**Figure 6**), highest proportions were again detected in the HOS sample (86 and 77%, respectively) and an increased level in WTPout (82 and 60%, respectively) compared to the WTPin sample (71 and 33%, respectively). Thus, we observed an 11 and 27% higher abundance of highly and extremely multiresistant strains, respectively, in the WTPout sample compared to WTPin. The most frequently isolated genera of highly multiresistant species were *Pseudomonas* and *Escherichia/Shigella* which were present at all three locations. *Enterococcus* and *Brevundimonas* were present in HOS. While the former was otherwise only detected in WTPin, the latter was only found in WTPout. *Acinetobacter* was prevalent in both WTPin and WTPout and a very dominant and highly multiresistant genus in the WTPout sample was *Sphingobacterium*.

PREVALENCE OF PLASMIDS AND OF *sul* GENES IN SELECTED WASTEWATER ISOLATES

The extraction of plasmid DNA from 129 of the 169 selected wastewater isolates showed that 58, 77, and 46% of the strains isolated from HOS, WTPin, and WTPout were harboring plasmids. For

Table 2 | Genera of Sul/Trm/Str resistant bacteria isolated from HOS, WTPin, and WTPout according to partial 16S rRNA gene sequences and their numerical distribution in the different wastewater compartments.

Genus	HOS	WTPin	WTPout	STEP water	STEP sed	DP water	DP sed	Total
Achromobacter	2							2
Acidovorax		8					1	9
Acinetobacter		5	4		1			10
Aeromonas	1	1	1	6		2		11
Arthrobacter					1			1
Azospira			1					1
Bacillus					14		7	21
Bosea						3		3
Brevundimonas	8		4		9			21
Brochothrix		2						2
Chryseobacterium			2					2
Comamonas	2	2						4
Delftia	2							2
Enterococcus	5	3			1			9
Escherichia/Shigella	1	7	12					20
Flavobacterium	1	2						3
Lysinibacillus					1			1
Microbacterium					1			1
Phyllobacterium						3		3
Pigmentiphaga	2							2
Pseudomonas	15	11	7					33
Pseudorhodoferax	1							1
Rheinheimera							3	3
Rhizobium		3	1					4
Solibacillus				3	5		15	23
Sphingobacterium		1	14					15
Sphingobium	1							1
Sphingomonas	1							1
Sporosarcina					1		2	3
Stenotrophomonas		4	4		1			9
Tatumella	1							1
Thermohalobacter	1							1
Thermomonas			1					1
Tolumonas		1		1				2
Uruburella		1	1					2
Variovorax						5		5
Xanthobacter	3	1	1					5
Unidentified	5	2	4	5	10	1	9	36
n	52	54	57	15	45	14	37	274

many of the tested strains agarose gel electrophoresis of the plasmid DNA extract revealed multiple bands in the low kilobit size range, indicating multiple plasmids per strain. Replicon typing of 78 plasmid positive extracts revealed only a low number of specific amplifications for $IncP_{1\alpha,\beta}$ ($n = 3$), $IncP_7$ ($n = 8$), IncQ ($n = 17$), IncW ($n = 2$), and no amplicons were detected for IncN. Though the yield of positives for the tested Inc groups was quite low, the results nevertheless gave further indications for the presence of multiple plasmids (of replicon types IncQ and $IncP_7$) in three of the tested strains. All plasmid extracts, independent of their band pattern, were screened for presence or absence of sulfonamide resistance genes *sul1*, *sul2*, and *sul3*. *Sul* genes were amplified in 76 out of 129 plasmid extracts, accounting for 77, 40, and 52% of the investigated bacterial isolates from HOS, WTPin, and WTPout, respectively.

DISCUSSION

Our study focuses on a wastewater flow path in Lausanne, Switzerland. Switzerland is a country with comparatively low antibiotic consumption (Filippini et al., 2006). However, the impact of hospital effluents and wastewater treatment on the dissemination of resistant bacteria and resistance genes in the receiving (aquatic)

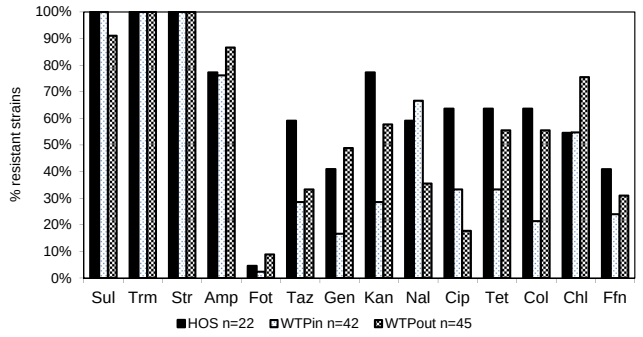


FIGURE 5 | Percentage of bacterial isolates from HOS, WTPin, and WTPout that were classified as resistant to the listed antibiotics.

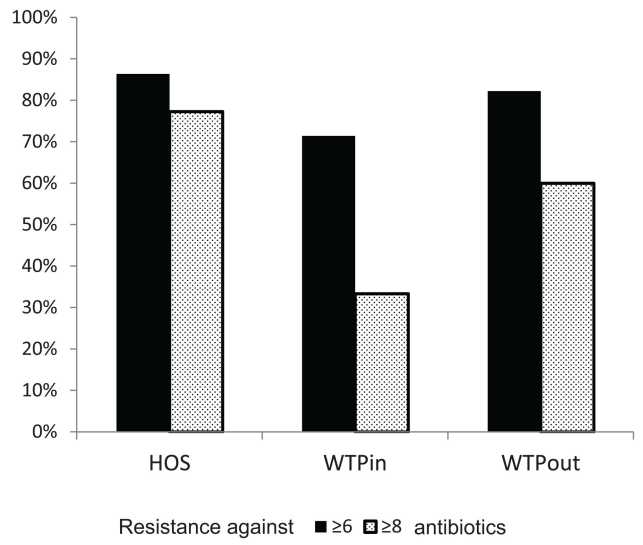


FIGURE 6 | Comparison of percentages of bacteria inheriting more than six ("highly multiresistant") and more than eight resistances ("extremely multiresistant") at the three sampling sites HOS, WTPin, and WTPout.

environments is of global relevance. Several studies have documented high incidence of antibiotic resistance in such wastewaters all over the world (Reinthaler et al., 2003; Kümmerer, 2004; Baquero et al., 2008; Martinez, 2009). Our results confirmed our expectation that compared to the other tested water sources hospital effluents are most strongly contaminated with MRB and ARG. These findings are in agreement with the assumption that MRB are mainly selected in clinical environments (Kümmerer, 2009). Nevertheless, the overall impact of discharging untreated hospital effluents into the sewer system is still under debate. On the one hand, it has been demonstrated that significantly higher proportions of resistant bacteria were isolated from influents of WTPs receiving hospital wastewaters than from influents only treating municipal wastewater (Reinthaler et al., 2003; Garcia et al., 2007). On the other hand, Guardabassi et al. (2002) could not detect significantly elevated levels of resistant *Acinetobacter* species in wastewater downstream of a hospital. Taking into account that, typically, hospital effluent accounts for less than 1% of the total sewage arriving at the WTP (Kümmerer, 2008), its impact on the receiving environment remains open. In Lausanne, hospital wastewater contributes to approximately 0.4% of the total sewage volume processed at the WTP (calculated from release data of the main building, which accounts for 71% of the "CHUV" (Blanc, 2010). The reduced level of resistance at the WTPin site compared to HOS, is therefore expected. However the high levels of MRB and ARGs observed in the WTP inflow in this study were still high, indicating that municipal wastewater is probably the bigger net source of resistances entering the WTP. Nonetheless, a separate treatment of hospital sewage with the goal of removing MRB (as well as the pharmaceutical load, see below) to prevent their mixing with communal effluents and the environment is desirable.

Our observations indicate that the WTP of Lausanne strongly reduces the total number of MRB. This could, however, be primarily an effect of the reduction of the total bacterial load in the WTP effluent as we did not universally observe a strong reduction of the relative abundance of MRB according to the plate count analysis. In one case (Cla/Tet) we even observed an increase. The culture-independent approach also indicated a less efficient removal, respectively increase, of *sul* resistance genes during passage of the WTP. This data would suggest that there may be some selection for resistance factors during passage of the WTP. Finally, our results showed increased proportions of highly and extremely MRB among the isolated Sul/Trm/Str resistant strains in the sample of treated wastewater compared to the WTP inlet sample. As the respective bacterial genera of these strains were partly also detected in raw sewage but partly belong to genera only found at the outlet, we assume that there is both a poor removal of certain extremely multiresistant strains (pseudomonads and *E. coli*) during treatment, as well as a positive selection on multiresistance in other taxa, e.g., *Brevundimonas* (only detected in hospital sewage and WTPout).

As *Sphingobacteria* were quite abundant among the isolated highly MRB in the WTP it would be interesting to further investigate their potential as key players in the accumulation and subsequent transfer of highly multiresistant genotypes into the lake. *Sphingobacteria* are members of the Cytophaga/Flavobacteria/Bacteroidetes (CFB) group which are well known as regular members of freshwater aquatic microbial communities (Zwart et al., 2002). Close relatives of *Sphingobacteria* have been found in freshwater lakes (Grossart et al., 2008). The high degree of resistance to antimicrobial agents of the group is well known (Holmes, 2006). Another important genus we identified that carried multiple resistance to the lake is *Brevundimonas* which is infrequently isolated from clinical settings (Han and Andrade, 2005) but is typically established in fresh water communities (Zwart et al., 2002) and drinking water (World Health Organization, 2003), which would indicate a potential of these MRB to become established in the receiving water. These organisms might thus play a role in preserving and disseminating clinical multiresistant determinants in lakes and even promote their return to clinical settings via drinking water.

The ambivalent role of WTPs has been discussed in several reviews (Kim and Aga, 2007; Jury et al., 2010, 2011) and previous studies. The evidence presented so far is however in the majority based on using culture-dependent approaches. Some of these studies observed a general decrease in the prevalence of resistance against antimicrobial agents (Iwane et al., 2001; Guardabassi et al., 2002; Vilanova et al., 2002; Garcia et al., 2007) but still stated that the removal efficiency was unsatisfactory (Reinthaler et al., 2003; Duong et al., 2008; Prado et al., 2008) which is in line with our own findings. Auerbach et al. (2007) quantified various tetracycline resistance genes in influent and effluent of several WTPs and observed a general decrease in *tet* gene prevalence after sewage treatment. However, others observed a significant increase in antibiotic resistance prevalence, and concluded a selective effect for resistance genes during wastewater treatment (Da Silva et al., 2006; Silva et al., 2006; Zhang et al., 2009). In summary, the picture that emerges is that at least some WTPs select for certain resistance genes and certain multiresistant species, whereas for other species, or under different conditions, a significant removal may be possible. The factors governing the efficiency of removal remain to be determined.

An important factor fostering the selective effect of WTPs is the occurrence of HGT, which can be mediated via mobile genetic elements such as plasmids (conjugation), bacteriophages (transduction), or by uptake of free DNA from the surrounding environment (transformation). In the extreme case these processes might transfer resistance factors from multiresistant pathogens to bacteria which are further disseminated into the receiving aquatic environment. If these organisms subsequently become established in this environment, they will increase the resistance pool in the environment in the long term, which may allow other pathogens to recruit new resistances more easily. It has often been suggested that WTPs might be hot spots for HGT due to the favorable conditions which prevail in this environment, such as high bacterial densities, high oxygen, and high nutrient concentrations (Kim and Aga, 2007). We found a high prevalence of plasmids, partly identified as broad-host range plasmids such as IncP, IncQ, and IncW, in the studied multiresistant wastewater isolates. Detection of *sul* genes in the plasmid fraction indicated that plasmids carried transferrable antibiotic resistance determinants. This provides some evidence that the occurrence of HGT events and associated spread of ARGs is likely in the WTP environment.

Resistance to important new antibiotic classes such as fluoroquinolones and new generation cephalosporins were in general less prevalent at all our test sites compared to resistances against older antibiotics, such as sulfonamides. A similar outcome of a screening we conducted in 2009 (data not shown) supports this conclusion. Only Gram-positive bacteria showed some degree of tolerance against these substances. However, it remains unclear whether this is due to intrinsic resistance or whether Gram-positives have developed resistance to fluoroquinolones and cephalosporins more successfully. Moreover, whereas for most of the tested substances we found an increased tolerance among selected bacterial isolates at the outlet of the WTP, we could not confirm this pattern for fluoroquinolones. Fluoroquinolone resistance can be mediated by chromosomal mutations that are thought to be transferred vertically and the plasmid-borne *qnr* genes, which have been described to occur in Gram-negative bacteria (Martínez-Martínez et al., 1998; Cattoir et al., 2007, 2008). We assume that plasmid-mediated resistance is still a less prevalent mechanism at our site compared to chromosomal mutations. Hence, resistance to fluoroquinolones might not be as easily transferable to other bacteria, even in presence of high CIP concentrations in the effluents (**Table 3**). For instance, among the extremely multiresistant and highly prevalent strains of *Sphingobactaria* isolated at the outlet, none showed elevated tolerance to fluoroquinolones. It will be interesting to observe whether these resistances become more prevalent in wastewater and environmental communities over the next decades.

The role of lakes as potential reservoirs of antibiotic resistance has been addressed only infrequently (Jones et al., 1986; Auerbach et al., 2007). Most comparable studies have focused on rivers (Castiglioni et al., 2008; Cummings et al., 2010). One goal of our study was to gain first insights on the impact of discharged wastewaters on the prevalence of MRB and ARGs in the receiving water body, the Vidy Bay of Lake Geneva. In this study we evaluated two sites, one directly at the WTP discharge point and a second site a few kilometers away, close to the drinking water pump of Lausanne. Our data indicate pollution of water and sediments with ARB and resistance genes close to the outlet of the WTP compared to the remote site. Both sites have been analyzed in preliminary experiments in 2009 (data not shown) which revealed a similar outcome as for the data presented here.

However, at both sites the sediments exhibited higher numbers of MRB, than the overlying water column. In the extreme case no MRB were isolated from the water column but isolates were always obtained from the sediments, as, e.g., for Nor/Cef resistant bacteria. As expected, the total bacterial counts were also higher in sediments. Thus, lake sediments appeared as sites where resistance traits persisted and accumulated. The potential of the heavily polluted (e.g., with heavy metals) sediments in Vidy Bay to preserve fecal indicator bacteria has been demonstrated in previous studies (Haller et al., 2009a,b; Poté et al., 2009b) and the impact of the WTP on contamination of the Vidy Bay and its bacterial community is a well-established fact (Pardos et al., 2004; Poté et al., 2008; Bravo et al., 2011; Haller et al., 2011; Thevenon et al., 2011). It is likely that selection and persistence of elevated levels of ARB and resistance genes in close proximity to the WTP outlet are favored, e.g., due to co-selection of antibiotic and heavy metal resistance (Wireman et al., 1997; Stepanauskas et al., 2006). The spatial distribution of

Table 3 | Concentrations of ciprofloxacin and sulfamethoxazole determined in raw hospital sewage released from the main building of the CHUV (HOS), at sampling points WTPin and WTPout of Lausanne's WTP (Blanc, 2010) as well as lake water above the outlet of the WTP discharge pipe (STEP) and a reference point (Bonvin et al., 2011), which is located southwest of STEP, 1.5 km from the DP (Swiss coordinates: 533048/150920).

	HOS (ng/l)	WTPin (ng/l)	WTPout (ng/l)	STEP (ng/l)	Ref point (ng/l)
Sulfamethoxazole	1116	248	61	4	0.4
Ciprofloxacin	18281	3259	807	6	0.9

resistances in the sediment is studied in more detail in ongoing research.

The question remains open whether the level of ARGs at the point close to the drinking water pump is also influenced by anthropogenic pollution (discharge of wastewaters) or represents typical natural levels of these genes in fresh water environments. Compared to rivers (Iwane et al., 2001; Vilanova et al., 2002; Li et al., 2009), addressing this question in lakes is not an easy task as transport of contaminated water masses and progress of dilution depends on constantly changing wind and temperature regimes, as well as bathymetric characteristics that determine currents and mixing. Tracer experiments releasing bacteriophages from the WTP outlet revealed their transport to within 1.5 km of the drinking water pump within 5–6 h during holomixing in winter time (Goldscheider et al., 2007). A similar tracer study carried out in 1997 even revealed bacteriophages released from the STEP in lake water in the immediate vicinity of the drinking water pump (Wildi and Rossi, 1997) within 48 h. Studies on the transport and fate of antibiotics and other pharmaceuticals in Vidy Bay, which can be ascribed to the WTP discharge with some certainty, were also detected at 1.5 km distance from the DP (reference point, compare **Table 3**) in direction of the drinking water pump at concentrations in the low nanogram per liter range (Bonvin et al., 2011). Thus, it cannot be ruled out that MRB and ARG released from the WTP might reach the DP and that the detected resistance levels in the present study might result or at least partly result from the impact of anthropogenic pollution and/or selection of resistance due to the presence of low antibiotic concentrations (Gullberg et al., 2011). In addition to direct contamination there is the question whether the chronic release of elevated levels of resistant bacteria, and transfer of resistance vectors into natural microbial populations can alter the natural resistance background in the long term. Studies in soil have indicated that such long-term trends may exist (Knapp et al., 2009). Currently however, we lack data on the natural resistance background and an understanding of the impact of elevated antibiotic resistance in natural environments, which leaves us unable to assess the risks associated with the levels observed here. This is clearly an important goal for research in the future.

In the present study we have analyzed a sample set of different aquatic compartments along a wastewater flow using different methodologies, which can be mainly differentiated into culture-based and culture-independent tools. The culture-based approach nicely allowed us to link antibiotic resistance to active (viable) bacterial cells present in the different environments and identify the bacterial taxa carrying resistances. Further, we were able to characterize detected strains with respect to multiple resistances and mobile genetic elements, such as plasmids and the genetic base for the observed resistance. The great limitation of this method is that only a small proportion of the natural bacterial community is accessible to culturing. It is well known that natural aquatic environments contain large proportions of non-culturable but viable bacteria (Oliver, 2005). Less than 1% of environmental bacteria are culturable, and large parts of the bacterial diversity have so far not been cultured at all (Amann et al., 1995). Even lab strains fail to be cultured when living under harsh conditions such as in fresh water environments (Arana et al., 1997). Large differences (2–4 log units) between numbers obtained from heterotrophic plate counts and FC have been described, e.g., for raw water samples by Hoefel et al. (2003).

The advantages of culture-independent approaches are evident particularly when dealing with oligotrophic environments such as fresh water and other natural ecosystems. Clearly, comparing plate counts to bacterial 16S rRNA gene copy numbers and flow-cytometric cell counts, results obtained from plate counts alone would have underestimated the actual prevalence of antibiotic resistance in the wastewater and especially at the two investigated sites in Lake Geneva. Of course biases for these methods exist as well, e.g., with respect to the method used for DNA extraction, PCR efficiencies, presence of PCR inhibitors or the gating parameters in the flow cytometer. Application of the culture-independent methods, FC, and qPCR, alone would have given no information on biological activity of the quantified bacterial traits (though for FC there exist procedures for-live-dead staining of cells) and no linkage of resistance genes and bacterial taxonomic identity. Determination of multiresistances and detection of the presence of mobile genetic elements within intact cells would likewise not have been possible. As such it has to be taken into account that for the high numbers (compared to the plate counts) of resistance genes and 16S RNA gene copy numbers or flow-cytometric counts, a certain fraction might result from disrupted or dead cells. The presence of multicopy plasmids carrying resistance genes, or the presence of abundant but non-culturable environmental strains may further contribute to the observed disparity between the two approaches. Taking the advantages and short-comings of the discussed methods into account it is perhaps surprising that, studies applying both culture-based and molecular tools in parallel are still limited in number although they can provide substantial information on the actual situation and related processes of antibiotic resistance in different compartments. Most previous studies discussing, e.g., the role of hospital effluents on potential contamination of nearby water sheds, focused on culture-based approaches (Esiobu et al., 2002; Novais et al., 2005; Schwartz et al., 2006; Duong et al., 2008; Manaia et al., 2010). Volkmann et al. (2004) could, e.g., confirm the contamination of different wastewater samples with genes conferring resistance to β-lactam antibiotics and vancomycin, whereas the staphylococci-specific methicillin resistance gene was found in significantly lower amounts. The result supported previous culture-based approaches in which methicillin resistant staphylococci were only isolated from clinical wastewaters (Schwartz et al., 2003).

Due to logistical constraints, particularly the high workload associated with culturing, it was not feasible to replicate the sampling, nor to sample all water sources at the same time, which of course implies that one should be careful in generalizing from the results presented here. Similar limitations are frequently encountered in comparable studies (Schwartz et al., 2006; Auerbach et al., 2007; Szczepanowski et al., 2009). Care was taken to sample under comparable circumstances (i.e., avoiding rainfall) and time of day, particularly in the actual sewage system, to obtain comparable samples. Temporal variation in total bacterial load and resistance counts might of course influence the general trends observed in our study. However, in two comparable studies where temporal variations were taken into account this seemed not to be the case. Da Silva et al. (2006) sampled a WTP in Chile during four separate

sampling campaigns, representing spring, summer, autumn, and winter. They found higher fractions of resistant organisms in the warmer periods compared to the winter months, but the numbers usually varied within the same order of magnitude for all sampling events. The effect of treatment was consistent over time, i.e., treated effluents always exhibited higher resistance fractions compared to raw sewage. In contrast, Zhang et al. (2009) reported higher levels of resistance among *Acinetobacter* isolates from a WTP and its receiving river in the US during a cold and low flow sampling event compared to a warm and high flow sampling event. Still the trend of increased resistance from influent to effluent was observed during both campaigns. Finally, another study by Li et al. (2009) determined total cell counts (DAPI staining) and culturable bacteria (on TSA) of $3.3 \pm 1.9 \times 10^7$ cells ml^{-1} and of $4.5 \pm 2.1 \times 10^4$ CFU ml^{-1}, respectively (averaged over three sampling campaigns carried out in December, April, and August) in the effluent of a WTP treating wastewater from an oxytetracycline production facility in China, indicating a seasonal variability of around 50%. We have carried out a similar campaign to that compared in the present study in 2009 which resulted in comparable trends, further supporting the validity of the data presented here (Nadine Czekalski, unpublished data).

The molecular methods proved valuable and generally the trends observed were in good agreement with the cultivation-based data. Therefore, a more thorough time-resolved and replicated study based on this methodology would be a good approach to overcome the noted limitations.

For future studies aiming to provide deeper insights into resistance background of natural aquatic environments as well as their pollution with resistant bacteria from anthropogenic sources, the use of advanced culture-independent tools, such as metagenomics and high throughput sequencing will be of great value. With respect to linking activity, mobility, and functional traits to bacterial taxa single cell sequencing approaches might probably become a helpful tool too, potentially replacing and even improving on current culture-based approaches.

ACKNOWLEDGMENTS

We would like to thank Francisco Vazquez for substantial support of our lab work, Holger Heuer for kindly providing *E. coli* strains carrying *sul* resistance genes, Frederik Hammes for the opportunity to conduct and his support during flow-cytometric measurements, Janine Halder for providing CTD profiles, Alois Zwyssig, Michael Schurter, Philippe Arpagaus for assistance during field campaigns, and Luca Rossi and Nathalie Chèvre for organizing access to the CHUV and WTP sampling sites and providing background data. We would like to thank all members of the ProDoc project for helpful discussions. Laetitia Stoykowski, Pascal Kistler, and Sandro Brunner for assisting with the plate count experiment. Funding was provided by the Swiss National Science Foundation grant PFMP2-123048 for research module 4 of the ProDoc project "Scientific Concepts for the Sustainable Management of Mid-sized Lakes in the 21st Century."

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at http://www.frontiersin.org/Antimicrobials,_Resistance_and_Chemotherapy/10.3389/fmicb.2012.00106/abstract

REFERENCES

Alder, A. C., McArdell, C. S., Golet, E. M., Ibric, S., Molnar, E., Nipales Norriel, S., and Giger, W. (2001). "Occurrence and fate of fluoroquinolone, macrolide, and sulfonamide antibiotics during wastewater treatment and in ambient waters in Switzerland," in *Pharmaceuticals and Care Products in the Environment: Scientific and Regulatory Issues*, *ACS Symposium Series*, Vol. 791, C. G. Daughton and T. L. Jones-Lepp (Washington, DC: American Chemical Society), 56–69.

Alonso, A., Sánchez, P., and Martínez, J. L. (2001). Environmental selection of antibiotic resistance genes. *Environ. Microbiol.* 3, 1–9.

Altschul, S., Gish, W., Miller, W., Myers, E., and Lipman, D. (1990). Basic local alignment search tool. *J. Mol. Biol.* 215, 403–410.

Amalfitano, S., and Fazi, S. (2008). Recovery and quantification of bacterial cells associated with streambed sediments. *J. Microbiol. Methods* 75, 237–243.

Amann, R. I., Ludwig, W., and Schleifer, K. H. (1995). Phylogenetic identification and in-situ detection of individual microbial-cells without cultivation. *Microbiol. Rev.* 59, 143–169.

Aminov, R. I. (2009). The role of antibiotics and antibiotic resistance in nature. *Environ. Microbiol.* 11, 2970–2988.

Arana, I., Pocino, M., Muela, A., Fernandez-Astorga, A., and Barcina, I. (1997). Detection and enumeration of viable but non-culturable transconjugants of *Escherichia coli* during the survival of recipient cells in river water. *J. Appl. Microbiol.* 83, 340–346.

Auerbach, E. A., Seyfried, E. E., and Mcmahon, K. D. (2007). Tetracycline resistance genes in activated sludge wastewater treatment plants. *Water Res.* 41, 1143–1151.

Baquero, F., Martínez, J.-L., and Cantón, R. (2008). Antibiotics and antibiotic resistance in water environments. *Curr. Opin. Biotechnol.* 19, 260–265.

Berney, M., Vital, M., Hülshoff, I., Weilenmann, H.-U., Egli, T., and Hammes, F. (2008). Rapid, cultivation-independent assessment of microbial viability in drinking water. *Water Res.* 42, 4010–4018.

Blanc, L. (2010). *Characterisation of Hospital Wastewater: The Case of the CHUV*. MSc thesis, Cranfield University, Cranfield.

Blasco, M. D., Esteve, C., and Alcaide, E. (2009). Multiresistant waterborne pathogens isolated from water reservoirs and cooling systems. *J. Appl. Microbiol.* 105, 469–475.

Bonvin, F., Rutler, R., Che'Vre, N., Halder, J., and Kohn, T. (2011). Spatial and temporal presence of a wastewater-derived micropollutant plume in Lake Geneva. *Environ. Sci. Technol.* 45, 4702–4709.

Bravo, A. G., Bouchet, S., Amouroux, D., Pote, J., and Dominik, J. (2011). Distribution of mercury and organic matter in particle-size classes in sediments contaminated by a waste water treatment plant: Vidy Bay, Lake Geneva, Switzerland. *J. Environ. Monit.* 13, 974–982.

Carattoli, A., Bertini, A., Villa, L., Falbo, V., Hopkins, K. L., and Threlfall, E. J. (2005). Identification of plasmids by PCR-based replicon typing. *J. Microbiol. Methods* 63, 219–228.

Castiglioni, S., Pomati, F., Miller, K., Burns, B. P., Zuccato, E., Calamari, D., and Neilan, B. A. (2008). Novel homologs of the multiple resistance regulator marA in antibiotic-contaminated environments. *Water Res.* 42, 4271–4280.

Cattoir, V., Poirel, L., Aubert, C., C.-J., S., and Nordmann, P. (2008). Unexpected occurrence of plasmid-mediated quinolone resistance determinants in environmental *Aeromonas* spp. *Emerg. Infect. Dis.* 14, 231–237.

Cattoir, V., Poirel, L., Rotimi, V., Soussy, C.-J., and Nordmann, P. (2007). Multiplex PCR for detection of plasmid-mediated quinolone resistance qnr genes in ESBL-producing enterobacterial isolates. *J. Antimicrob. Chemother.* 60, 394–397.

Chèvre, N., Guignard, C., Rossi, L., Pfeiffer, H.-R., Bader, H.-P., and Scheidegger, R. (2011). Substance flow analysis as a tool for urban water management. *Water Sci. Technol.* 63, 8.

Cummings, D. E., Archer, K. F., Arriola, D. J., Baker, P. A., Faucett, K. G., Laroya, J. B., Pfeil, K. L., Ryan, C. R., Ryan, K. R. U., and Zuill, D. E. (2010). Broad dissemination of plasmid-mediated quinolone resistance genes in sediments of two urban coastal wetlands. *Environ. Sci. Technol.* 45, 447–454.

Da Silva, M. F., Tiago, I., Veríssimo, A., Boaventura, R. A., Nunes, O. C., and Manaia, C. M. (2006). Antibiotic resistance of enterococci and related bacteria in an urban wastewater treatment plant. *FEMS Microbiol. Ecol.* 55, 322–329.

DeSantis, T. Z., Hugenholtz, P., Larsen, N., Rojas, M., Brodie, E. L., Keller, K., Huber, T., Dalevi, D., Hu, P., and Andersen, G. L. (2006). Greengenes, a chimera-checked 16S rRNA gene database and workbench compatible with ARB. *Appl. Environ. Microbiol.* 72, 5069–5072.

Deutsches Institut Für Normung e. V. (DIN). (2004). Medizinische Mikrobiologie-Empfindlichkeitsprüfung von Mikrobiellen Krankheitserregern gegen Chemotherapeutika-Teil 4: Bewertungsstufen für die minimale Hemmkonzentration; MHK-Grenzwerte von antibakteriellen Wirkstoffen; DIN 58940-4, Bbl, 1:2004-2. Berlin: Beuth Verlag.

Duong, H. A., Pham, N. H., Nguyen, H. T., Hoang, T. T., Pham, H. V., Pham, V. C., Berg, M., Giger, W., and Alder, A. C. (2008). Occurrence, fate and antibiotic resistance of fluoroquinolone antibacterials in hospital wastewaters in Hanoi, Vietnam. *Chemosphere* 72, 968–973.

Escher, B. I., Baumgartner, R., Koller, M., Treyer, K., Lienert, J., and McArdell, C. S. (2011). Environmental toxicology and risk assessment of pharmaceuticals from hospital wastewater. *Water Res.* 45, 75–92.

Esiobu, N., Armenta, L., and Ike, J. (2002). Antibiotic resistance in soil and water environments. *Int. J. Environ. Health Res.* 12, 133–144.

European Committee on Antimicrobial Susceptibility Testing (EUCAST). (2011). Data from the EUCAST MIC and zone diameter distribution website. Available at: http://www.eucast.org [accessed January 20, 2011].

Filippini, M., Masiero, G., and Moschetti, K. (2006). Socioeconomic determinants of regional differences in outpatient antibiotic consumption: evidence from Switzerland. *Health Policy (New York)* 78, 77–92.

Garcia, S., Wade, B., Bauer, C., Craig, C., Nakaoka, K., and Lorowitz, W. (2007). The effect of wastewater treatment on antibiotic resistance in *Escherichia coli* and *Enterococcus* sp. *Water Environ. Res.* 79, 2387–2395.

Giger, W., Alder, A. C., Golet, E. M., Kohler, H. P. E., McArdell, C. S., Molnar, E., Siegrist, H., and Suter, M. J. F. (2003). Occurrence and fate of antibiotics as trace contaminants in wastewaters, sewage sludges, and surface waters. *Chimia (Aarau)* 57, 485–491.

Goldscheider, N., Haller, L., Pote, J., Wildi, W., and Zopfi, J. (2007). Characterizing water circulation and contaminant transport in Lake Geneva using bacteriophage tracer experiments and limnological methods. *Environ. Sci. Technol.* 41, 5252–5258.

Gotz, A., Pukall, R., Smit, E., Tietze, E., Prager, R., Tschape, H., Van Elsas, J., and Smalla, K. (1996). Detection and characterization of broad-host-range plasmids in environmental bacteria by PCR. *Appl. Environ. Microbiol.* 62, 2621–2628.

Grossart, H.-P., Jezbera, J., Hornák, K., Hutalle, K. M. L., Buck, U., and Šimek, K. (2008). Top-down and bottom-up induced shifts in bacterial abundance, production and community composition in an experimentally divided humic lake. *Environ. Microbiol.* 10, 635–652.

Guardabassi, L., Lo Fo Wong, D. M. A., and Dalsgaard, A. (2002). The effects of tertiary wastewater treatment on the prevalence of antimicrobial resistant bacteria. *Water Res.* 36, 1955–1964.

Gullberg, E., Cao, S., Berg, O. G., Ilbäck, C., Sandegren, L., Hughes, D., and Andersson, D. I. (2011). Selection of resistant bacteria at very low antibiotic concentrations. *PLoS Pathog.* 7, e1002158. doi:10.1371/journal.ppat.1002158

Haller, L., Amedegnato, E., Poté, J., and Wildi, W. (2009a). Influence of freshwater sediment characteristics on persistence of fecal indicator bacteria. *Water Air Soil Pollut.* 203, 217–227.

Haller, L., Pote, J., Loizeau, J. L., and Wildi, W. (2009b). Distribution and survival of faecal indicator bacteria in the sediments of the Bay of Vidy, Lake Geneva, Switzerland. *Ecol. Indic.* 9, 540–547.

Haller, L., Tonolla, M., Zopfi, J., Peduzzi, R., Wildi, W., and Poté, J. (2011). Composition of bacterial and archaeal communities in freshwater sediments with different contamination levels (Lake Geneva, Switzerland). *Water Res.* 45, 1213–1228.

Han, X. Y., and Andrade, R. A. (2005). Brevundimonas diminuta infections and its resistance to fluoroquinolones. *J. Antimicrob. Chemother.* 55, 853–859.

Heuer, H., Focks, A., Lamshöft, M., Smalla, K., Matthies, M., and Spiteller, M. (2008). Fate of sulfadiazine administered to pigs and its quantitative effect on the dynamics of bacterial resistance genes in manure and manured soil. *Soil Biol. Biochem.* 40, 1892–1900.

Heuer, H., and Smalla, K. (2007). Manure and sulfadiazine synergistically increased bacterial antibiotic resistance in soil over at least two months. *Environ. Microbiol.* 9, 657–666.

Hoefel, D., Grooby, W. L., Monis, P. T., Andrews, S., and Saint, C. P. (2003). Enumeration of water-borne bacteria using viability assays and flow cytometry: a comparison to culture-based techniques. *J. Microbiol. Methods* 55, 585–597.

Holmes, B. (2006). "The genera *Flavobacterium*, *Sphingobacterium* and *Weeksella*," in *The Prokaryotes*, 3rd Edn, eds. M. Dworkin, S. Falkow, E. Rosenberg, K.-H. Schleifer, and E. Stackebrandt (New York: Springer), 539–548.

Hönerlage, W., Hahn, D., and Zeyer, J. (1995). Detection of mRNA of nRPM in Bacillus megaterium ATTC 14581 grown in soil by whole-cell hybridization. *Arch. Microbiol.* 163, 235–241.

Iwane, T., Urase, T., and Yamamoto, K. (2001). Possible impact of treated wastewater discharge on incidence of antibiotic resistant bacteria in river water. *Water Sci. Technol.* 43, 91–99.

Jones, J. G., Gardener, S., Simon, B. M., and Pickup, R. W. (1986). Antibiotic resistant bacteria in Windermere and two remote upland tarns in the English Lake District. *J. Appl. Microbiol.* 60, 443–453.

Jury, K. L., Khan, S. J., Vancov, T., Stuetz, R. M., and Ashbolt, N. J. (2011). Are sewage treatment plants promoting antibiotic resistance? *Crit. Rev. Environ. Sci. Technol.* 41, 243–270.

Jury, K. L., Vancov, T., Stuetz, R. M., Khan, S. J. (2010). "Antibiotic resistance dissemination and sewage treatment plants," in *Current Research, Technology and Education Topics in Applied Microbiology and Microbial Biotechnology*, ed. A. Mendez-Vilas (Badajoz: Formatex Research Center), 509–519.

Kemper, N. (2008). Veterinary antibiotics in the aquatic and terrestrial environment. *Ecol. Indic.* 8, 1–13.

Kim, S., and Aga, D. S. (2007). Potential ecological and human health impacts of antibiotics and antibiotic-resistant bacteria from wastewater treatment plants. *J. Toxicol. Environ. Health B Crit. Rev.* 10, 559–573.

Knapp, C. W., Dolfing, J., Ehlert, P. A. I., and Graham, D. W. (2009). Evidence of increasing antibiotic resistance gene abundances in archived soils since 1940. *Environ. Sci. Technol.* 44, 580–587.

Kümmerer, K. (2004). Resistance in the environment. *J. Antimicrob. Chemother.* 54, 311–320.

Kümmerer, K. (2008). *Pharmaceuticals in the Environment. Sources, Effects and Risk*. Berlin/Heidelberg: Springer.

Kümmerer, K. (2009). Antibiotics in the aquatic environment – a review – part II. *Chemosphere* 75, 435–441.

Lane, D. J. (1991). "16S/23S rRNA sequencing," in *Nucleic Acid Techniques in Bacterial Systematics*, eds E. Stackebrandt and M. Goodfellow (New York, NY: John Wiley and Sons), 115–175.

Lanz, R., Kuhnert, P., and Boerlin, P. (2003). Antimicrobial resistance and resistance gene determinants in clinical *Escherichia coli* from different animal species in Switzerland. *Vet. Microbiol.* 91, 73–84.

Levchuk, A., Bulyga, I., Izmalkova, T., Sevast'ianovich, I., Kosheleva, I., Thomas, C., and Titok, M. (2006). Nah-plasmids of IncP-9 group from natural strains of *Pseudomonas*. *Mol. Biol.* 40, 835–843.

Li, D., Yu, T., Zhang, Y., Yang, M., Li, Z., Liu, M., and Qi, R. (2009). Antibiotic resistance characteristics of environmental bacteria from an oxytetracycline production wastewater treatment plant and the receiving river. *Appl. Environ. Microbiol.* 76, 3444–3451.

Life Technologies Corporation. (2012). Available at: http://www.invitrogen.com/site/us/en/home/Products-and-Services/Applications/PCR/real-time-pcr/qpcr-education/real-time-pcr-troubleshooting-tool/gene-expression-quantitation-troubleshooting/poor-pcr-efficiency.html [accessed January 30, 2012].

Lindahl, V. (1996). Improved soil dispersion procedures for total bacterial counts, extraction of indigenous bacteria and cell survival. *J. Microbiol. Methods* 25, 279–286.

Loizeau, J.-L., Pardos, M., Monna, F., Peytremann, C., Haller, L., and Dominik, J. (2004). The impact of a sewage treatment plant's effluent on sediment quality in a small bay in Lake Geneva (Switzerland–France). Part 2: Temporal evolution of heavy metals. *Lakes Reserv. Res. Manag.* 9, 53–63.

Manaia, C., Novo, A., Coelho, B., and Nunes, O. (2010). Ciprofloxacin resistance in domestic wastewater

treatment plants. *Water Air Soil Pollut.* 208, 335–343.

Martinez, J. L. (2008). Antibiotics and antibiotic resistance genes in natural environments. *Science* 321, 365–367.

Martinez, J. L. (2009). Environmental pollution by antibiotics and by antibiotic resistance determinants. *Environ. Pollut.* 157, 2893–2902.

Martínez-Martínez, L., Pascual, A., and Jacoby, G. A. (1998). Quinolone resistance from a transferable plasmid. *Lancet* 351, 797–799.

McArdell, C. S., Molnar, E., Suter, M. J. F., and Giger, W. (2003). Occurrence and fate of macrolide antibiotics in wastewater treatment plants and in the Glatt Valley watershed, Switzerland. *Environ. Sci. Technol.* 37, 5479–5486.

Newcombe, D., Stuecker, T., La Duc, M., and Venkateswaran, K. (2005). "Q-PCR based bioburden assessment of drinking water throughout treatment and delivery to the International Space Station," in *35th International Conference on Environmental Systems (ICES); 8th European Symposium on Space Environmental Control Systems (ESSECS)* (Rome, Italy/Pasadena, CA: Jet Propulsion Laboratory, National Aeronautics and Space Administration).

Novais, C., Coque, T. M., Ferreira, H., Sousa, J. C., and Peixe, L. (2005). Environmental contamination with vancomycin-resistant enterococci from hospital sewage in Portugal. *Appl. Environ. Microbiol.* 71, 3364–3368.

NRP49. (2007). *National Research Programme NRP 49. Antibiotic Resistance. Final Report*, eds F. Baggi, J. C. Piffaretti, F. Lurati, (Bern: Schweizerischer Nationalfonds zur Förderung der wissenschaftlichen Forschung. Abteilung IV, Orientierte Forschung, Nationale Forschungsprogramme).

Oliver, J. D. (2005). The viable but nonculturable state in bacteria. *J. Microbiol.* 43, 93–100.

Pardos, M., Benninghoff, C., De Alencastro, L. F., and Wildi, W. (2004). The impact of a sewage treatment plant's effluent on sediment quality in a small bay in Lake Geneva (Switzerland–France). Part 1: Spatial distribution of contaminants and the potential for biological impacts. *Lakes Reserv. Res. Manag.* 9, 41–52.

Pei, R., Kim, S.-C., Carlson, K. H., and Pruden, A. (2006). Effect of river landscape on the sediment concentrations of antibiotics and corresponding antibiotic resistance genes (ARG). *Water Res.* 40, 2427–2435.

Perreten, V., and Boerlin, P. (2003). A new sulfonamide resistance gene (sul3) in *Escherichia coli* is widespread in the pig population of Switzerland. *Antimicrob. Agents Chemother.* 47, 1169–1172.

Picao, R. C., Poirel, L., Demarta, A., Silva, C. S. F., Corvaglia, A. R., Petrini, O., and Nordmann, P. (2008). Plasmid-mediated quinolone resistance in *Aeromonas allosaccharophila* recovered from a Swiss lake. *J. Antimicrob. Chemother.* 62, 948–950.

Poté, J., Goldscheider, N., Haller, L., Zopfi, J., Khajehnouri, F., and Wildi, W. (2009a). Origin and spatial–temporal distribution of faecal bacteria in a bay of Lake Geneva, Switzerland. *Environ. Monit. Assess.* 154, 337–348.

Poté, J., Haller, L., Kottelat, R., Sastre, V., Arpagaus, P., and Wildi, W. (2009b). Persistence and growth of faecal culturable bacterial indicators in water column and sediments of Vidy Bay, Lake Geneva, Switzerland. *J. Environ. Sci. (China)* 21, 62–69.

Poté, J., Haller, L., Loizeau, J.-L., Garcia Bravo, A., Sastre, V., and Wildi, W. (2008). Effects of a sewage treatment plant outlet pipe extension on the distribution of contaminants in the sediments of the Bay of Vidy, Lake Geneva, Switzerland. *Bioresour. Technol.* 99, 7122–7131.

Prado, T., Pereira, W. C., Silva, D. M., Seki, L. M., Carvalho, A. P. D. A., and Asensi, M. D. (2008). Detection of extended-spectrum β-lactamase-producing *Klebsiella pneumoniae* in effluents and sludge of a hospital sewage treatment plant. *Lett. Appl. Microbiol.* 46, 136–141.

Reasoner, D. J., and Geldreich, E. E. (1985). A new medium for the enumeration and subculture of bacteria from potable water. *Appl. Environ. Microbiol.* 49, 1–7.

Reinthaler, F. F., Posch, J., Feierl, G., Wüst, G., Haas, D., Ruckenbauer, G., Mascher, F., and Marth, E. (2003). Antibiotic resistance of *E. coli* in sewage and sludge. *Water Res.* 37, 1685–1690.

Schwartz, T., Kohnen, W., Jansen, B., and Obst, U. (2003). Detection of antibiotic-resistant bacteria and their resistance genes in wastewater, surface water, and drinking water biofilms. *FEMS Microbiol. Ecol.* 43, 325–335.

Schwartz, T., Volkmann, H., Kirchen, S., Kohnen, W., Schon-Holz, K., Jansen, B., and Obst, U. (2006). Real-time PCR detection of *Pseudomonas aeruginosa* in clinical and municipal wastewater and genotyping of the ciprofloxacin-resistant isolates. *FEMS Microbiol. Ecol.* 57, 158–167.

Sengelov, G., Agerso, Y., Halling-Sorensen, B., Baloda, S. B., Andersen, J. S., and Jensen, L. B. (2003). Bacterial antibiotic resistance levels in Danish farmland as a result of treatment with pig manure slurry. *Environ. Int.* 28, 587–595.

Silva, J., Castillo, G., Callejas, L., López, H., and Olmos, J. (2006). Frequency of transferable multiple antibiotic resistance amongst coliform bacteria isolated from a treated sewage effluent in Antofagasta, Chile. *Electron. J. Biotechnol.* 9, 533–540.

Stepanauskas, R., Glenn, T. C., Jagoe, C. H., Tuckfield, R. C., Lindell, A. H., King, C. J., and Mcarthur, J. V. (2006). Coselection for microbial resistance to metals and antibiotics in freshwater microcosms. *Environ. Microbiol.* 8, 1510–1514.

Suzuki, M. T., Taylor, L. T., and Delong, E. F. (2000). Quantitative analysis of small-subunit rRNA genes in mixed microbial populations via 5′-nuclease assays. *Appl. Environ. Microbiol.* 66, 4605–4614.

Szczepanowski, R., Linke, B., Krahn, I., Gartemann, K.-H., Gützkow, T., Eichler, W., Pühler, A., and Schlüter, A. (2009). Detection of 140 clinically relevant antibiotic-resistance genes in the plasmid metagenome of wastewater treatment plant bacteria showing reduced susceptibility to selected antibiotics. *Microbiology* 155, 2306–2319.

Thevenon, F., Graham, N. D., Herbez, A., Wildi, W., and Poté, J. (2011). Spatio-temporal distribution of organic and inorganic pollutants from Lake Geneva (Switzerland) reveals strong interacting effects of sewage treatment plant and eutrophication on microbial abundance. *Chemosphere* 84, 609–617.

Troussellier, M., Courties, C., and Zettelmaier, S. (1995). Flow cytometric analysis of coastal lagoon bacterioplankton and picophytoplankton: fixation and storage effects. *Estuar. Coast. Shelf Sci.* 40, 621–633.

Vilanova, X., Manero, A., Cerdà-Cuéllar, M., and Blanch, A. R. (2002). The effect of a sewage treatment plant effluent on the faecal coliforms and enterococci populations of the reception river waters. *J. Appl. Microbiol.* 92, 210–214.

Vioget, P., Jaquerod, C.-A., Ruegg, E., and Poget, E. (2011). *Bilans 2010 de l'épuration vaudoise.* Orbe, Savigny, Switzerland: Service des eaux, sols et assainissement, Canton de Vaud.

Volkmann, H., Schwartz, T., Bischoff, P., Kirchen, S., and Obst, U. (2004). Detection of clinically relevant antibiotic-resistance genes in municipal wastewater using real-time PCR (TaqMan). *J. Microbiol. Methods* 56, 277–286.

Wang, Q., Garrity, G. M., Tiedje, J. M., and Cole, J. R. (2007). Naive Bayesian classifier for rapid assignment of rRNA sequences into the new bacterial taxonomy. *Appl. Environ. Microbiol.* 73, 5261–5267.

Wegmüller, S., and Schmid, S. (2009). *Plasmid DNA isolation from the Gram-positive Bacterium Pediococcus damnosus Using a Modified PureYield™Plasmid Midiprep System Protocol Promega.* Available at: http://www.promega.com/resources/articles/pubhub/enotes/plasmid-dna-isolation-from-the-gram-positive-bacterium-pediococcus-damnosus/ (retrieved April 7, 2012).

Wildi, W., and Rossi, P. (1997). *Assainissement de la baie de Vidy: tarcage par bactériophages 19-25 février 1997 6-11 mai 1997* (Lausanne: Ville de Lausanne, Direction de la sécurité cociale et de lénvironnement, Service d'assainissement, Station d'épuration de Vidy, Université de Genève, Institut F.A. Forel, Section des sciences de la terre, Université Neuchâtel, Laboratoire de Microbiologie).

Wireman, J., Liebert, C., Smith, T., and Summers, A. (1997). Association of mercury resistance with antibiotic resistance in the Gram-negative fecal bacteria of primates. *Appl. Environ. Microbiol.* 63, 4494–4503.

World Health Organization. (2003). "Relationships between common water bacteria and pathogens in drinking-water," in *Heterotrophic Plate Counts and Drinking-water Safety*, eds. J. Bertram, J. Cotruvo, M. Exner, C. Fricker, and A. Glasmacher (London: IWA Publishing), 80–118.

Young, H.-K. (1993). Antimicrobial resistance spread in aquatic environments. *J. Antimicrob. Chemother.* 31, 627–635.

Zhang, Y., Marrs, C. F., Simon, C., and Xi, C. (2009). Wastewater treatment contributes to selective increase of antibiotic resistance among *Acinetobacter* spp. *Sci. Total Environ.* 407, 3702–3706.

Zwart, G., Crump, B. C., Agterveld, M. P. K.-V., Hagen, F., and Han, S.-K. (2002). Typical freshwater bacteria: an analysis of available 16S rRNA gene sequences from plankton of lakes and rivers. *Aquat. Microb. Ecol.* 28, 141–155.

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 07 November 2011; accepted: 02 March 2012; published online: 22 March 2012.
Citation: Czekalski N, Berthold T, Caucci S, Egli A and Bürgmann H (2012) Increased levels of multiresistant bacteria and resistance genes after wastewater treatment and their dissemination into Lake Geneva, Switzerland. Front. Microbio. ***3****:106. doi: 10.3389/fmicb.2012.00106*
This article was submitted to Frontiers in Antimicrobials, Resistance and Chemotherapy, a specialty of Frontiers in Microbiology.

APPENDIX

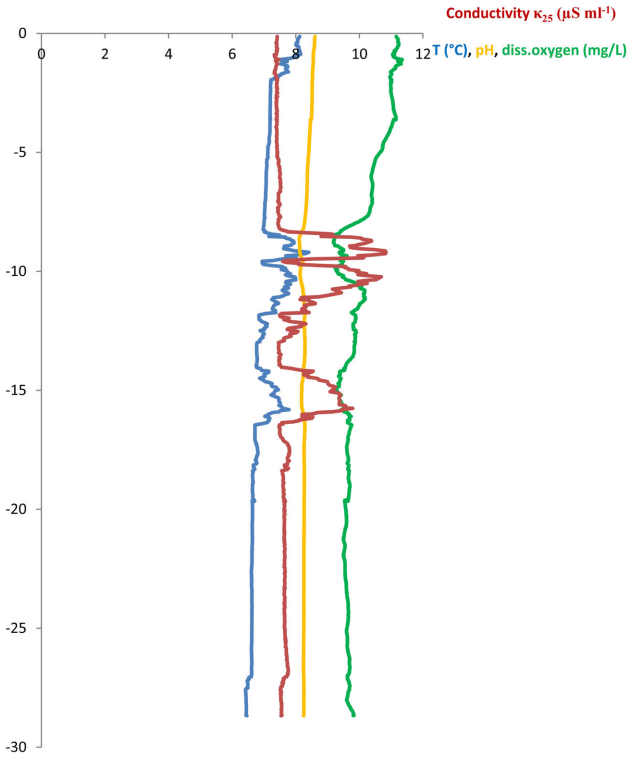


FIGURE A1 | CTD-profile from the Vidy Bay, Lake Geneva above the STEPoutlet, recorded on April 7, 2011.

frontiers in
MICROBIOLOGY

REVIEW ARTICLE
published: 22 February 2012
doi: 10.3389/fmicb.2012.00067

Distribution of quinolones, sulfonamides, tetracyclines in aquatic environment and antibiotic resistance in Indochina

***Satoru Suzuki*[1]* and *Phan Thi Phuong Hoa*[2]**

[1] *Center for Marine Environmental Studies, Ehime University, Matsuyama, Ehime, Japan*
[2] *Vietnam Type Culture Collection, Institute of Microbiology and Biotechnology, Vietnam National University, Hanoi, Vietnam*

Edited by:
David W. Graham, Newcastle University, UK

Reviewed by:
Charles Knapp, University of Strathclyde, UK
Veljo Kisand, University of Tartu, Estonia
Tong Zhang, The University of Hong Kong, Hong Kong

***Correspondence:**
Satoru Suzuki, Center for Marine Environmental Studies, Ehime University, Matsuyama, Ehime 790-8577, Japan.
e-mail: ssuzuki@ehime-u.ac.jp

Southeast Asia has become the center of rapid industrial development and economic growth. However, this growth has far outpaced investment in public infrastructure, leading to the unregulated release of many pollutants, including wastewater-related contaminants such as antibiotics. Antibiotics are of major concern because they can easily be released into the environment from numerous sources, and can subsequently induce development of antibiotic-resistant bacteria. Recent studies have shown that for some categories of drugs this source-to-environment antibiotic resistance relationship is more complex. This review summarizes current understanding regarding the presence of quinolones, sulfonamides, and tetracyclines in aquatic environments of Indochina and the prevalence of bacteria resistant to them. Several noteworthy findings are discussed: (1) quinolone contamination and the occurrence of quinolone resistance are not correlated; (2) occurrence of the *sul* sulfonamide resistance gene varies geographically; and (3) microbial diversity might be related to the rate of oxytetracycline resistance.

Keywords: Indochina, environment, quinolone, sulfonamide, tetracycline, resistance gene, bacteria

INTRODUCTION

Since antibiotics were first discovered in 1928, they have been widely used in human and animal medicine. However, most antibiotics are excreted and enter the soil or water environments through wastewater and fertilization with manure (Kümmerer, 2003). Antibiotic contamination of the natural environment has been documented in many countries, including those of Europe, North America, and East Asia, and areas of antibiotic contamination are widespread throughout the region (Segura et al., 2009). But not much information is available for Southeast Asia and Africa.

The pathways whereby residual antibiotics enter the environment are varied and include wastewater effluent discharge and runoff and leaching from land fertilized with agricultural or human waste. An increasing number of reports show a rise in the occurrence and distribution of antibiotics in surface waters in Europe (Hirsch et al., 1999; McArdell et al., 2003), the USA (Lindsey et al., 2001; Kolpin et al., 2002; Kim and Carlson, 2007), and Canada (Metcalfe et al., 2004). Cases of antibiotic contamination of wastewater are also well-known (Göbel et al., 2005; and others). Some antibiotics can be found in ground water (Baquero et al., 2008). In China, antibiotics have been found in animal wastewater, pond water, animal farm effluents, and rivers. The most frequently detected antibiotics in China include sulfamethazine (75%), oxytetracycline (64%), tetracycline (60%), sulfadiazine (55%), and sulfamethoxazole (51%), which were found at maximum concentrations of 211, 72.9, 10.3, 17.0, and 63.6 ppb, respectively (Wei et al., 2011). In Korea, environmental contamination with tetracyclines and sulfonamides was reported at the ppb level (Ok et al., 2011). Recent excellent reviews by Kümmerer (2009a,b) summarize contamination of the environment with antibiotics and the spread of antibiotic resistance.

This manuscript will focus on Indochina environment having unique situation as following.

WHY INDOCHINA? – SPECIFIC SITUATION OF INDOCHINA

Unlike the socioeconomic-unified continents of Europe and North America, Asia is more geographically and culturally disjunct, and cannot be readily grouped as a single geopolitical entity. It therefore remains difficult to obtain a generalized theory of antibiotic and antibiotic resistance contamination across all Asian countries. This manuscript focuses specifically on the region of Indochina and the major antibiotics in this area, including quinolones, sulfonamides, and tetracyclines. Countries of "Indochina" include Vietnam, Thailand, Cambodia, Lao PDR, and Myanmar. Of these, Vietnam and Thailand have particularly become a center of rapid industrial development and economic growth; however this growth has far outpaced investment in public infrastructure, leading chemical pollution and sanitary issues.

Although Indochina is globally one of the most active regions of industrial growth and development, there remains few studies on antibiotic contamination relative to their Western and East Asian counterparts (Le and Munekage, 2004; Nhiem et al., 2006; Managaki et al., 2007; Duong et al., 2008; Zhang et al., 2009; Hoa et al., 2011; Takasu et al., 2011). From Asian aquatic environments contamination levels are highly variable, ranging from scales of ppm to ppt (Le and Munekage, 2004; Managaki et al., 2007; Duong et al., 2008; Hoa et al., 2011; Takasu et al., 2011). **Table 1** summarizes the various antibiotic contaminants that have been detected in Indochina aquatic environments. Until now for antibiotic resistance in this area, major reports consisted of studies on *Salmonella* isolates (Ogasawara et al., 2008; Sirichote et al., 2010; and others).

Table 1 | Antibiotics detected in aquatic environments of Indochina countries.

Antibiotic classes	Compounds	Habitat	Concentration range (ppt)	Reference
Sulfonamides	Sulfamethoxazole	Shrimp pond	0.01–6.06 × 10^6	Le and Munekage (2004)
		Urban drainage	190–330	Managaki et al. (2007)
		Canal	37–174	Managaki et al. (2007)
			0.02–4330	Hoa et al. (2011)
		Mekong river	20–33	Managaki et al. (2007)
		Pig farm	n.q.–227	Managaki et al. (2007)
			68.2–422	Hoa et al. (2011)
	Sulfamethazine	Urban drainage	110–251	Managaki et al. (2007)
		Canal	62–328	Managaki et al. (2007)
			16.1–66.2	Hoa et al. (2011)
		Mekong river	15–28	Managaki et al. (2007)
		Pig farm	18512–19152	Managaki et al. (2007)
			6.78–6662	Hoa et al. (2011)
Trimethoprim	Trimethoprim	Shrimp pond	0.08–1.04 × 10^6	Le and Munekage (2004)
			n.d.–85	Hoa et al. (2011)
		Urban drainage	27–46	Managaki et al. (2007)
		Canal	315–44	Managaki et al. (2007)
			23–1808	Hoa et al. (2011)
		Mekong river	7–19	Managaki et al. (2007)
		Pig farm	30–84	Managaki et al. (2007)
			n.d.–34.6	Hoa et al. (2011)
Macrolides	Erythromycin	Urban drainage	29–39	Managaki et al. (2007)
		Canal	31–41	Managaki et al. (2007)
			61.1–2246	Hoa et al. (2011)
		Mekong river	9–12	Managaki et al. (2007)
		Pig farm	n.q.	Managaki et al. (2007)
			n.d.–63.9	Hoa et al. (2011)
Fluroquinolones	Norfloxacin	Shrimp pond	0.06–6.06 × 10^6	Le and Munekage (2004)
		Hospital wastewater	1.5–15.2 × 10^3	Duong et al. (2008)
			4.62–2560	Takasu et al. (2011)
	Oxolinic acid	Shrimp pond	0.01–2.5 × 10^6	Le and Munekage (2004)
	Ciprofloxacin	Hospital wastewater	1.2–10.9 × 10^3	Duong et al. (2008)
			328	Takasu et al. (2011)
	Ofloxacin	Canal	185–782	Takasu et al. (2011)

n.q., not quantifiable due to overlapped interfering peak.

Large amounts of antibiotics are used for human medicine, livestock farming, and aquaculture in Southeast Asia. In addition, we observed that antibiotics are used as additives in ice to prevent decay of harvested fish in fish markets. It is believed that high concentrations of antibiotics applied in this manner will remain in the fish, and will then be taken in by people upon consumption. Such widespread antibiotic contamination of the environment and food supply likely promotes the development of antibiotic-resistant bacteria, both in the environment and within the human intestine. Prior to the early 2000s, few studies addressed antibiotic contamination in Indochina (Le and Munekage, 2004), although the occurrence of antibiotic-resistant bacteria in the region was reported (Kim et al., 2004; Le et al., 2005; Zhang et al., 2009). However, recent studies on drug contamination, combined with detection of antibiotic-resistant bacteria and/or resistance genes, have provided a clearer picture of the problem in this area of Asia (Hoa et al., 2011; Takasu et al., 2011).

In Indochina, an integrated agriculture–aquaculture farming system known as VAC (Vegetation, Aquaculture, and Cage) is common. The VAC system is a recycling farm, typically consisting of a vegetable field, an aquaculture pond, and caged animals, and has been practiced since the 1980s. In the VAC system, livestock manure (usually from pigs, chickens, and ducks) is directly transported to fish ponds and to vegetable and rice fields. This untreated sewage and wastewater from the livestock operations is used for fish culture and for fertilization of the vegetable fields. The animal manure contributes to the eutrophication of pond water, which enhances phytoplankton growth. The VAC system is considered a very economical method of recycling farming (Hop, 2003); however, the heavy use of antibiotics in livestock increases the prevalence of enteric antibiotic-resistant bacteria and the potential for antibiotic-resistant pathogenic bacteria to arise. Subsequent discharge of antibiotic residues in farm waste likely increases the number of antibiotic-resistant bacteria in the environment, which

in turn promotes the further selection and transfer of antibiotic resistance genes within the microbial community in the surrounding environment (Petersen and Dalsgaard, 2003; Heuer and Smalla, 2007). Recent study by Dang et al. (2011) reported that enteric bacteria of pigs in VAC showed high resistance to nalidixic acid and enrofloxacin, which would be dispersed to water environment. Tetracycline resistance was found at all times with high frequency (80%<). The VAC system can be thought as a "bazaar of antibiotic resistance." That is, the abundance of antibiotic-resistant bacteria and genes initially increases in the intestinal tracts of livestock as a result of exposure to high concentrations of various antibiotics. Next, horizontal gene transfer occurs among various bacteria in the environment surrounding the VAC operation. Antibiotic resistance genes persist and spread among environmental bacteria through acquisition and recombination. Finally, the antibiotic resistance genes move to other environments via water use and the food supply. The antibiotic resistance genes are brought together, exchanged, and spread in VAC systems. If the antibiotic-resistant bacteria come into contact with human pathogens and commensal bacteria, the risk to human health may increase significantly. Although there are many snapshot reports of antibiotic resistance (reviewed in Zhang et al., 2009), further dynamic study is needed to evaluate the risks posed by the presence of antibiotic resistance genes in the aquatic environments of developing countries in Asia.

QUINOLONE RESISTANCE

Fluoroquinolones (FQs) are fully synthetic antibiotics that are widely used in humans, animals, and fish (Grave et al., 1999; Le and Munekage, 2004; Samanidou et al., 2005; Kemper, 2008). The antibacterial mechanism of FQs is based on inhibition of bacterial DNA gyrase or topoisomerase IV, which are enzymes essential for DNA replication. First-generation FQs are piromidic acid, oxolinic acid, and nalidixic acid, which were widely used in aquaculture in the 1970s in Japan. In tropical Asian countries, oxolinic acid still appears to be one of the major drugs used (Gräslund et al., 2003; Le and Munekage, 2004). Second-generation (ciprofloxacin, CIP and norfloxacin, NOR) and third-generation (levofloxacin, LEV and its enantiomer ofloxacin, OFL) compounds are used in hospitals and animal husbandry in Indochina (Takasu et al., 2011).

The FQs are photo-degradable, with a half-life in pure water of 105 and 90 min for NOR and CIP, respectively (Burhenne et al., 1997). However, FQs in the environment are relatively stable in water and sediment (Kümmerer, 2004; Le and Munekage, 2004), which might be due to sorption onto particulates (Nowara et al., 1997). Lai and Lin (2009) reported that oxolinic acid and flumequine could be retained in sediment for 9.5–15 and 3.6–6.4 days, respectively. Such long half-lives in the environment pose a selective pressure for environmental bacteria. Although the bioavailability of antibiotics is suspected to decrease upon adsorption on clay and humic substances, no supporting evidence has been reported.

Holmström et al. (2003) reported that 74% of Thailand shrimp aquaculture farmers use antibiotics, primarily NOR. Le and Munekage (2004) reported detecting NOR at 0.5–3.5 ppm in the water column and 200–1500 ppm in the sediment of intensive ponds and improved extensive ponds in Vietnam. Oxolinic acid could be detected in the water column at a concentration similar to that of NOR, but not in the sediment. The "water column concentration" indicates present inflow, while the "sediment concentration" indicates the value integrated over time (Takasu et al., 2011). The presence of antibiotics in both samples suggests that the compounds being used at present and are retained in the sediment.

Ciprofloxacin is commonly used with other drugs, such as griseofulvin, rifampicin, and oxytetracycline for shrimp larvae in Vietnam (Thuy et al., 2011). The most recent quantification of FQs in Indochina aquatic environments showed that the average concentrations were higher in Thailand (OFL, 7400 ppb; NOR, 209 ppb; CIP, 328 ppb; LOM, 67.4 ppb) than in Vietnam (OFL, 255 ppb; NOR, 41.1 ppb; CIP, 162 ppb; LOM, 25.3 ppb; Takasu et al., 2011). Both OFL and NOR were confirmed as major environmental contaminants in both countries; however, contamination at aquaculture sites was lower than at VAC farms and city canals in both countries. A recent decrease in drug application and/or dilution effects may explain the improved contamination situation in aquaculture settings. Since FQs are not natural compounds, it is believed that bacteria do not possess FQ resistance genes. However, bacteria resistant to FQs can be found easily. Several resistance mechanisms have been reported, including mutation of DNA gyrase (Fukuda et al., 1990), impermeability to FQs due to loss of porins, and extrusion by overexpressed efflux pumps (Hirai et al., 1986, 1987). A number of plasmid-mediated quinolone resistance (PMQR) genes (*qnrA*, *qnrB*, and *qnrS*) have also been identified (Robicsek et al., 2006). These PMQR genes are transferred horizontally among bacteria and encode a protein of the pentapeptide repeat family that has been shown to block the actions of FQs on purified DNA gyrase and topoisomerase IV (Robicsek et al., 2006). The origin of these genes is thought to be chromosomes in aquatic bacteria (Poirel et al., 2005a,b; Cattoir et al., 2008). Thus, the aquatic environment is hypothesized to be a natural reservoir of FQ-resistant bacteria and resistance genes.

As mentioned above, the environmental concentration of FQs was found to be much higher in Thailand than in Vietnam. Despite the lower level of contamination, the occurrence rate of FQ-resistant bacteria was found to be higher in Vietnam than Thailand (Takasu et al., 2011). Takasu et al. (2011) demonstrated that there is no relationship between the concentration of FQs in the environment and the rate of bacterial resistance. Kümmerer (2003) reported that exposure to a sub-inhibitory concentration induces the emergence of resistant bacteria in aquatic environments. It was reported that FQs at concentrations 10–100 times lower than the MIC induce the expression of a functional gene (Herold et al., 2005), mutations (Gillespie et al., 2005), and morphological changes (Loubeyre et al., 1993) at the single-cell level. Low concentrations are thus effective inducers of drug-resistant bacteria. The effect of disinfectants on bacteria is considerably reduced in the presence of organic matter (Kawamura-Sato et al., 2008), suggesting that the actual active concentration in the environment might be lower than analysis suggests. The importance of low concentrations of FQs to the development of antibiotic resistance should not be ignored, and additional research involving both instrumental analyses and bioassays is needed to estimate the actual concentration of FQs acting upon environmental bacteria.

A broad range of bacteria can acquire resistance to FQs, including enteric bacteria (*Escherichia coli*), pathogenic bacteria (e.g., *Acinetobacter*), and aquatic bacteria (e.g., *Brevundimonus*). Proteobacteria and Actinobacteria are the major taxa of FQ-resistant bacteria (Takasu et al., 2011), indicating that FQ-resistant bacteria are not limited to specific groups. It was hypothesized that bacteria from humans and animals as well as natural aquatic bacteria are potential reservoirs of FQ resistance genes. A number of resistance genes have been identified in FQ-resistant bacteria, including *qnrA*, *qnrB*, *qnrS*, and *aac(6′)-Ib-cr* (Takasu et al., 2011). Since *qnrA*, *qnrB*, and *qnrS* are thought to have originated from the chromosomes of water-dwelling bacteria (Poirel et al., 2005a,b), it is possible that these genes can be transferred from aquatic bacteria to human bacteria (Hernández et al., 2011). To date, PMQR genes including *aac (6′)-Ib-cr*, which are linked with other drug resistance genes and are transferable, have only been discovered in Gammaproteobacteria such as *Vibrio* (Poirel et al., 2005a), *Shewanella* (Poirel et al., 2005b), and *Aeromonas* (Cattoir et al., 2008; Picao et al., 2008) in the environment. The number of FQ-resistant *Salmonella enterica* isolates found in Thailand increased in the late 1990s, and the resistant isolates possessed the same *gyrA* region, suggesting rapid spread of the resistant bacteria (Hakanen et al., 2001). The *gyrA* mutation is located on the chromosome, but recent research indicates that *gyrA* can also be transferred (Ferrandiz et al., 2000). Spreading of both resistant bacteria and chromosomal resistance genes might be a cause of the observed widespread drug resistance in Asia. However, precisely how FQ resistance genes are spread among various environments is not known, although mutation-based resistance and transferable genes should be considered. The dynamics of FQ resistance genes are of interest and importance from the viewpoints of human clinical and gene evolution studies.

SULFONAMIDE RESISTANCE

Sulfonamides, which are also synthetic antibiotics, have been widely used to treat bacterial and protozoan infections in humans, domestic animals, and aquaculture species since their introduction to clinical practice in 1935 (Perreten and Boerlin, 2003; Le and Munekage, 2004; Blahna et al., 2006). Sulfonamides inhibit folate biosynthesis by competing with the natural substrate *p*-aminobenzoic acid for binding to dihydropteroate synthase (DHPS), an enzyme in the folic acid synthesis pathway. While the use of sulfonamides in humans has decreased in developed countries, they are still frequently used in developing Asian countries due to their low cost (Macrolides are 1–3 $/tablet, whereas sulfonamides are 2 cent/tablet in Vietnam). Sulfamethoxazole (SMX), a commonly used sulfonamide analog, was detected at ppm levels in shrimp ponds in Vietnam in the late 1990s (Le and Munekage, 2004). Although such levels are extremely high, SMX levels in rivers in Japan and Vietnam are typically around 100 ppt in recent years (Managaki et al., 2007). This was confirmed in various locations in Vietnam (Hoa et al., 2011), where concentrations were in the 600–4000 ppt range throughout the year in a city canal. Trimethoprim was detected in the 20–1800 ppt range as well, probably because it is often used with SMX. In livestock farm wastewater, the concentration of SMX was lower, in the 68–600 ppt range, whereas that of sulfamethazine was much higher (400–6000 ppt). Other sulfonamides were either not detected or found at trace levels, suggesting that SMX and sulfamethazine are the major sulfonamides in use in Vietnam at present.

Since sulfonamides inhibit the formation of dihydrofolic acid (Perreten and Boerlin, 2003), bacterial resistance to sulfonamides can occur through mutations in the chromosomal DHPS gene (*folP*) or through acquisition of an alternative DHPS gene (*sul*) whose product has a low affinity for sulfonamides (Perreten and Boerlin, 2003). Acquisition of sulfonamide resistance through *sul* genes is the most prevalent mechanism (Enne et al., 2002; Perreten and Boerlin, 2003). Sulfonamide resistance is globally prevalent among human and animal pathogens (Kerrn et al., 2002; Perreten and Boerlin, 2003; Antunes et al., 2005); however, the presence of *sul* genes is not equally distributed among bacterial populations (Kerrn et al., 2002; Antunes et al., 2005; Hammerum et al., 2006). Reports from Asia are not uniform regarding the distribution of *sul* genes. The *sul2* gene, which is related to class I integron, is common in *Acinetobacter* isolated from fish farms and chicken manure in Thailand (Agersø and Petersen, 2007). However, Hoa et al. (2008) reported that *sul1* was the major *sul* gene among SMX-resistant bacteria isolated from VAC farms, city canals, and aquaculture sites in Vietnam. Most of these reports were based on isolated bacteria, which may bias interpretation of results due to the processes of isolation; therefore, we cannot obtain an accurate assessment of the distribution of antibiotic resistance genes from culturable bacteria alone. A direct quantification approach indicated that *sul1* is the major sulfonamide resistance gene in lagoon and river waters in the USA (Pruden et al., 2006). It is likely that such non-culture monitoring of antibiotic resistance genes will increase in the near future (for example, Pei et al., 2006). A culture-dependent study of SMX-resistant bacteria revealed that *Acinetobacter* is one of the major reservoirs of *sul* genes in Asian aquatic environments (Agersø and Petersen, 2007; Hoa et al., 2011). Since *Acinetobacter*, especially *A. baumannii*, is known to be an important opportunistic pathogen with multi-drug resistance (Vila and Pachón, 2011), the risk posed by environmental *A. baumannii* to human health should be examined further. Low concentration of SMX can affect on bacterial nitrate metabolism and select bacterial species (Underwood et al., 2011), suggesting importance of trace contamination by SMX.

TETRACYCLINE RESISTANCE

Tetracyclines are a family of broad-spectrum antibiotics that includes tetracycline, oxytetracycline (OTC), chlortetracycline, doxycycline, and minocycline (Chopra and Roberts, 2001). These antibiotics inhibit protein synthesis in Gram-positive and Gram-negative bacteria by preventing the binding of aminoacyl-tRNA molecules to the 30S ribosomal subunit (Geigenmuller and Nierhaus, 1986; Ross et al., 1998). Owing to their broad-spectrum activity and low toxicity, tetracyclines are used in the treatment of a number of human skin and dental diseases. Tetracyclines are also used in agriculture as growth promoters in farm animals and are used widely as prophylaxes in plant agriculture and aquaculture around the world (Chopra and Roberts, 2001), including Southeast Asia (Thuy et al., 2011). The first-generation tetracyclines include OTC, chlorotetracycline, and 6-demethylchlorotetracycloine, which were developed in the 1940s.

Minocycline and doxycycline were launched as second-generation drugs in the 1960s (Thaker et al., 2010). A new glycylcycline derivative, tigecycline, which is active against most OTC-resistant bacteria, *Acinetobacter*, MRSA, and extended-spectrum β-lactamase (ESBL) producers, was licensed in 2005 (Livermore, 2005). Despite the variety of available analogs, OTC is still commonly used in animal production and aquaculture (Holmström et al., 2003; Nonaka et al., 2007; and others), although use as growth promoters has been banned in EU until 2006 (Castanon, 2007). As tetracycline-resistant bacteria, high occurrence of tetracycline-resistant *Salmonella* from human (50%) and animals (pig 34% and poultry 76%) are reported in Vietnam (Vo et al., 2010), as well as *Enterococcus* and *E. coli* (both 80%<; Dang et al., 2011). Similar results are known in Thailand, e.g., chicken (42%) and chicken meat (45%), and pork (11%) and pork meat (20%; Ogasawara et al., 2008).

Tetracycline resistance genes, *tet* series, from the resistant bacteria have been well-studied (Roberts, 2005; and others). The *tet* genes are classified by mechanism of action. Studies have indicated that some *tet* genes are persistent in aquaculture site without selective pressure (Tamminen et al., 2011), pristine environments and in animals (Gilliver et al., 1999; Rahman et al., 2008a,b), suggesting that they have a natural origin. Tracking of *tet* genes in aquatic environments, including pristine areas, has been examined in the USA (Pruden et al., 2006; Storteboom et al., 2010a,b), Japan (Kim et al., 2004; Nonaka et al., 2007), and other countries (Zhang and Zhang, 2011); however, similar research in Indochina aquatic environments is limited (Kobayashi et al., 2007a; Suzuki et al., 2008). Monitoring and phylogenetic analyses are needed if we are to obtain a thorough understanding of the origin and spread dynamics of *tet* genes. Kobayashi et al. (2007a) detected *tet(M)*, *tet(S)*, and *tet(W)* genes throughout the Mekong River Delta. They found that *tet(S)* and *tet(W)* have only one genotype each, while *tet(M)* has at least two genotypes. One *tet(M)* genotype is identical to the gene encoded in various plasmids and transposons of Gram-positive and Gram-negative bacteria, and another is similar to a gene encoded in Tn1545 of *Enterococcus faecalis* (99% identity in PCR product, 170 bp/171 bp). Since the two types could be found in the same sample, it was assumed that more than one source of the gene exists. This hypothesis was partly confirmed by microbial diversity analysis using denatured gradient gel electrophoresis analysis, which indicated a positive relationship between the Shannon index (H′) value and the *tet* gene detection- and OTC-resistant bacteria occurrence-rates across a wide area, ranging from the Mekong River and Tonle Sap Lake, Cambodia to the South China Sea (Suzuki et al., 2008).

Gene exchange and horizontal transfer among various bacterial species in natural environments have been demonstrated (Smets and Barkay, 2005). Ribosomal protection protein (RPP) genes, including *tet(M)*, *tet(S)*, and *tet(W)*, are transferred among bacteria (Chopra and Roberts, 2001), and this phenomenon was experimentally confirmed using combinations of marine bacteria (gene donors) and *E. coli* (Neela et al., 2009). However, another hypothesis was proposed by Kobayashi et al. (2007b), which holds that the RPP genes were derived through duplication and divergence of an ancient GTPase before the divergence of the three domains. The RPPs may have originally provided ribosomal protection against other chemical substances in the environment. It is known that exposing microbial assemblages to one toxicant can result in indirect selection for bacteria with resistance to multiple, chemically unrelated toxicants (Baker-Austin et al., 2006; Baker et al., 2006). For example, exposure to Cd, Ni, ampicillin, and tetracycline significantly increases the frequency of bacterial resistance to multiple, chemically unrelated metals and antibiotics (Stepanauskas et al., 2006). Metals and antibiotics are known not only for being associated to similar efflux pumps, but also for inducing co-resistance (Baker-Austin et al., 2006; Baker et al., 2006). Aquatic environments in Indochina are highly contaminated with many kinds of persistent organic pollutants, metals (Suzuki and Takada, 2009), and antibiotics (Managaki et al., 2007; Hoa et al., 2011). Under such conditions, environmental bacteria can acquire drug resistance naturally over time as well as through horizontal gene transfer. Both transferable paralogous resistance genes and canonical resistance genes might be present in natural environments. From the viewpoint of both drug resistance and evolutionary biology, RPP genes are thus of considerable interest (Kobayashi et al., 2007b).

CONCLUSION

Indochina aquatic environments are particularly vulnerable to the development of antibiotic-resistant bacteria due to pollution with antibiotics and other chemicals in the absence of adequate wastewater treatment systems. Recent important findings concerning antibiotic resistance are as follows: (1) sub-lethal concentrations of antibiotics accelerate the development of antibiotic resistance (Yeh et al., 2009; Gullberg et al., 2010), and (2) chemicals other than antibiotics can promote development of antibiotic resistance (Alonso et al., 2001; Baker-Austin et al., 2006). Both of these scenarios are suspected to occur in natural environments, especially in Asian countries. Correlation between the number of antibiotic drugs detected and the occurrence of multi-drug-resistant bacteria in Vietnam was found (Hoa et al., 2011), indicating that multi-drug contamination is almost certain to result in an increase in the prevalence of multi-drug-resistant bacteria in aquatic environments. It is important to note that contamination with various antibiotics correlates strongly with induction of multi-drug resistance, even if the contaminant concentration is low. Multi-drug resistance is a serious issue, particularly in Asia, and is likely to become of even greater concern in the future. Furthermore, contamination of antibiotics with trace concentrations can alter microbial ecosystem in terms of metabolism and diversity (Underwood et al., 2011), indicating the importance to consider unexpectedly effect than antimicrobial resistance. There is still a considerable gap in knowledge regarding the environmental chemistry and microbiology in the environment. Chemical and microbial monitoring in aquatic environments should thus be accelerated worldwide.

ACKNOWLEDGMENTS

This work was partly supported by the 21COE and GCOE Programs, Ehime University, MEXT, Japan, and Grant-in-Aids for Scientific Research from JSPS (19405004, 22241014, 22254001).

REFERENCES

Agersø, Y., and Petersen, A. (2007). The tetracycline resistance determinant Tet 39 and the sulphonamide resistance gene sulII are common among resistant *Acinetobacter* spp. isolated from integrated fish farms in Thailand. *J. Antimicrob. Chemother.* 59, 23–27.

Alonso, A., Sánchez, P., and Martínez, J. L. (2001). Environmental selection of antibiotic resistance genes. *Environ. Microbiol.* 3, 1–9.

Antunes, P., Machado, J., Sousa, J. C., and Peixe, L. (2005). Dissemination of sulfonamide resistance genes (sul1, sul2, and sul3) in Portuguese *Salmonella enterica* strains and relation with integrons. *Antimicrob. Agents Chemother.* 49, 836–839.

Baker, B. J., Wright, M. S., Stepanauskas, R., and McArthur, J. V. (2006). Co-selection of antibiotic and metal resistance. *Trends Microbiol.* 14, 176–182.

Baker-Austin, C., Wright, M. S., Stepanauskas, R., and McArthur, J. V. (2006). Co-selection of antibiotic and metal resistance. *Trends Microbiol.* 14, 176–182.

Baquero, F., Martínez, J.-L., and Cantón, R. (2008). Antibiotics and antibiotic resistance in water environments. *Curr. Opin. Biotechnol.* 19, 1–6.

Blahna, M. T., Zalewski, C. A., Reuer, J., Kahlmeter, G., Foxman, B., and Marrs, C. F. (2006). The role of horizontal gene transfer in the spread of trimethoprim–sulfamethoxazole resistance among uropathogenic *Escherichia coli* in Europe and Canada. *J. Antimicrob. Chemother.* 57, 666–672.

Burhenne, J., Ludwig, M., Nikoloudis, P., and Spiteller, M. (1997). Primary photoproducts and half-lives. *Environ. Sci. Pollut. Res. Int.* 4, 10–15.

Castanon, J. I. R. (2007). History of the use of antibiotic as growth promoters in European poultry feeds. *Poult. Sci.* 86, 2466–2471.

Cattoir, V., Poirel, L., Aubert, C., Soussy, C. J., and Nordmann, P. (2008). Unexpected occurrence of plasmid-mediated quinolone resistance determinants in environmental *Aeromonas* spp. *Emerging Infect. Dis.* 14, 231–237.

Chopra, I., and Roberts, M. (2001). Tetracycline antibiotics: mode of action, applications, molecular biology, and epidemiology of bacterial resistance. *Microbiol. Mol. Biol. Rev.* 65, 232–260.

Dang, S. T. T., Petersen, A., Truong, D. V., Chu, H. T. T., and Dalsgaard, A. (2011). Impact of medicated feed on the development of antimicrobial resistance in bacteria at integrated pig-fish farms in Vietnam. *Appl. Environ. Microbiol.* 77, 4494–4498.

Duong, H. A., Pham, N. H., Nguyen, H. T., Hoang, T. T., Pham, H. V., Pham, V. C., Berg, M., Giger, W., and Alder, A. C. (2008). Occurrence, fate and antibiotic resistance of fluoroquinolone antibacterials in hospital wastewaters in Hanoi, Vietnam. *Chemosphere* 72, 968–973.

Enne, V. I., King, A., Livermore, D. M., and Hall, L. M. C. (2002). Sulfonamide resistance in Haemophilus influenzae mediated by acquisition of sul2 or a short insertion in chromosomal folP. *Antimicrob. Agents Chemother.* 46, 1934–1939.

Ferrandiz, M. J., Fenoll, A., Linares, J., and De La Campa, A. G. (2000). Horizontal transfer of parC and gyrA in fluoroquinolone-resistant clinical isolates of *Streptococcus pneumoniae*. *Antimicrob. Agents Chemother.* 44, 840–847.

Fukuda, H., Hosaka, M., Hirai, K., and Iyobe, S. (1990). New norfloxacin resistance gene in *Pseudomonas aeruginosa* PAO. *Antimicrob. Agents Chemother.* 34, 1757–1761.

Geigenmuller, U., and Nierhaus, K. H. (1986). Tetracycline can inhibit tRNA binding to the ribosomal P site as well as to the A site. *Eur. J. Biochem.* 161, 723–726.

Gillespie, S. H., Basu, S., Dickens, A. L., O'Sullivan, D. M., and McHugh, T. D. (2005). Effect of subinhibitory concentrations of ciprofloxacin on *Mycobacterium fortuitum* mutation rates. *J. Antimicrob. Chemother.* 56, 344–348.

Gilliver, M. A., Bennett, M., Begon, M., Hazel, S. M., and Hart, C. A. (1999). Antibiotic resistance found in wild rodents. *Nature* 401, 233–234.

Göbel, A., Thomsen, A., McArdell, C. S., Joss, A., and Giger, W. (2005). Occurrence and sorption behavior of sulfonamides, macrolides, and trimethoprim in activated sluge treatment. *Environ. Sci. Technol.* 39, 3981–3989.

Gräslund, S., Holmström, K., and Wahlström, A. (2003). A field survey of chemicals and biological products used in shrimp farming. *Mar. Pollut. Bull.* 46, 81–90.

Grave, K., Lingaas, E., Bangen, M., and Rønning, M. (1999). Surveillance of the overall consumption of antibacterial drugs in humans, domestic animals and farmed fish in Norway in 1992 and 1996. *J. Antimicrob. Chemother.* 43, 243–252.

Gullberg, E., Cao, S., Berg, O. G., Ilba, C., Sandegren, L., Hughes, D., and Andersson, D. I. (2010). Selection of resistant bacteria at very low antibiotic concentrations. *PLoS Pathog.* 7, e1002158. doi:10.1371/journal.ppat.1002158

Hakanen, A., Kotilainen, P., Huovinen, P., Helenius, H., and Siitonen, A. (2001). Reduced fluoroquinolone susceptibility in *Salmonella enterica* serotypes in travelers returning from Southeast Asia. *Emerging Infect. Dis.* 7, 996–1003.

Hammerum, A. M., Sandvang, D., Andersen, S. R., Seyfarth, A. M., Porsbo, L. J., Frimodt-Møller, N., and Heuer, O. E. (2006). Detection of sul1, sul2 and sul3 in sulphonamide resistant *Escherichia coli* isolates obtained from healthy humans, pork and pigs in Denmark. *Int. J. Food Microbiol.* 106, 235–237.

Hernández, H. A., Sánchez, M. B., and Martínez, J. L. (2011) Quinolone resistance: much more than predicted. *Front. Microbiol.* 2:22. doi:10.3389/fmicb.2011.00022

Herold, S., Siebert, J., Huber, A., and Schmidt, H. (2005). Global expression of prophage genes in *Escherichia coli* O157:H7 strain EDL933 in response to Norfloxacin. *Antimicrob. Agents Chemother.* 49, 931–944.

Heuer, H., and Smalla, K. (2007). Manure and sulfadiazine synergistically increased bacterial antibiotic resistance in soil over at least two months. *Environ. Microbiol.* 9, 657–666.

Hirai, H., Aoyama, H., Suzue, S., Irikura, T., Iyobe, S., and Mitsuhashi, S. (1986). Isolation and characterization of norfloxacin-resistant mutants of *Escherichia coli* K-12. *Antimicrob. Agents Chemother.* 30, 248–253.

Hirai, H., Suzue, S., Irikura, T., Iyobe, S., and Mitsuhashi, S. (1987). Mutations producing resistance to Norfloxacin in *Pseudomonas aeruginosa*. *Antimicrob. Agents Chemother.* 31, 582–586.

Hirsch, R., Ternes, T., Haberer, K., and Kratz, K.-L. (1999). Occurrence of antibiotics in the aquatic environment. *Sci. Total Environ.* 225, 109–118.

Hoa, P. T. P., Nonaka, L., Viet, P. H., and Suzuki, S. (2008). Detection of the sul1, sul2, and sul3 genes in sulfonamide-resistant bacteria from wastewater and shrimp ponds of north Vietnam. *Sci. Total Environ.* 405, 377–384.

Hoa, P.T. P., Managaki, S., Nakada, N., Takada, H., Shimizu, A., Anh, D. H., Viet, P. H., and Suzuki, S. (2011). Antibiotic contamination and occurrence of antibiotic-resistant bacteria in aquatic environments of northern Vietnam. *Sci. Total Environ.* 409, 2894–2901.

Holmström, K., Gräslund, S., Wahlstrom, A., Poungshompoo, S., Bengtsson, B.-E., and Kautsky, N. (2003). Antibiotic use in shrimp farming and implications for environmental impacts and human health. *Int. J. Food Sci. Technol.* 38, 255–266.

Hop, L. T. (2003). Programs to improve production and consumption of animal source foods and malnutrition in Vietnam. *J. Nutr.* 133, 4006S–4009S.

Kawamura-Sato, K., Wachino, J., Kondo, T., Ito, H., and Arakawa, Y. (2008). Reduction of disinfectant bactericidal activities in clinically isolated *Acinetobacter* species in the presence of organic material. *J. Antimicrob. Chemother.* 61, 568–576.

Kemper, N. (2008). Veterinary antibiotics in the aquatic and terrestrial environment. *Ecol. Indic.* 8, 1–13.

Kerrn, M. B., Klemmensen, T., Frimodt-Møller, N., and Espersen, F. (2002). Susceptibility of Danish *Escherichia coli* strains isolated from urinary tract infections and bacteraemia, and distribution of *sul* genes conferring sulphonamide resistance. *J. Antimicrob. Chemother.* 50, 513–516.

Kim, S.-C., and Carlson, K. (2007). Temporal and spatial trends in the occurrence of human and veterinary antibiotics in aqueous and river sediment matrices. *Environ. Sci. Technol.* 41, 50–57.

Kim, S.-R., Nonaka, L., and Suzuki, S. (2004). Occurrence of tetracycline resistance genes tet(M) and tet(S) in bacteria from marine aquaculture sites. *FEMS Microbiol. Lett.* 237, 147–156.

Kobayashi, T., Nonaka, L., Maruyama, F., and Suzuki, S. (2007a). Molecular evidence for the ancient origin of the ribosomal protection protein that mediates tetracycline resistance in bacteria. *J. Mol. Evol.* 65, 228–235.

Kobayashi, T., Suehiro, F., Tuyen, B. C., and Suzuki, S. (2007b). Distribution and diversity of tetracycline resistance genes encoding ribosomal protection proteins in Mekong river sediments in Vietnam. *FEMS Microbiol. Ecol.* 59, 729–737.

Kolpin, D. W., Furlong, E. T., Meyer, M. T., Thurman, E. M., Zaugg, S. D., Barber, L. B., and Buxton, H. T. (2002). Pharmaceuticals, hormones, and other organic wastewater contaminants in U.S. streams, 1999–2000: a national reconnaissance. *Environ. Sci. Technol.* 36, 1202–1211.

Kümmerer, K. (2003). Significance of antibiotics in the environment. *J. Antimicrob. Chemother.* 52, 5–7.

Kümmerer, K. (2004). Resistance in the environment. *J. Antimicrob. Chemother.* 54, 311–320.

Kümmerer, K. (2009a). Antibiotics in the aquatic environment – a review – part I. *Chemosphere* 75, 417–434.

Kümmerer, K. (2009b). Antibiotics in the aquatic environment – a review – part II. *Chemosphere* 75, 435–441.

Lai, H.-T., and Lin, J.-J. (2009). Degradation of oxolinic acid and flumequine in aquaculture pond waters and sediments. *Chemosphere* 75, 462–468.

Le, T. X., and Munekage, Y. (2004). Residues of selected antibiotics in water and mud from shrimp ponds in mangrove areas in Vietnam. *Mar. Pollut. Bull.* 49, 922–929.

Le, T. X., Munekage, Y., and Kato, S.-I. (2005). Antibiotic resistance in bacteria from shrimp farming in mangrove areas. *Sci. Total Environ.* 349, 95–105.

Lindsey, M. E., Meyer, M., and Thurman, E. M. (2001). Analysis of trace levels of sulfonamide and tetracycline antimicrobials in groundwater and surface water using solid-phase extraction and liquid chromatography/mass spectrometry. *Anal. Chem.* 73, 4640–4646.

Livermore, D. M. (2005). Tigecycline: what is it, and where should it be used? *J. Antimicrob. Chemother.* 56, 611–614.

Loubeyre, C., Desnottes, J. F., and Moreau, N. (1993). Influence of subinhibitory concentrations of antibacterials on the surface properties and adhesion of *Escherichia coli*. *J. Antimicrob. Chemother.* 31, 37–45.

Managaki, S., Murata, A., Takada, H., Tuyen, B. C., and Chiem, N. H. (2007). Distribution of macrolides, sulfonamides and trimethoprim in tropical waters: ubiquitous occurrence of veterinary antibiotics in the Mekong delta. *Environ. Sci. Technol.* 41, 8004–8010.

McArdell, C. S., Molnar, E., Suter, M. J.-F., and Giger, W. (2003). Occurrence and fate of macrolide antibiotics in wastewater treatment plants and in the Glatt valley watershed, Switzerland. *Environ. Sci. Technol.* 37, 5479–5486.

Metcalfe, C., Miao, X.-S., Hua, W., Letcher, R., and Servos, M. (2004). "Pharmaceuticals in the Canadian environment," in *Pharmaceuticals in the Environment-Sources, Fate, Effects and Risks*, 2nd Edn, ed. K. Kümmerer (Berlin: Springer-Verlag), 67–90.

Neela, F. A., Nonaka, L., Rahman, M. H., and Suzuki, S. (2009). Transfer of the chromosomally encoded tetracycline resistance gene tet(M) from marine bacteria to *Escherichia coli* and *Enterococcus faecalis*. *World J. Microbiol. Biotechnol.* 25, 1095–1101.

Nhiem, D. V., Paulsen, P., Suriyasathaporn, W., Smulders, F. J. M., Kyule, M. N., Baumann, M. P. O., Zessin, K. H., and Ngan, P. H. (2006). Preliminary analysis of tetracycline residues in marketed pork in Hanoi, Vietnam. *Ann. N. Y. Acad. Sci.* 1081, 534–542.

Nonaka, L., Ikeno, K., and Suzuki, S. (2007). Distribution of tetracycline resistance gene, tet(M), in gram-positive and gram-negative bacteria isolated from sediment and seawater at a coastal aquaculture site in Japan. *Microbes Environ.* 22, 355–364.

Nowara, A., Burhenne, J., and Spiteller, M. (1997). Binding of fluoroquinolone carboxylic acid derivatives to clay minerals. *J. Agric. Food Chem.* 45, 1459–1463.

Ogasawara, N., Tran, T. P., Ly, T. L. K., Nguyen, T. T., Iwata, T., Okatani, A. T., Watanabe, M., Taniguchi1, T., Hirota, Y., and Hayashidani, H. (2008). Antimicrobial susceptibilities of *Salmonella* from domestic animals, food and human in the Mekong Delta, Vietnam. *J. Vet. Med. Sci.* 70, 1159–1164.

Ok, Y.-S., Kim, S.-C., Kim, K.-R., Lee, S.-S., Moon, D.-H., Lim, K.-J., Sung, J.-K., Hur, S.-O., and Yang, J.-E. (2011). Monitoring of selected veterinary antibiotics in environmental compartments near a composting facility in Gangwon Province, Korea. *Environ. Monit. Assess.* 174, 693–701.

Pei, R., Kim, S.-C., Carlson, K. H., and Pruden, A. (2006). Effect of river landscape on the sediment concentrations of antibiotics and corresponding antibiotic resistance genes (ARG). *Water Res.* 40, 2427–2435.

Perreten, V., and Boerlin, P. (2003). A new sulfonamide resistance gene (sul3) in *Escherichia coli* is widespread in the pig population of Switzerland. *Antimicrob. Agents Chemother.* 47, 1169–1172.

Petersen, A., and Dalsgaard, A. (2003). Species composition and antimicrobial resistance genes of *Enterococcus* spp., isolated from integrated and traditional fish farms in Thailand. *Environ. Microbiol.* 5, 395–402.

Picao, R. C., Poirel, L., Demarta, A., Silva, C. S. F., Corvaglia, A. R., Petrini, O., and Nordmann, P. (2008). Plasmid-mediated quinolone resistance in *Aeromonas allosaccharophila* recovered from a Swiss lake. *J. Antimicrob. Chemother.* 62, 948–950.

Poirel, L., Liard, A., Rodriguez-Martinez, J. M., and Nordmann, P. (2005a). Vibrionaceae as a possible source of Qnr-like quinolone resistance determinants. *J. Antimicrob. Chemother.* 56, 1118–1121.

Poirel, L., Liard, A., Rodriguez-Martinez, J. M., Mammeri, H., Liard, A., and Nordmann, P. (2005b). Origin of plasmid-mediated quinolone resistance determinant QnrA. *Antimicrob. Agents Chemother.* 49, 3523–3525.

Pruden, A., Pei, R., Storteboom, H., and Carlson, K. H. (2006). Antibiotic resistance genes as emerging contaminants: studies in northern Colorado. *Environ. Sci. Technol.* 40, 7445–7450.

Rahman, M. H., Nonaka, L., Tago, R., and Suzuki, S. (2008a). Occurrence of two genotypes of tetracycline (TC) resistance gene tet(M) in the TC-resistant bacteria in marine sediments of Japan. *Environ. Sci. Technol.* 42, 5055–5061.

Rahman, M. H., Sakamoto, K. Q., Nonaka, L., and Suzuki, S. (2008b). Occurrence and diversity of the tetracycline resistance gene tet(M) in enteric bacteria of Antarctic Adelie penguins. *J. Antimicrob. Chemother.* 62, 627–628.

Roberts, M. C. (2005). Update on acquired tetracycline resistance genes. *FEMS Microbiol. Lett.* 245, 195–203.

Robicsek, A., Jacoby, G. A., and Hooper, D. C. (2006). The worldwide emergence of plasmid-mediated quinolone resistance. *Lancet Infect. Dis.* 6, 629–640.

Ross, J. I., Eady, E. A., Cove, J. H., and Cunliffe, W. J. (1998). 16S rRNA mutation associated with tetracycline resistance in a gram-positive bacterium. *Antimicrob. Agents Chemother.* 42, 1702–1705.

Samanidou, V. F., Christodoulou, E. A., and Papadoyannis, I. N. (2005). Determination of fluoroquinolones in edible animal tissue samples by high performance liquid chromatography after solid phase extraction. *J. Sep. Sci.* 28, 555–565.

Segura, P. A., François, M., Gagnon, C., and Sauve, S. (2009). Review of the occurrence of anti-infectives in contaminated wastewaters and natural and drinking waters. *Environ. Health Perspect.* 117, 675–684.

Sirichote, P., Bangtrakulnonth, A., Tianmanee, K., Unahalekhaka, A., Oulai, A., Chittaphithakchai, P., Kheowrod, P., and Hendriksen, R. S. (2010). Serotypes and antimicrobial resistance of *Salmonella enteritica* ssp in central Thailand, 2001-1006. *Southeast Asian Trop. J. Med. Public Health* 41, 1405–1415.

Smets, B. F., and Barkay, T. (2005). Horizontal gene transfer: perspectives at a crossroads of scientific disciplines. *Nat. Rev. Microbiol.* 3, 675–678.

Stepanauskas, R., Glenn, T. C., Jagoe, C. H., Tuckfield, R. C., Lindell, A. H., King, C. J., and McArthur, V. J. (2006). Coselection for microbial resistance to metals and antibiotics in freshwater microcosms. *Environ. Microbiol.* 8, 1510–1514.

Storteboom, H., Arabi, M., Davis, J. G., Crimi, B., and Pruden, A. (2010a). Identification of antibiotic-resistance-gene molecular signatures suitable as tracers of pristine river, urban, and agricultural sources. *Environ. Sci. Technol.* 44, 1947–1953.

Storteboom, H., Arabi, M., Davis, J. G., Crimi, B., and Pruden, A. (2010b). Tracking antibiotic resistance genes in the South Platte River basin using molecular signatures of urban, agricultural, and pristine sources. *Environ. Sci. Technol.* 44, 7397–7404.

Suzuki, S., Kobayashi, T., Suehiro, F., Tuyen, B. C., and Tana, S. T. (2008). High occurrence rate of tetracycline (TC)-resistant bacteria and TC resistant genes relates to microbial diversity in sediment of Mekong river main waterway. *Microbes Environ.* 23, 149–152.

Suzuki, S., and Takada, H. (eds). (2009). *Chemical Pollution in Indochina, Contamination Status, Ecosystem Impact and Remediation Technology*. Hadano: Tokai University Press, 200.

Takasu, H., Suzuki, S., Reungsang, A., and Viet, P. H. (2011). Fluoroquinolone (FQ) contamination does not correlate with occurrence of FQ-resistant bacteria in aquatic environments of Vietnam and Thailand. *Microbes Environ.* 26, 135–143.

Tamminen, M., Karkman, A., Lõhmus, A., Muziasari, W. I., Takasu, H., Wada, S., Suzuki, S., and Virta, M. (2011). Tetracycline resistance genes persist at aquaculture farms in the absence of selection pressure. *Environ. Sci. Technol.* 45, 386–391.

Thaker, M., Spanogiannopoulos, P., and Wright, G. D. (2010). The tetracycline resistome. *Cell. Mol. Life Sci.* 67, 419–431.

Thuy, H. T. T., Nga, L. P., and Loan, T. T. C. (2011). Antibiotic contaminants

in coastal wetlands from Vietnamese shrimp farming. *Environ. Sci. Pollut. Res. Int.* 18, 835–841.

Underwood, J. C., Harvey, R. W., Metge, D. W., Repert, D. A., Baumgartner, L. K., Smith, R. L., Roane, T. M., and Barber, L. B(2011). Effect of the antimicrobial sulfamethoxazole on groundwater bacterial enrichment. *Environ. Sci. Technol.* 45, 3096–3101.

Vila, J., and Pachón, J. (2011). *Acinetobacter baumannii* resistant to everything: what should we do? *Clin. Microbiol. Infect.* 17, 955–956.

Vo, A. T. T., van Duijkeren, E., Gaastra, W., and Fluit, A. C. (2010). Antimicrobial resistance, class 1 integrons, and genomic island 1 in *Salmonella* isolated from Vietman. *PLoS ONE* 5, e9440. doi:10.1371/journal.pone.0009440

Wei, R., Ge, F., Huang, S., Chen, M., and Wanga, R. (2011). Occurrence of veterinary antibiotics in animal wastewater and surface water around farms in Jiangsu Province, China. *Chemosphere* 82, 1408–1414.

Yeh, P. J., Hegrenes, M. J., Aiden, P. A., and Kishony, R. (2009). Drug interactions and the evolution id antibiotic resistance. *Nat. Rev. Microbiol.* 7, 460–466.

Zhang, X.-X., and Zhang, T. (2011). Occurrence, abundance, and diversity of tetracycline resistance genes in 15 sewage treatment plants across China and other global locations. *Environ. Sci. Technol.* 45, 2598–2604.

Zhang, X.-X., Zhang, T., and Fang, H. P. P. (2009). Antibiotic resistance genes in water environment. *Appl. Microbiol. Biotechnol.* 82, 397–414.

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 11 November 2011; accepted: 07 February 2012; published online: 22 February 2012.

Citation: Suzuki S and Hoa PTP (2012) Distribution of quinolones, sulfonamides, tetracyclines in aquatic environment and antibiotic resistance in Indochina. Front. Microbio. ***3****:67. doi: 10.3389/fmicb.2012.00067*

This article was submitted to Frontiers in Antimicrobials, Resistance and Chemotherapy, a specialty of Frontiers in Microbiology.

frontiers in
MICROBIOLOGY

ORIGINAL RESEARCH ARTICLE
published: 10 April 2012
doi: 10.3389/fmicb.2012.00139

Excretion of antibiotic resistance genes by dairy calves fed milk replacers with varying doses of antibiotics

Callie H. Thames[1], Amy Pruden[2]*, Robert E. James[1], Partha P. Ray[1] and Katharine F. Knowlton[1]

[1] *Department of Dairy Science, Virginia Tech, Blacksburg, VA, USA*
[2] *Department of Civil and Environmental Engineering, Virginia Tech, Blacksburg, VA, USA*

Edited by:
Rustam I. Aminov, University of Aberdeen, UK

Reviewed by:
Jun Lin, The University of Tennessee, USA
Ludek Zurek, Kansas State University, USA
Peter Pristas, Slovak Academy of Sciences, Slovak Republic
Jun Liu, Mount Sinai School of Medicine, USA

****Correspondence:***
Amy Pruden, Department of Civil and Environmental Engineering, Virginia Tech, 403 Durham Hall (0246), Blacksburg, VA 24061, USA.
e-mail: apruden@vt.edu

Elevated levels of antibiotic resistance genes (ARGs) in soil and water have been linked to livestock farms and in some cases feed antibiotics may select for antibiotic resistant gut microbiota. The purpose of this study was to examine the establishment of ARGs in the feces of calves receiving milk replacer containing no antibiotics versus subtherapeutic or therapeutic doses of tetracycline and neomycin. The effect of antibiotics on calf health was also of interest. Twenty-eight male and female dairy calves were assigned to one of the three antibiotic treatment groups at birth and fecal samples were collected at weeks 6, 7 (prior to weaning), and 12 (5 weeks after weaning). ARGs corresponding to the tetracycline (*tet*C, *tet*G, *tet*O, *tet*W, and *tet*X), macrolide (*erm*B, *erm*F), and sulfonamide (*sul*1, *sul*2) classes of antibiotics along with the class I integron gene, *int*I1, were monitored by quantitative polymerase chain reaction as potential indicators of direct selection, co-selection, or horizontal gene transfer of ARGs. Surprisingly, there was no significant effect of antibiotic treatment on the absolute abundance (gene copies per gram wet manure) of any of the ARGs except *erm*F, which was lower in the antibiotic-treated calf manure, presumably because a significant portion of host bacterial cells carrying *erm*F were not resistant to tetracycline or neomycin. However, relative abundance (gene copies normalized to 16S rRNA genes) of *tet*O was higher in calves fed the highest dose of antibiotic than in the other treatments. All genes, except *tet*C and *int*I1, were detectable in feces from 6 weeks onward, and *tet*W and *tet*G significantly increased ($P < 0.10$), even in control calves. Overall, the results provide new insight into the colonization of calf gut flora with ARGs in the early weeks. Although feed antibiotics exerted little effect on the ARGs monitored in this study, the fact that they also provided no health benefit suggests that the greater than conventional nutritional intake applied in this study overrides previously reported health benefits of antibiotics. The results suggest potential benefit of broader management strategies, and that cost and risk may be avoided by minimizing incorporation of antibiotics in milk replacer.

Keywords: antibiotic resistance genes, manure, dairy calves, milk replacer

INTRODUCTION

The extensive use of antibiotics in animal agriculture and the development of antibiotic resistant bacteria have been cause for increasing concern. Livestock operations are often cited as a reservoir for resistant bacteria and antibiotic resistance genes (ARGs; Chee-Sanford et al., 2001; Smith et al., 2004; Sawant et al., 2007; McKinney et al., 2010); and antibiotic use has implications for both animal and human health. Antibiotics are fed to livestock at subtherapeutic levels for both growth promotion and disease prevention, because their use can reduce morbidity and mortality (e.g., Wileman et al., 2009). However, the unintentional selection of bacteria that are resistant to antibiotics could have important human health consequences, with several studies noting identical resistance elements in both humans and food animals (e.g., Boerlin et al., 2001; Lauderdale et al., 2002; Ho et al., 2010).

Based on concerns about human health effects, subtherapeutic use of antibiotics was banned in the European Union in the late 1990s (Boerlin et al., 2001). A major trigger for earlier bans in Denmark and other Nordic countries, was linkage of avoparcin use in broiler chickens and swine to vancomycin resistant *Enterococcal* infections (VRE) in humans (Bager et al., 1997; Bates, 1997). In the United States (U.S.), the Food and Drug Administration (FDA) recently issued new rules limiting antibiotic use in milk replacers for calves (21CFR §520.1484; 21CFR §520.1660d). The precise effects of antibiotics in milk replacer is currently unknown, and the general consequences of antibiotic amendment in livestock feed are complex (Marshall and Levy, 2011). In the 3-years following the European ban, a slight decrease in antibiotic resistance was observed in rectal swabs collected from food animals at slaughter, and a documentable decrease in acquired fecal enterococci resistance in humans was also observed (Casewell et al., 2003). Following the Taiwan avoparcin ban in chickens in 2000, the incidence of VRE decreased significantly (Lauderdale et al., 2007). However, VRE did still persist following the ban and resistance to

other classes of antibiotics, including tetracyclines and macrolides, stayed the same or even increased. In support of this observation, vancomycin and macrolide ARGs have been noted to be present together on the same genetic element from isolates obtained from Danish pig herds (Aarestrup, 2000). Thus, it is critical to consider co-selection of resistance across antibiotic classes in evaluating impacts of feed antibiotics.

Effects of subtherapeutic antibiotic use on animal health have been difficult to assess. Based on medicated feed prescription rates, decreased rates of respiratory infections were observed in Swiss piglets and fattening pigs in the 3-years following the antibiotic ban relative to the 3-years prior, but gastrointestinal tract infections appeared to go up (Arnold et al., 2004). A marked increase in necrotic enteritis was noted in broiler chickens in Norway following the avoparcin ban, but this seemed to be ameliorated by the subsequent approval of narisin as a feed additive (Grave et al., 2004). Also, while therapeutic antibiotic use rates did indeed decrease in Switzerland (Arnold et al., 2004), Norway, and Sweden (Grave et al., 2006) following the E.U. ban, therapeutic use increased in weaning piglets in Denmark (Grave et al., 2006). Generally, a comprehensive examination of banning subtherapeutic antibiotic use in piglets, beef cattle, and poultry in Sweden, indicated that although temporary health problems and increases in antibiotic uses were noted, strategic management practices make possible competitive animal production and reduced antibiotic use (Wierup, 2001). Few controlled studies have examined the effects of antibiotic use on health (Berge et al., 2009a) or antibiotic resistance (Langford et al., 2003; Berge et al., 2006a; Kaneene et al., 2008; Pereira et al., 2011) in pre-weaned calves, and none have incorporated culture-independent techniques to quantify the establishment of ARGs in calf manure.

The objective of this study was to compare the establishment of a cross-section of ARGs in the feces of calves receiving milk replacer dosed with subtherapeutic and therapeutic antibiotics and their effect on overall health. In particular, the effect of oxytetracycline and neomycin in the milk replacer and weaning to starter grain containing monensin were of interest as typical practices in the U.S. The inclusion of oxytetracycline and neomycin in milk replacer for young calves is one of the most common uses of subtherapeutic antibiotics in the U.S. dairy industry. Neomycin is absorbed only very slightly by the animal (Aschbacher and Feil, 1994) and thus remains in the digestive tract where it controls growth of pathogenic organisms. Oxytetracycline, in contrast, is highly absorbed, so is considered useful for prevention and treatment of respiratory illnesses. One intentional contrast to conventional dairy practice incorporated in this study was feeding calves >2 times more protein than in traditional feeding programs, and >1.5 times more fat. This is because while conventional U.S. milk replacer feeding rates generally provide sufficient nutrients only for maintenance and modest growth [National Research Council (NRC), 2001], which could bias the evaluation of the health benefits of antibiotics.

In this study, 28 male and female dairy calves were assigned to control, subtherapeutic, or therapeutic antibiotic treatment groups at birth and fecal samples were collected at weeks 6, 7 (prior to weaning), and 12 (5 weeks after weaning). The abundance of a representative range of tetracycline (*tet*O, *tet*W, *tet*C, *tet*G, and *tet*X), sulfonamide (*sul*1 and *sul*2), and macrolide (*erm*B and *erm*F) ARGs as well as a class 1 transferable genetic element (*int*I1) was quantified in the calf feces as a culture-independent assessment of resistance. The specific ARGs examined were selected as indicators of direct antibiotic selection (i.e., *tet* ARGs) as well as potential co-selection of resistance to clinically relevant antibiotics (i.e., *sul* and *erm* ARGs) and overall horizontal gene transfer potential (i.e., *int*I1). Quantitative polymerase chain reaction (qPCR) was incorporated to provide a culture-independent assessment of the overall resistance potential incurred by these ARGs. Identifying factors that affect fecal excretion of ARGs by dairy calves will help assess the contribution of dairy farms to the overall loading of anthropogenic sources of antibiotic resistance to the environment and provide insight into effective management strategies for limiting the spread of antibiotic resistance.

MATERIALS AND METHODS

EXPERIMENTAL TREATMENTS

Forty-one newborn calves were blocked according to breed (Holstein or crossbred), birth order (two blocks), and gender. Crossbred calves were either Holstein- or Jersey-sired and out of Jersey × Holstein, Jersey × Holstein × Brown Swiss, or Jersey × Holstein × Swedish Red dams. Within each block, calves were randomly assigned to treatments at birth, resulting in the assignments summarized in **Table A1** in Appendix. Treatments were control (containing no antibiotics in the milk replacer), subtherapeutic (neomycin sulfate and oxytetracycline hydrochloride each fed at 10 mg/calf/day provided from day 1 until weaning), and therapeutic (no antibiotics in the milk replacer until day 36, then neomycin sulfate and oxytetracycline hydrochloride each fed at 1000 mg/calf/day for 14 day). All protocols and procedures were approved by the Virginia Tech Institutional Animal Care and Use Committee.

MANAGEMENT OF NEWBORN CALVES

Within 2 h of birth, calves were moved from the calving pen to individual stalls in an enclosed barn. Calf navels were dipped in a 7% tincture of iodine, and calves were vaccinated intranasally with Nasalgen (Merck Animal Health, Millsboro, DE). Birth date, weight, and identity of the dam were recorded. Calves received 1.89 l of high quality thawed colostrum as soon as possible after birth, usually within 2 h. A second feeding of 1.89 l of colostrum was administered 6 h after the first feeding and calf navels were dipped a second time.

FEEDING AND HOUSING

Calves were enrolled and treatments were imposed at 1 day of age. Calves were fed milk replacer (Cow's Match, Land O'Lakes Animal Products Co., Arden Hills, MN, USA) twice daily, at 6:00 AM and 6:00 PM. Milk replacer contained 28% crude protein and 20% fat, with whey protein as the protein source and human grade edible lard as the fat source. Milk replacer powder was reconstituted with water to contain 17.6% solids and fed individually to calves via nipple buckets. Amount of liquid fed provided 1.1–0.68 kg of dry matter per calf, depending upon birth weight, resulting in feeding 2.1–3.4 times more protein and 1.5–2.4 times more fat than traditional feeding programs. This feeding rate has been

termed "intensive feeding" is higher than conventional calf feeding programs, which deliver only 0.454 kg of milk replacer dry matter containing 20% fat and 20% protein. The intensive feeding program thus more closely approaches amounts calves would consume if allowed free choice to liquid diets. Antibiotic treatments were added directly to the nipple buckets, which were used exclusively for a specific treatment to avoid cross-contamination. Calves were supervised during feeding, and 15 min was allowed for consumption. After feeding, all equipment was thoroughly cleaned. Calves were fed starter grain (Intensity −22% crude protein, 10.13 mg/kg of monensin, Southern States Cooperative, Richmond, VA, USA) beginning at 1 day of age.

Calves were housed at the Virginia Tech Dairy Cattle Center in individual fiberglass or plastic hutches (1.83 m × 1.37 m) with metal hog panels to create a fenced area (∼2.5 m^2). Bull calves were housed in hutches until 39 day, when they were moved into metabolism crates to facilitate total feces and urine collection (described below). After the total collection period was complete, bull calves were moved back to their original hutches until weaned.

Weaning was initiated at day 50 by reducing milk replacer offered by 50% and feeding only once daily at 6:00 AM. Calves were initially fed 0.22 kg of starter grain, and the amount offered increased in 0.22 kg increments as consumption increased. When starter grain consumption reached 1.81 kg daily, calves were fully weaned (59 ± 2 day of age). Post-weaning, calves were housed in the calf hutches for 7 days then were housed in small groups in a 3-sided barn with access to a drylot. Weaned calves were group-fed first cutting alfalfa hay and 22% crude protein starter grain ad lib. Starter grain contained monensin at a concentration of 10.13 mg/kg.

SAMPLE AND DATA COLLECTION

Following the 6:00 AM feeding, body temperatures, fecal scores, and respiratory scores (Larson et al., 1977) were recorded daily. Calves were weighed at birth and at weaning to calculate average daily gain.

Bull calves were housed in metabolism crates (1.2 m × 0.6 m) to facilitate total collection of feces beginning at 39 day of age, for 3 day of adaptation to the stall followed by 7 day of total collection. Trays were placed under the metabolism crates to collect excreted feces. Feces were collected daily, weighed, and subsampled. Because heifer calves are anatomically ill-suited for total collection of feces uncontaminated by urine, grab samples of feces were collected from heifer calves at 6:00 AM, 2:00 PM, 8:00 PM, and 12:00 AM for 7 day beginning at 6 weeks of age. Fecal samples were collected from the gravel surface if a fresh sample was available at collection time. If no fresh sample was available, calves were rectally stimulated to produce a fresh sample. After weaning, samples were collected weekly from calves (now group-housed) via rectal palpation.

REMOVED CALVES

A total of 13 calves were removed from the study (3, 7, and 3 were on control, subtherapeutic, and therapeutic treatments, respectively), because of health problems requiring clinical antibiotic treatment.

SAMPLE ANALYSIS

Feces samples were frozen immediately at −20°C after collection. Samples collected from heifer calves were thawed and pooled by date collected on an equal wet weight basis. Pooling was not necessary for bull calf feces samples because one sample was collected of each day's total excretion. DNA was extracted from 500 mg of wet feces per calf from week 6, 7, and 12. The FastDNA® Spin Kit for soil was applied across all samples. The FastPrep® instrument (MP Biomedicals, Santa Ana, CA, USA) was employed with homogenization for 40 s at a speed of 6.0. The DNA was suspended in purified water and aliquots were stored in 0.5 ml cryovials at −80°C.

Previously reported qPCR protocols were implemented to quantify the following ARGs: *erm*B, *erm*F (Chen et al., 2007), *sul*1, *sul*2 (Pei et al., 2006), *tet*C, *tet*G (Aminov et al., 2002), *tet*O, *tet*W (Aminov et al., 2001), and *tet*X (Ng et al., 2001) as well as *int*I1 (Hardwick et al., 2008) and 16S rRNA (Suzuki et al., 2000) genes. Primer sequences are provided in **Table A2** in Appendix. The 9-μl per reaction master mix consisted of 2.8 μl of sterile water, 0.6 μl (5 M) of forward primer, 0.6 μl (5 M) of reverse primer, and 5 μl of SsoFastEvaGreen (Bio-Rad, Hercules, CA, USA) with 1 μl of diluted DNA extract added. A dilution series qPCR was conducted for each gene and each sample matrix, through which it was determined that a 1:70 dilution provided the most consistent results across all samples. All qPCR were conducted in triplicate on a CFX-96 (Bio-Rad). Calibration curves were constructed from serial dilutions of positive controls over seven orders of magnitude. Positive controls were obtained from cloned PCR products of target genes verified by DNA sequencing. Gene copies for any samples with numbers of gene copies below the limit of quantification were recorded as 0 for the purposes of statistical analysis.

STATISTICAL ANALYSIS

Fecal scores, respiratory scores, and body temperatures were analyzed using the Mixed procedure of SAS (9.2, 2008) with the model:

$$Y = \mu + D_i + T_j + B_k + G_l + BG_{kl} + TB_{jk} + TG_{jl} + e_{ijkl}$$

where:

μ = overall mean;

D = effect of day (i = 1–65);

T = effect of treatment (j = control, subtherapeutic, therapeutic);

B = effect of breed (k = crossbred, Holstein);

G = effect of gender (l = bull, heifer); and

e = error (interaction of day, treatment, breed, and gender).

Average daily gains were analyzed using the Mixed procedure of SAS (9.2, 2008) with the model:

$$Y = \mu + T_i + B_j + G_k + BG_{jk} + TB_{ij} + TG_{ik} + e_{ijk}$$

where:

μ = overall mean;

T = effect of treatment (i = control, subtherapeutic, therapeutic);

B = effect of breed (j = crossbred, Holstein);

G = effect of gender (k = bull, heifer); and
e = error (interaction of treatment, breed, and gender).

Because they were not normally distributed, ARG data were log-transformed prior to statistical analysis to determine significance of main effects and interactions. Log-transformed values were used to calculate LSM. All gene data were analyzed using the GLIMMIX procedure of SAS (9.2, 2008) with the model:

$$Y = \mu + T_i + B_j + G_k + W_l + TB_{ij} + TG_{ik} + BG_{jk} + WT_{il} + WB_{jl} + WG_{kl} + e_{ijkl}$$

where:
μ = overall mean;
T = effect of treatment (i = control, subtherapeutic, therapeutic);
B = effect of breed (j = crossbred, Holstein);
G = effect of gender (k = bull, heifer);
W = effect of week (l = 6, 7, 12); and
e = error (interaction of treatment, breed, gender, and week).

After the log-transformed data set was used to determine significance, contrasts were performed to separate means for significant effects and interactions. All data are reported as LSM ± SE. Significance was determined at $P < 0.10$.

RESULTS

EFFECT OF ANTIBIOTICS ON GROWTH AND HEALTH

No effects of antibiotic treatment, breed or gender were observed on average daily gain (1.30 ± 0.13 kg/day), fecal scores (2.5 ± 0.17), respiratory scores (1.2 ± 0.04), or body temperature (102.2° ± 0.95).

EFFECT OF ANTIBIOTICS ON ARGs IN FECES

Two of the analyzed genes (*tet*C and *int*I1) were not detected in fecal samples of any calves. Four genes encoding resistance to tetracyclines were detected in feces (*tet*G, *tet*O, *tet*W, *tet*X; **Figure 1**) as were two genes encoding resistance to sulfonamides (*sul*1, *sul*2) and two genes encoding resistance to erythromycin (*erm*B, *erm*F). All calves harbored at least one of the *tet* genes in the feces by week 12, and multiple ARGs related to tetracyclines were present in nearly all of the fecal samples analyzed. ARGs corresponding to antibiotics not fed to calves were less abundant across all three treatments (**Figure 2**). About one third of the calves did not have *sul*1 present in their feces at any individual sampling point.

Feeding milk replacer with subtherapeutic or therapeutic doses of neomycin and oxytetracycline had no effect on the absolute abundance (log copy per gram wet weight) of *tet*G, *tet*O, *tet*W, *tet*X, *sul*1, *sul*2, or *erm*B in manure, but calves fed medicated milk replacers yielded reduced abundance of *erm*F as compared to control calves (**Table 1**). The relative abundance (ARG per 16S rRNA; normalized) of *erm*F was not different between antibiotic fed and non-antibiotic control groups (**Table 2**). Calves fed the higher (therapeutic) dose of antibiotics resulted in increased relative abundance of *tet*O as compared to subtherapeutic and control calves (**Table 2**).

EFFECT OF OTHER FACTORS ON ARG ABUNDANCE

Abundance of the ARGs analyzed was not different between genders or between breeds, but abundance of all *tet* ARGs except *tet*O increased with time in all treatments (**Figure 1**), including the no antibiotic control. For *tet*G and *tet*X, gene abundance increased between weeks 6 and 12. For *tet*W, the increase in abundance occurred during the period following weaning, and was detected at the week 12 sampling point. Effects of time on *tet* ARGs were no longer observed when the data were

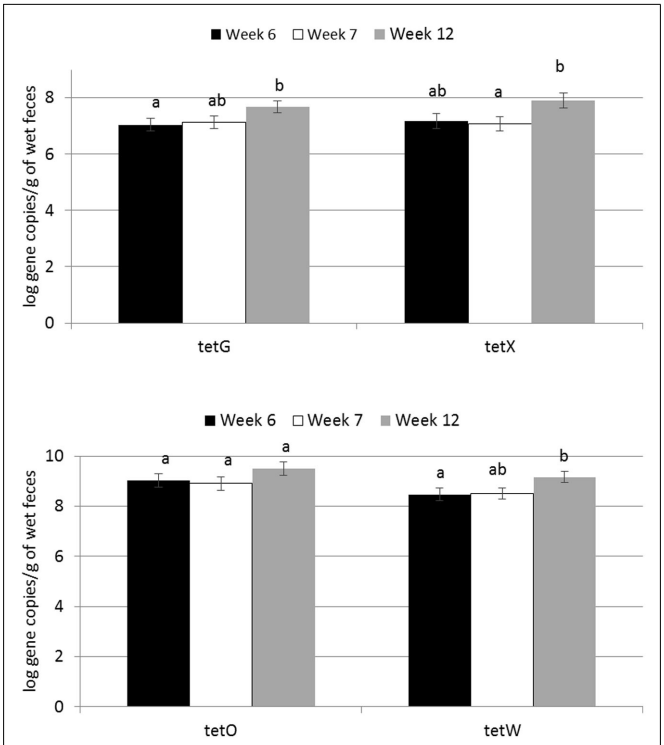


FIGURE 1 | Effect of time on abundance of tetracycline ARGs in calf feces. Bars represent the pooled averages across calves fed medicated or non-medicated milk replacers. Letters above the bars indicate significantly different LSM groupings ($P < 0.10$).

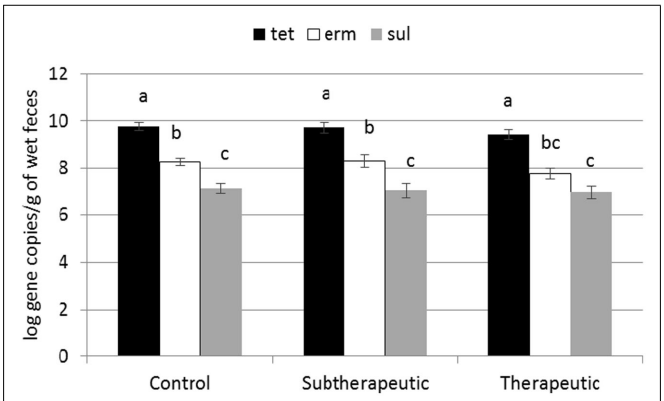


FIGURE 2 | Effect of antibiotics in milk replacer on abundance of tetracycline (*tet*), macrolide (*erm*), and sulfonamide (*sul*) classes of ARGs. Bars represent the pooled averages of the total ARGs quantified within each class. Letters above the bars indicate significantly different LSM groupings ($P < 0.10$).

Table 1 | Effect of milk replacer medication, breed, gender, week, and the interaction of milk replacer with these on abundance of selected antibiotic resistance genes in the feces of dairy calves.

	Log gene copies per gram wet feces			SE[4]	*P* <				
	Control[1]	Subtherapeutic[2]	Therapeutic[3]		Trt	Breed	Gender	Day	Interactions[5]
n	12	7	9						
*tet*G	7.40	7.29	7.17	0.18	NS	NS	NS	0.06	Trt*gender; breed*gender
*tet*O	9.18	9.10	9.13	0.34	NS	NS	NS	NS	NS
*tet*W	8.89	8.80	8.43	0.28	NS	NS	NS	0.07	NS
*tet*X	7.47	7.70	6.99	0.34	NS	NS	NS	NS	NS
*sul*1	6.29	5.88	6.34	0.28	NS	0.10	NS	NS	Trt*gender; gender*week
*sul*2	6.78	6.64	6.61	0.23	NS	NS	NS	NS	NS
*erm*B	7.20	7.31	6.82	0.37	NS	NS	NS	NS	NS
*erm*F	7.91	7.36	6.95	0.32	0.04	NS	NS	0.003	NS

[1] *Control milk replacer containing no antibiotics fed from day 2 to weaning (59± 2 day).*

[2] *Medicated milk replacer containing 10 mg/calf/day of tetracycline and neomycin fed from day 2 to weaning.*

[3] *Medicated milk replacer containing 1000 mg/calf/day of tetracycline and neomycin fed from day 36 to weaning.*

[4] *SE for treatment LS means.*

[5] *Significant (P < 0.10) two and three way interactions of treatment, breed, gender, and week.*

Table 2 | Effect of milk replacer medication, breed, gender, week, and the interaction of milk replacer with these on selected antibiotic resistance genes in the feces of dairy calves.

	Gene copies/16S rRNA			SE[4]	*P* <				
	Control[1]	Subtherapeutic[2]	Therapeutic[3]		Trt	Breed	Gender	Day	Interactions[5]
*tet*G	0.65	0.90	13.5	14.3	NS	NS	NS	NS	NS
*tet*O	2.27	2.13	5.35	1.53	0.02	NS	NS	NS	NS
*tet*W	10.3	4.00	1.84	9.13	NS	NS	0.07	NS	NS
*tet*X	0.50	0.69	0.88	0.68	NS	0.03	NS	NS	Trt*gender; breed*gender; breed*week
*sul*1	0.13	0.12	1.11	0.58	NS	NS	NS	NS	Gender*week
*sul*2	0.06	0.06	0.13	0.12	NS	NS	NS	NS	Breed*week
*erm*B	0.89	0.78	0.67	0.99	NS	NS	NS	NS	NS
*erm*F	0.66	0.09	0.16	0.75	NS	NS	NS	0.004	NS

[1] *Control milk replacer containing no antibiotics fed from day 2 to weaning (59± 2 day).*

[2] *Medicated milk replacer containing 10 mg/calf/day of tetracycline and neomycin fed from day 2 to weaning.*

[3] *Medicated milk replacer containing 1000 mg/calf/day of tetracycline and neomycin fed from day 36 to weaning.*

[4] *SE for treatment LS means.*

[5] *Other significant (P < 0.10) two and three way interactions of treatment, breed, gender, and week.*

expressed per unit of 16S rRNA gene (**Table 2**). However, relative abundance of *erm*F was observed to increase with time across treatments, only when expressed per unit of 16S rRNA gene (**Figure 3**).

For the majority of the ARGs, breed, gender, and week did not have any effect except that crossbred and bull calves harbored reduced relative abundance of *tet*X and *tet*W, respectively (**Table 2**). Interaction of treatment with breed, gender, and week of treatment had no significant effects on gene abundance for the majority of the analyzed genes. The interactions of treatment and gender were significant for *tet*G and *sul*1 (**Table 1**). Among control calves, heifers harbored higher abundance of *tet*G more than did bulls; that was reversed in the subtherapeutic group but the difference was not significant (**Figure 4**). There was no effect of gender on abundance of *sul*1 among calves fed the subtherapeutic milk replacer, but among control calves, heifers had far more copies of *sul*1 in their feces than did bulls (**Figure 5**). The interaction of week and gender was significant for *sul*1 (**Table 1**). Heifers had higher abundance of *sul*1 at week 6 than did bulls, (**Figure 6**). The interaction of breed with gender was significant for *tet*G (**Table 1**). Crossbred bulls had the higher abundance of *tet*G than crossbred heifers; there was no effect of gender among Holstein calves (**Figure 7**).

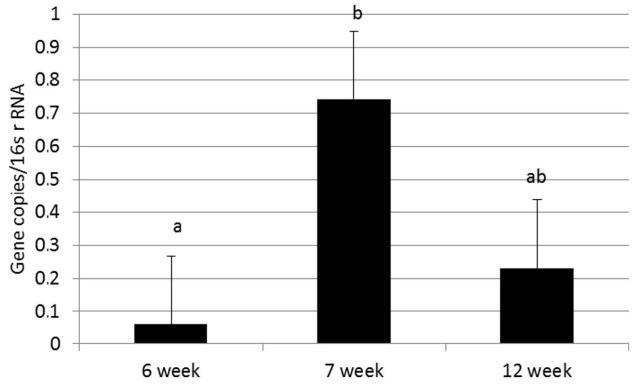


FIGURE 3 | Effect of time on relative abundance of *ermF* ARGs in calf feces. Bars represent the pooled averages across calves fed medicated or non-medicated milk replacers. Letters above the bars indicate significantly different LSM groupings ($P < 0.10$).

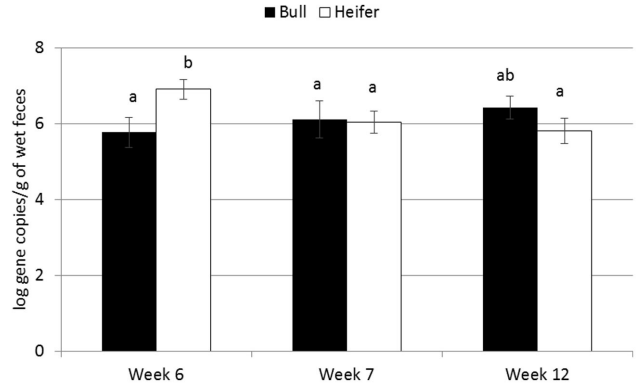


FIGURE 6 | Effect of the gender by time interaction on abundance of *sul1* ARGs in calf feces. Bars represent the pooled averages across calves fed medicated or non-medicated milk replacers. Letters above the bars indicate significantly different LSM groupings ($P < 0.10$).

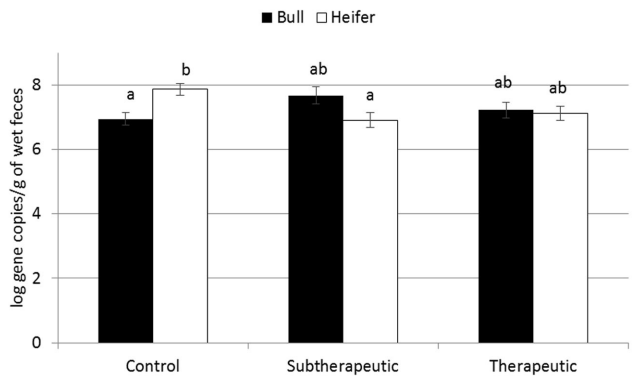


FIGURE 4 | Effect of the gender and antibiotic treatment interaction on abundance of *tetG* ARGs in calf feces. Bars represent the pooled averages across the three sampling events. Letters above the bars indicate significantly different LSM groupings ($P < 0.10$).

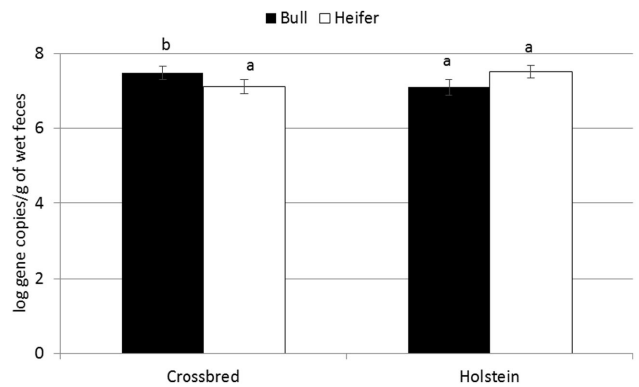


FIGURE 7 | Effect of gender by breed interaction on abundance of *tetG* ARGs in calf feces. Bars represent the pooled averages across calves fed medicated or non-medicated milk replacers and over the three sampling events. Letters above the bars indicate significantly different LSM groupings ($P < 0.10$).

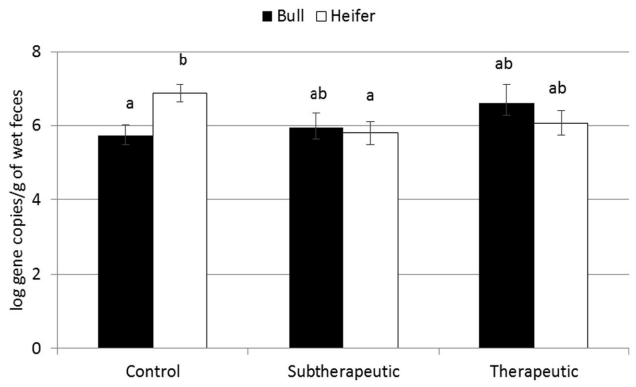


FIGURE 5 | Effect of the gender and antibiotic treatment interaction on abundance of *sul1* ARGs in calf feces. Bars represent the pooled averages across the three sampling events. Letters above the bars indicate significantly different LSM groupings ($P < 0.10$).

DISCUSSION

EFFECT OF ANTIBIOTICS ON GROWTH AND HEALTH

The lack of effect of milk replacer medication on growth and health is in contrast to some observations (Quigley and Drew, 2000; Berge et al., 2005; Stanton et al., 2010), probably because calves in the current study were fed a more nutrient-dense diet with higher intake than in most early research. Consequently, calves completing the course of the study were relatively healthy and grew quickly. Notably, of the 13 calves that were removed due to health problems, 7 were in the subtherapeutic treatment group. Health problems that were observed in calves in the current study were largely due to protozoan and viral microorganisms, which are not susceptible to antibiotics. Langford et al. (2003) observed no effect of penicillin content of milk in calves fed *ad libitum*; they also attributed the lack of effect of antibiotic to the nutritional benefits to the calf of offering an unlimited supply of milk. Interestingly, Berge et al. (2009a) actually observed antibiotic addition to pasteurized waste

milk to be associated with a higher percentage of diarrhea days in calves compared to no antibiotic addition, while respiratory scores were equivalent between the two treatments. They partially attributed this effect to adequate passage of immunity via colostrum, as advocated in their parallel studies (Berge et al., 2009b). Their study also highlights potentially negative effects of antibiotics on gastrointestinal flora. Kaneene et al. (2008) similarly observed no health benefits of adding oxytetracycline and neomycin to milk replacer and also noted substantial colostrum provisions to the calves. Thus, nutritional status and overall health may override benefits provided by subtherapeutic antibiotic treatment.

EFFECT OF ANTIBIOTICS ON ABUNDANCE OF ARGs IN FECES

The present study represents the first controlled, culture-independent evaluation of the effect of antibiotic addition in milk replacer on antibiotic resistance in calf feces through weaning. The results provide an indication of the effects of subtherapeutic and therapeutic tetracycline and neomycin treatments typical in the U.S. Among the eight ARGs examined across three classes, *tet*O was the only one to exhibit an increase in response to antibiotic treatment, but only when normalized to 16S rRNA genes. The results were somewhat unexpected, given reports of feed antibiotics increasing resistance in manure of mature cattle (Alexander et al., 2009) and other livestock (Marshall and Levy, 2011). In a prior study of calves fed "waste milk" containing penicillin residues, resistance of fecal bacteria to penicillin increased with increasing dose fed (Langford et al., 2003). One possible cause for discrepancy is that of the 25 known tetracycline ARGs (Aminov et al., 2001, 2002; Yang et al., 2004), only five were quantified in this study. Although these represented the three main classes of resistance (efflux, ribosomal protection, and degradation) and have previously been reported to be found in cattle waste (Storteboom et al., 2007; Alexander et al., 2009; Dahshan et al., 2010; McKinney et al., 2010), it is quite possible that key tetracycline ARGs responding to antibiotic treatment in this study were overlooked. Interestingly, though *tet*X was previously reported to be confined within the genus *Bacteroides* (Yang et al., 2004), its abundance was comparable with that of *tet*G, with recent reports suggesting it is more widespread than previously thought (Ghosh et al., 2009). Regardless, the results of this study suggest that removal of chlortetracycline and neomycin from milk replacer will not eliminate shedding of ARGs into the environment. McKinney et al. (2010) similarly observed only a very modest reduction of *tet* and *sul* ARG abundance in waste lagoons on organic dairy farms relative to those of conventional dairies.

The reduced abundance of *erm*F observed in feces of calves fed antibiotics as compared to control calves was intriguing. Macrolide and sulfonamide ARGs were examined in this study as potential indicators of co-selection of resistance to antibiotics other than the oxytetracycline and neomycin administered in the milk replacer. In particular, *sul*1 is carried within the 3′ conserved region of class 1 integrons (Mazel, 2006), while tetracycline resistance has been noted to be prevalent in macrolide-resistant *Streptococcus pyogenes* (Nielsen et al., 2004). In a study by Berge et al. (2006a) it was found that milk replacer containing neomycin sulfate and tetracycline HCl selected for *E. coli* resistant to classes of antimicrobials not used, including: aminoglycosides, chloramphenicol, and sulfonamides. The reduced abundance of *erm*F in the current study may be explained by effects of medicated milk replacer on specific populations of excreted bacteria. For example, Berge et al. (2006b) observed that medicated milk replacer decreased fecal shedding of *Salmonella enterica* by dairy calves. Decreased fecal shedding of certain bacteria could explain the decreased numbers of gene copies of *erm*F with antibiotic feeding; the species of bacteria carrying erythromycin resistance being shed in the feces was unknown in this study. It seems probable that one of the two antibiotics fed in the milk replacer were effective against fecal bacteria carrying the *erm*F gene. Interestingly, the effect of antibiotic treatment was no longer observed when *erm*F was normalized to 16S rRNA genes (**Table 2**). Normalization to the 16S rRNA gene, present in all bacteria, provides an indication of the proportion of the bacterial community carrying the ARG of interest and also aids in accounting for minor variations in sample processing. The similarity in relative abundance (ARGs per 16S rRNA; normalized) of *erm*F between antibiotic fed and non-antibiotic control groups suggests that there was a decrease in overall fecal bacterial shedding in antibiotic-treated groups. Reduced 16S rRNA genes in the manure of antibiotic-treated calves would also be consistent with the *tet*O increase only being significant when normalized to 16S rRNA genes. However, the range of 16S rRNA gene concentrations encountered among the fecal samples was wide, and there was no significant effect found of antibiotic treatment on overall 16S rRNA gene abundance.

ESTABLISHMENT OF ARGs IN CALF FECES WITH TIME

The increased absolute abundance of *tet* and relative *erm*F ARGs with time in all calves in the current study (including calves not fed antibiotics) suggests that the calf gut environment itself was amenable to the establishment of resistance regardless of antibiotic content of the feed. Berge et al. (2006a) similarly reported an age-related increase in antibiotic resistance of *E. coli* (measured phenotypically) even in non-treated calves. On the other hand, no effect of time on *tet* ARGs was observed when data were expressed per unit of 16S rRNA. This suggests that the overall bacterial population density increased in corresponding samples with time and that the bacteria carrying *tet* ARGs were neither negatively nor positively impacted. However, the range of 16S rRNA gene concentrations was again wide and a statistically significant increase with time was not identified as a general phenomenon.

Few studies have specifically characterized the establishment of antibiotic resistance in calf manure with time. Similar to the present study, increased overall resistance to 12 antimicrobials was noted among fecal *E. coli* isolates over the first 4 months of life in calves not receiving antibiotics (Berge et al., 2006a). A recent study by Pereira et al. (2011) examined the antibiotic resistance patterns of *E. coli* isolates on day 2 and day 6 of calves from two farms that did not administer antibiotics in the feed milk as compared to a third farm that administered both chlortetracycline and sulfamethazine. Odds of resistance to most antibiotics observed for the farm administering antibiotics was higher compared to control farms. The inclusion of sulfonamide, rather than neomycin, could have been the driving factor in the effects observed relative to the present study. While neomycin was included in this study because of its widespread implementation in the U.S., *neo* ARGs

(i.e., aminoglycoside phosphotransferases) were not monitored. Nonetheless, as neomycin is rarely used in humans, the present study did provide insight into potential effects of common practice on resistance to three classes of antibiotics of high clinical relevance, in addition to the clinically relevant integron, *int*I1 (Hall and Collis, 1998). Also important to consider, in comparison to the Pereira et al. (2011) study, is that in a study design in which the antibiotic treatments are geographically isolated, the actual established microbiota of the farm, rather than the antibiotic present in the milk, could be an overarching factor. The present study thus has value in its focus of the effects of the three controlled treatments on a single farm.

Of note, as is routine practice in the U.S., monensin was included in the grain fed to all calves. While very little grain was consumed during the early weeks, the monensin-containing grain became the sole source of nutrition post-weaning (7 weeks). *Tet* ARGs did generally increase with time, *tet*G and *tet*W prior to weaning and *tet*X 5 weeks post-weaning. It is not possible to determine from this study whether monensin was related to the observed increases of *tet* ARGs; however, all significant increases with time were lost when normalized to 16S rRNA genes. Therefore, it can be concluded that monensin did not generally suppress the total bacterial density to produce the observed effect. Co-selection of bacteria carrying *tet* ARGs by monensin is one possible explanation for their increase with time; however, acquired resistance of bacteria to monensin has not previously been observed (Butaye et al., 2001). Furthermore, bacteria that are tolerant of monensin have been observed to be no more or less sensitive to medically important antibiotics than monensin-susceptible bacteria (Houlihan and Russell, 2003).

OTHER FACTORS AND THEIR INTERACTIONS

The effects of interactions of time, treatment, gender, and/or breed are not easily explained. They may be attributed to the small number of observations associated with each interaction, although each individual group was comprised of *n* ranging from six to eight. If the effects observed across the gender and breed interactions are indeed real, they highlight the complexity of factors governing the microbial ecology of the bovine gut and the establishment of antibiotic resistance.

ADVANTAGES AND LIMITATIONS OF MOLECULAR-BASED APPROACH

To our knowledge, this study provides the first insight into the establishment of antibiotic resistance in calf feces in the early weeks using qPCR to directly quantify ARGs. This approach is advantageous in that it circumvents biases associated with culturing and thus enables broad quantification of resistance elements, including those harbored by unculturable strains. However, molecular techniques possess their own limitations, which must be considered in interpreting the findings of this study. Firstly, although 10 genes were quantified by qPCR, these likely only scratch the surface of the full array of resistance elements present in calf feces. Indeed, recent examination of the human gut microbiome has revealed a vast array of resistance elements, most of which not previously described (Sommer et al., 2009). Thus, it is quite possible that ARGs not monitored or detectable in this study did in fact respond strongly to antibiotic treatment. Secondly, qPCR is limited by the quality of the DNA extraction. While no ideal DNA extraction method exists, we viewed the FastDNA® Spin Kit for soil to be the best option. While kits specifically formulated to manure are available, head to head comparison has actually indicated downstream detection of a broader range of bacteria when the soil kit was applied to extract DNA from feces (Ariefdjohan et al., 2010). Further, the FastPrep® instrument applied in this study was noted to result in the least DNA damage, relative to other cell lysis techniques. The FastDNA® Spin Kit for soil was successfully applied for qPCR analysis of a range of manures by Layton et al. (2006), although they employed a 1:10 dilution prior to extraction, while our study did not. A potential consequence of undiluted samples is overloading of the extraction column and thus lower overall DNA yield. Normalization to 16S rRNA genes should help account for such variation. Also important to note is that our methods were able to detect significant absolute difference in ARGs in several instances, which would not be expected to be discernible if extraction column overloading was the dominant factor driving the results.

MULTIPLE DRUG RESISTANCE VERSUS ABUNDANCE OF MULTIPLE ARGs

Up to 80% of *E. coli* isolates obtained from mature heifers have been observed to be multi-drug resistant (resistant to three to six drugs), typically displaying resistance to tetracycline, ampicillin, ceftiofur, florfenicol, chloramphenicol, spectinomycin, and streptomycin (Sawant et al., 2007). However, the effect of antimicrobial use on multiple antimicrobial resistance has only recently been studied in calves. Pereira et al. (2011) noted that 81% of the *E. coli* isolates obtained from both medicated and non-medicated calves were resistant to three or more antibiotics already within the first 6 days of life. Berge et al. (2006a) observed that tetracycline and neomycin in the feed were associated with higher levels of multiple antimicrobial resistant fecal *E. coli* in calves monitored up to 4 weeks of age. The same research group (Berge et al., 2010) further noted that fecal *E. coli* were more likely to be resistant to multiple antibiotics in older calves (14- or 28-2) than in newborn calves if calves were fed antimicrobials in milk. In the current study, 8 of the 10 target ARGs were detected in the feces of the experimental calves. Fecal samples from all calves carried multiple types of ARGs within the same sample. Also, feces of all calves carried at least one gene encoding resistance to each of the three classes of antibiotics of interest (tetracyclines, sulfonamides, erythromycins), but class 1 integrons (*int*I1) were not detected in any of the fecal samples. In contrast, bacterial strains carrying class 1 integrons were isolated from dairy manure-impacted soil, but not from a corresponding unimpacted soil (Srinivasan et al., 2008). Class 1 integrons were also detected in 10% of samples from scouring calves (Ahmed et al., 2009); calves in the current study were not diarrheic during sample collections. Wu et al. (2011) observed that sulfonamide use was associated with higher occurrence of *int*I1 genes among tetracycline resistant *E. coli* from beef cattle. That *int*I1 was not detectable but that multiple ARGs were present in feces of all calves is intriguing. It is plausible that the ARGs identified were carried by different bacterial species in the feces sample, but also the analyzed genes may be carried by the same

bacterial species, without linkage to a transferable element such as *int*I1. Thus, conclusive evidence of multiple drug resistance in the calf manure was not obtained in this study. Nonetheless, multiple drug resistance has been observed to be significantly higher in dairy manure-impacted environments, such as soils (Srinivasan et al., 2008), thus the evolution of antibiotic resistance from calf to mature cow to the environment remains an important issue.

CONCLUSION

Improved calf health and growth commonly observed with feeding of medicated milk replacers were not observed, likely because the calves in this experiment were maintained on a high plane of nutrition. In situations where management or nutrition is not optimal, health differences may be more apparent. Genes related to tetracycline resistance increased over time regardless of treatment, thus the calf gut itself appears to be an environment conducive to the proliferation of certain ARGs. Genes encoding resistance to antibiotics that were not administered were less abundant, thus co-selection was not observed in this study. However, macrolide and sulfonamide ARGs were still present and persisted in the feces of these experimental calves. Other ARGs not examined were also likely present and may have responded to antibiotic treatment. The results have important management implications, indicating that if nutritional requirements are appropriately managed, the cost and risk of subtherapeutic antibiotic use may not be necessary. Nonetheless, ARGs across three classes persisted and increased with time, even in the feces of calves not administered antibiotics. Thus, comprehensive management strategies that limit cross-dissemination between animals and control transport of manure from farms, in addition to prudent use of antibiotics, are called for.

ACKNOWLEDGMENTS

Financial support was provided by the Virginia Agricultural Council. The authors wish to thank Chad McKinney for technical assistance.

REFERENCES

Aarestrup, F. M. (2000). Characterization of glycopeptide-resistant *Enterococcus faecium* (GRE) from broilers and pigs in Denmark: genetic evidence that persistence of GRE in pig herds is associated with coselection by resistance to macrolides. *J. Clin. Microbiol.* 38, 2774–2777.

Ahmed, A. M., Younis, E. E. A., Osman, S. A., Ishida, Y., El-khodery, S. A., and Shimamoto, T. (2009). Genetic analysis of antimicrobial resistance in *Escherichia coli* isolated from diarrheic neonatal calves. *Vet. Microbiol.* 136, 397–402.

Alexander, T. W., Reuter, T., Sharma, R., Yanke, L. J., Topp, E., and McAllister, T. A. (2009). Longitudinal characterization of resistant *Escherichia coli* in fecal deposits from cattle fed subtherapeutic levels of antimicrobials. *Appl. Environ. Microbiol.* 75, 7125–7134.

Aminov, R. I., Chee-Sanford, J. C., Garrigues, N., Teferedegne, B., Krapac, I. J., White, B. A., and Mackie, R. I. (2002). Development, validation and application of PCR primers for detection of tetracycline efflux genes of Gram-negative bacteria. *Appl. Environ. Microbiol.* 68, 1786–1793.

Aminov, R. I., Garrigues-Jeanjean, N., and Mackie, R. I. (2001). Molecular ecology of tetracycline resistance: development and validation of primers for detection of tetracycline resistance genes encoding ribosomal protection proteins. *Appl. Environ. Microbiol.* 67, 22–32.

Ariefdjohan, M. W., Savaiano, D. A., and Nakatsu, C. H. (2010). Comparison of DNA extraction kits for PCR-DGGE analysis of human intestinal microbial communities from fecal specimens. *Nutr. J.* 9, 23.

Arnold, S., Gassner, B., Giger, T., and wahlen, R. Z. (2004). Banning antimicrobial growth promoters in feedstuffs does not result in increased therapeutic use of antibiotics in medicated feed in pig farming. *Pharmacoepidemiol. Drug Saf.* 13, 323–331.

Aschbacher, P. W., and Feil, V. J. (1994). Neomycin metabolism in calves. *J. Anim. Sci.* 72, 683–689.

Bager, F., Madsen, M., Christensen, J., and Aarestrup, F. M. (1997). Avoparcin used as a growth promoter is associated with the occurrence of vancomycin-resistant *Enterococcus faecium* on Danish poultry and pig farms. *Prevent. Vet. Med.* 31, 95–112.

Bates, J. (1997). Epidemiology of vancomycin-resistant enterococci in the community and the relevance of farm animals to human infection. *J. Hosp. Infect.* 37, 89–101.

Berge, A. C., Epperson, W. B., and Pritchard, R. H. (2005). Assessing the effect of a single dose florfenicol treatment in feedlot cattle on the antimicrobial resistance patterns in fecal *Escherichia coli*. *Vet. Res.* 36, 723–734.

Berge, A. C., Hancock, D. D., Sischo, W. M., and Besser, T. E. (2010). Geographic, farm and animal factors associated with multiple antimicrobial resistance in fecal *Escherichia coli* isolates from cattle in the Western United States. *J. Am. Vet. Med. Assoc.* 236, 1338–1344.

Berge, A. C., Moore, D. A., Besser, T. E., and Sischo, W. M. (2009a). Targeting therapy to minimize antimicrobial use in preweaned calves: effects on health, growth, and treatment costs. *J. Dairy Sci.* 92, 4704–4714.

Berge, A. C., Besser, T. E., Moore, D. A., and Sischo, W. M. (2009b). Evaluation of the effects of oral colostrum supplementation during the first fourteen days on the health and performance of preweaned calves. *J. Dairy Sci.* 92, 286–295.

Berge, A. C., Moore, D. A., and Sischo, W. M. (2006a). Field trial evaluating the influence of prophylactic and therapeutic antimicrobial administration on antimicrobial resistance of fecal *Escherichia coli* in dairy calves. *Appl. Environ. Microbiol.* 72, 3872–3878.

Berge, A. C., Moore, D. A., and Sischo, W. M. (2006b). Prevalence and antimicrobial resistance patterns of *Salmonella enterica* in preweaned calves from dairies and calf ranches. *Am. J. Vet. Res.* 67, 1580–1588.

Boerlin, P., Wissing, A., Aarestrup, F. M., Frey, J., and Nicolet, J. (2001). Antimicrobial growth promoter ban and resistance to macrolides and vancomycin in enterococci from pigs. *J. Clin. Microbiol.* 39, 4193–4195.

Butaye, P., Devriese, L. A., and Haesebrouck, F. (2001). Differences in antibiotic resistance patterns of *Enterococcus faecalis* and *Enterococcus faecium* strains isolated from farm and pet animals. *Antimicrob. Agents Chemother.* 45, 1374–1378.

Casewell, M., Friis, C., Marco, E., McMullin, P., and Phillips, I. (2003). The European ban on growth-promoting antibiotics and emerging consequences for human and animal health. *J. Antimicrob. Chemother.* 52, 159–161.

Chee-Sanford, J. C., Aminov, R. I., Krapac, I. J., Garrigues-Jeanjean, N., and Mackie, R. I. (2001). Occurrence and diversity of tetracycline resistance genes in lagoons and groundwater underlying two swine production facilities. *Appl. Environ. Microbiol.* 67, 1494–1502.

Chen, J., Yu, Z., Michel, F. C., Wittum, T., and Morrison, M. (2007). Development and application of real-time PCR assays for quantification of erm genes conferring resistance to macrolides-lincosamides-streptogramin B in livestock manure and manure management systems. *Appl. Environ. Microbiol.* 73, 4407–4416.

Dahshan, H., Shahada, F., Chuma, T., Moriki, H., and Okamoto, K. (2010). Genetic analysis of multidrug-resistant *Salmonella enterica* serovars Stanley and Typhimurium from cattle. *Vet. Microbiol.* 145, 76–83.

Ghosh, S., Sadowsky, M. J., Roberts, M. C., Gralnick, J. A., and LaPara, T. M. (2009). *Sphingobacterium* sp strain PM2-P1-29 harbours a functional tet(X) gene encoding for the degradation of tetracycline. *J. Appl. Microbiol.* 106, 1336–1342.

Grave, K., Jensen, V. F., Odensvik, K., Wierup, M., and Bangen, M. (2006). Usage of veterinary therapeutic antimicrobials in Denmark, Norway and Sweden following termination of antimicrobial growth promoter use. *Prevent. Vet. Med.* 75, 123–132.

Grave, K., Kaldhusdal, M., Krused, H., Fevang Harre, L. M., and Flatlandsmo, K. (2004). What has happened in Norway after the ban of avoparcin? Consumption of antimicrobials by poultry. *Prevent. Vet. Med.* 62, 59–72.

Hall, R., and Collis, C. (1998). Antibiotic resistance in Gram-negative bacteria: the role of gene cassettes and integrons. *Drug Resist. Updat.* 1, 109–119.

Hardwick, S. A., Stokes, H. W., Findlay, S., Taylor, M., and Gillings, M. R. (2008). Quantification of class 1 integron abundance in natural environments using real-time quantitative PCR. *FEMS Microbiol. Lett.* 278, 207–212.

Ho, P. L., Wong, R. C., Lo, S. W., Chow, K. H., Wong, S. S., and Que, T. L. (2010). Genetic identity of aminoglycoside-resistance genes in *Escherichia coli* isolates from human and animal sources. *J. Med. Microbiol.* 59, 702–707.

Houlihan, A. J., and Russell, J. B. (2003). The susceptibility of ionophore-resistant *Clostridium aminophilum* F to other antibiotics. *J. Antimicrob. Chemother.* 52, 623–628.

Kaneene, J. B., Warnick, L. D., Bolin, C. A., Erskine, R. J., May, K., and Miller, R. A. (2008). Changes in tetracycline susceptibility of enteric bacteria following switching to nonmedicated milk replacer for dairy calves. *J. Clin. Microbiol.* 46, 1968–1977.

Langford, F. M., Weary, D. M., and Fisher, L. (2003). Antibiotic resistance in gut bacteria from dairy calves: a dose response to the level of antibiotics fed in milk. *J. Dairy Sci.* 86, 3963–3966.

Larson, L. L., Owen, F. G., Albright, J. L., Appleman, R. D., Lamb, R. C., and Muller, L. D. (1977). Guidelines toward more uniformity in measuring and reporting calf experimental data. *J. Dairy Sci.* 60, 989–991.

Lauderdale, T. L., McDonald, L. C., Shiau, Y. R., Chen, P. C., Wang, H. Y., Lai, J. F., and Ho, M. (2002). Vancomycin-resistant enterococci from humans and retail chickens in Taiwan with unique VanB phenotype-vanA genotype incongruence. *Antimicrob. Agents Chemother.* 46, 525–527.

Lauderdale, T. L., Shiau, Y. R., Wang, H. Y., Lai, J. F., Huang, I. W., Chen, P. C., Chen, H. Y., Lai, S. S., Liu, Y. F., and Ho, M. (2007). Effect of banning vancomycin analogue avoparcin on vancomycin-resistant enterococci in chicken farms in Taiwan. *Environ. Microbiol.* 9, 819–823.

Layton, A., McKay, L., Williams, D., Garrett, V., Gentry, R., and Sayler, G. (2006). Development of bacteroides 16S rRNA gene TaqMan-based real-time PCR assays for estimation of total, human, and bovine fecal pollution in water. *Appl. Environ. Microbiol.* 72, 4214–4224.

Marshall, B. M., and Levy, S. B. (2011). Food animals and antimicrobials: impacts on human health. *Clin. Microbiol. Rev.* 24, 718.

Mazel, D. (2006). Integrons: agents of bacterial evolution. *Nat. Rev. Microbiol.* 4, 608–620.

McKinney, C. W., Loftin, K. A., Meyer, M. T., Davis, J. G., and Pruden, A. (2010). *tet* and *sul* antibiotic resistance genes in livestock lagoons of various operation type, configuration, and antibiotic occurrence. *Environ. Sci. Technol.* 44, 6102–6109.

National Research Council (NRC). (2001). *Nutrient Requirements of Dairy Cattle*, 7th Edn. Washington, DC: The National Academies Press.

Ng, L. K., Martin, I., Alfa, M., and Mulvey, M. (2001). Multiplex PCR for the detection of tetracycline resistant genes. *Mol. Cell. Probes* 15, 209–215.

Nielsen, H. U. K., Hammerum, A. M., Ekelund, K., Bang, D., Pallesen, L. V., and Frimodt-Moller, N. (2004). Tetracycline and macrolide co-resistance in *Streptococcus pyogenes*: co-selection as a reason for increase in macrolide-resistant *S-pyogenes*? *Microb. Drug Resist.* 10, 231–238.

Pei, R., Kim, S. C., Carlson, K. H., and Pruden, A. (2006). Effect of river landscape on the sediment concentrations of antibiotics and corresponding antibiotic resistance genes (ARG). *Water Res.* 40, 2427–2435.

Pereira, R. V. V., Santos, T. M. A., Bicalho, M. L., Caixeta, L. S., Machado, V. S., and Bicalho, R. C. (2011). Antimicrobial resistance and prevalence of virulence factor genes in fecal *Escherichia coli* of Holstein calves fed milk with and without antimicrobials. *J. Dairy Sci.* 94, 4556–4565.

Quigley, J. D., and Drew, M. D. (2000). Effects of oral antibiotics or bovine plasma on survival, health and growth in dairy calves challenged with *Escherichia coli*. *Food Agric. Immunol.* 12, 311–318.

Sawant, A. A., Hegde, N. V., Straley, B. A., Donaldson, S. C., Love, B. C., Knabel, S. J., and Jayarao, B. M. (2007). Antimicrobial-resistant enteric bacteria from dairy cattle. *Appl. Environ. Microbiol.* 73, 156–163.

Smith, M. S., Yang, R. K., Knapp, C. W., Niu, Y., Peak, N., Hanfelt, M. M., Galland, J. C., and Graham, D. W. (2004). Quantification of tetracycline resistance genes in feedlot lagoons by real-time PCR. *Appl. Environ. Microbiol.* 70, 7372–7377.

Sommer, M. O., Danatas, A. G., and Church, G. M. (2009). Functional characterization of the antibiotic resistance reservoir in the human microflora. *Science* 325, 1128–1131.

Srinivasan, V., Nam, H.-M., Sawant, A. A., Headrick, S. I., Nguyen, L. I., and Oliver, S. P. (2008). Distribution of tetracycline and streptomycin resistance genes and class 1 integrons in Enterobacteriaceae isolated from dairy and nondairy farm soils. *Microb. Ecol.* 55, 184–193.

Stanton, A. L., Kelton, D. F., LeBlanc, S. J., Millman, S. T., Wormuth, J., Dingwell, R. T., and Leslie, K. E. (2010). The effect of treatment with long-acting antibiotic at postweaning movement on respiratory disease and on growth in commercial dairy calves. *J. Dairy Sci.* 93, 574–581.

Storteboom, H. N., Kim, S. C., Doesken, K., Davis, J., Carlson, K. H., and Pruden, A. (2007). Response of antibiotics and resistance genes to high-intensity and low-intensity manure management. *J. Environ. Qual.* 36, 1695–1703.

Suzuki, M. T., Taylor, L. T., and DeLong, E. F. (2000). Quantitative analysis of small-subunit rRNA genes in mixed microbial populations via 5′-nuclease assays. *Appl. Environ. Microbiol.* 66, 4605–4614.

Wierup, M. (2001). The Swedish experience of the 1986 year ban of antimicrobial growth promoters, with special reference to animal health, disease prevention, productivity, and usage of antimicrobials. *Microb. Drug Resist.* 7, 183–190.

Wileman, B. W., Thomson, D. U., Reinhardt, C. D., and Renter, D. G. (2009). Analysis of modern technologies commonly used in beef cattle production: conventional beef production versus nonconventional production using meta-analysis. *J. Anim. Sci.* 87, 3418–3426.

Wu, R. B., Alexander, T. W., Li, J. Q., Munns, K., Sharma, R., and McAllister, T. A. (2011). Prevalence and diversity of class 1 integrons and resistance genes in antimicrobial-resistant *Escherichia coli* originating from beef cattle administered subtherapeutic antimicrobials. *J. Appl. Microbiol.* 111, 511–523.

Yang, W. R., Moore, I. F., Koteva, K. P., Bareich, D. C., Hughes, D. W., and Wright, D. G. (2004). TetX is a flavin-dependent monooxygenase conferring resistance to tetracycline antibiotics. *J. Biol. Chem.* 279, 52346–52352.

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 02 November 2011; accepted: 23 March 2012; published online: 10 April 2012.

*Citation: Thames CH, Pruden A, James RE, Ray PP and Knowlton KF (2012) Excretion of antibiotic resistance genes by dairy calves fed milk replacers with varying doses of antibiotics. Front. Microbio. **3**:139. doi: 10.3389/fmicb.2012.00139*

This article was submitted to Frontiers in Antimicrobials, Resistance and Chemotherapy, a specialty of Frontiers in Microbiology.

APPENDIX

Table A1 | Gender and breed distribution across antibiotic treatment groups.

Calf	Treatment	Breed	Gender
1	Control	Crossbred	Heifer
2	Control	Crossbred	Heifer
3	Control	Crossbred	Heifer
4	Control	Holstein	Heifer
5	Control	Holstein	Heifer
6	Control	Holstein	Heifer
7	Control	Crossbred	Bull
8	Control	Crossbred	Bull
9	Control	Crossbred	Bull
10	Control	Holstein	Bull
11	Control	Holstein	Bull
12	Control	Holstein	Bull
13	Subtherapeutic	Crossbred	Heifer
14	Subtherapeutic	Crossbred	Heifer
15	Subtherapeutic	Holstein	Heifer
16	Subtherapeutic	Crossbred	Bull
17	Subtherapeutic	Crossbred	Bull
18	Subtherapeutic	Holstein	Heifer
19	Subtherapeutic	Holstein	Bull
20	Therapeutic	Crossbred	Heifer
21	Therapeutic	Crossbred	Heifer
22	Therapeutic	Holstein	Heifer
23	Therapeutic	Holstein	Heifer
24	Therapeutic	Holstein	Heifer
25	Therapeutic	Crossbred	Bull
26	Therapeutic	Crossbred	Bull
27	Therapeutic	Holstein	Bull
28	Therapeutic	Holstein	Bull

Table A2 | Primers and annealing temperatures used in this study.

Primer	Target gene	Primer sequence (5′–3′)	Annealing temperature (°C)	Reference
*sul*1-Fw	*sul*1	CGCACCGGAAACATCGCTGCAC	69.9	Pei et al. (2006)
*sul*1-Rv		TGAAGTTCCGCCGCAAGGCTCG		
*sul*2-Fw	*sul*2	TCCGGTGGAGGCCGGTATCTGG	67.5	Pei et al. (2006)
*sul*2-Rv		CGGGAATGCCATCTGCCTTGAG		
erm(B)-Fw	*erm(*B)	GATACCGTTTACGAAATTGG	58	Chen et al. (2007)
erm(B)-Rv		GAATCGAGACTTGAGTGTGC		
erm(F)-Fw	*erm*(F)	CGACACAGCTTTGGTTGAAC	56	Chen et al. (2007)
erm(F)-Rv		GGACCTACCTCATAGACAAG		
tet(C)-Fw	*tet*(C)	GCGGGATATCGTCCATTCCG	70	Aminov et al. (2002)
tet(C)-Rv		GCGTAGAGGATCCACAGGACG		
tet(G)-Fw	*tet*(G)	GCAGAGCAGGTCGCTGG	64.2	Aminov et al. (2002)
tet(G)-Rv		CCYGCAAGAGAAGCCAGAAG		
tet(O)-Fw	*tet*(O)	ACGGARAGTTTATTGTATACC	50.3	Aminov et al. (2001)
tet(O)-Rv		TGGCGTATCTATAATGTTGAC		
tet(W)-Fw	*tet*(W)	GAGAGCCTGCTATATGCCAGC	60	Aminov et al. (2001)
tet(W)-Rv		GGGCGTATCCACAATGTTAAC		
tet(X)-Fw	*tet*(X)	CAATAATTGGTGGTGGACCC	64.5	Ng et al. (2001)
tet(X)-Rv		TTCTTACCTTGGACATCCCG		
HS463a	*int*I1	CTGGATTTCGATCACGGCACG	60	Hardwick et al. (2008)
HS464		ACATGCGTGTAAATCATCGTCG		
1369F	16S rRNA	CGGTGAATACGTTCYCGG	60	Suzuki et al. (2000)
1492R		GGWTACCTTGTTACGACTT		

frontiers in
MICROBIOLOGY

ORIGINAL RESEARCH ARTICLE
published: 21 February 2012
doi: 10.3389/fmicb.2012.00062

Resident cats in small animal veterinary hospitals carry multi-drug resistant enterococci and are likely involved in cross-contamination of the hospital environment

Anuradha Ghosh[1], Kate KuKanich[2], Caitlin E. Brown[1] and Ludek Zurek[1]*

[1] *Department of Diagnostic Medicine and Pathobiology, College of Veterinary Medicine, Kansas State University, Manhattan, KS, USA*
[2] *Department of Clinical Sciences, College of Veterinary Medicine, Kansas State University, Manhattan, KS, USA*

Edited by:
Rustam I. Aminov, University of Aberdeen, UK

Reviewed by:
Gautam Dantas, Washington University in St. Louis, USA
Carla Novais, Fernando Pessoa University, Portugal
Sabeel Padinhara Valappil, University of Liverpool, UK

***Correspondence:**
Ludek Zurek, Department of Diagnostic Medicine and Pathobiology, College of Veterinary Medicine, Kansas State University, 221K Mosier Hall, Manhattan, KS 66506, USA.
e-mail: lzurek@vet.k-state.edu

In the USA, small animal veterinary hospitals (SAVHs) commonly keep resident cats living permanently as pets within their facilities. Previously, multi-drug resistant (MDR) enterococci were found as a contaminant of multiple surfaces within such veterinary hospitals, and nosocomial infections are a concern. The objectives of this study were to determine whether resident cats carry MDR enterococci and to compare the feline isolates genotypically to those obtained from SAVH surfaces in a previous study. Enterococcal strains ($n = 180$) were isolated from the feces of six healthy resident cats from different SAVHs. The concentration of enterococci ranged from 1.1×10^5 to 6.0×10^8 CFU g^{-1} of feces, and the population comprised *Enterococcus hirae* ($38.3 \pm 18.6\%$), *E. faecium* ($35.0 \pm 14.3\%$), *E. faecalis* ($23.9 \pm 11.0\%$), and *E. avium* ($2.8 \pm 2.2\%$). Testing of phenotypic resistance to 14 antimicrobial agents revealed multi-drug resistance (≥3 antimicrobials) in 48.9% of all enterococcal isolates with most frequent resistance to tetracycline (75.0%), erythromycin (50.0%), and rifampicin (36.1%). Vancomycin resistant *E. faecalis* (3.9%) with *vanB* not horizontally transferable in *in vitro* conjugation assays were detected from one cat. Genotyping with pulsed-field gel electrophoresis demonstrated a host-specific clonal population of MDR *E. faecalis* and *E. faecium*. Importantly, several feline isolates were genotypically identical or closely related to isolates from surfaces of cage door, thermometer, and stethoscope of the corresponding SAVHs. These data demonstrate that healthy resident cats at SAVHs carry MDR enterococci and likely contribute to contamination of the SAVH environment. Proper disposal and handling of fecal material and restricted movement of resident cats within the ward are recommended.

Keywords: *Enterococcus*, antimicrobial resistance, resident cats, small animal veterinary hospital, clonal diversity

INTRODUCTION

USA households own a large number of dogs (77.5 million) and cats (93.6 million) as pets (APPA, 2009/2010), leading to an average of 2–3 veterinary hospital visits per household per year (AVMA, 2007). Due to high traffic with healthy and sick patients and clients in and out of small animal veterinary hospitals (SAVHs), it is important to maintain good hygiene of these facilities. Previous studies have documented bacterial contamination of surfaces in SAVHs (Aksoy et al., 2010; KuKanich et al., 2012). There are several sources that may contribute to this contamination. Frequent use of antimicrobial agents in veterinary medicine likely results in the selection of resistant bacteria within the gastrointestinal tract of patients that can be spread in the hospital environment by fecal contamination. In addition, in the USA, many SAVHs keep resident pets that freely move around their premises and could act as a carrier/reservoir of resistant bacteria. Other possible sources of contamination include visiting patients, animal owners, hospital personnel as well as lack of efficient infection control measures.

Although enterococci are commensals in the gut microbiota of mammals; in the last decades they become one of the important nosocomial human pathogens (Roberts et al., 2009a). Enterococci can readily acquire genes leading to increased virulence, antimicrobial resistance (Gilmore et al., 2002; Arias and Murray, 2008), and have the ability to survive on inanimate dry hospital surfaces for up to 4 months (Kramer et al., 2006). Therefore, the appropriate use of disinfectants is critical for maintaining proper hygiene standards in hospitals and for reducing nosocomial infections (Hota, 2004). Healthy companion animals have been shown to carry antimicrobial resistant enterococcal lineages associated with human infections and thus may be considered a reservoir shedding these bacteria into the environment (Guardabassi et al., 2004; Damborg et al., 2009; Jackson et al., 2009a). Our recent study has revealed that the dogs leaving the veterinary intensive care unit (ICU) carried a very large population of multi-drug resistant (MDR) enterococci with the capacity for biofilm formation and horizontal transfer of resistance genes. Furthermore, genotyping of canine enterococcal isolates demonstrated that some of them belonged to the human nosocomial clonal complex-17 (CC-17) (Ghosh et al., 2011). In small animals, enterococci have also been implicated in diarrhea, mastitis, urinary tract and

catheter-associated infections, and cholangiohepatitis (Helie and Higgins, 1999; Manson et al., 2003; Pressel et al., 2005; Marsh-Ng et al., 2007). With the exception of rare case reports (Boerlin et al., 2001; Benedict et al., 2008), little is known about the nosocomial potential of enterococci in veterinary medicine. Considering the previous study documenting MDR and potentially virulent enterococci in the SAVH environment (KuKanich et al., 2012), further research is needed to better understand bacterial contamination in order to assess and mitigate nosocomial and public health risks.

In the current study, we hypothesized that resident cats in SAVHs acquire and carry antimicrobial resistant enterococci and play a role in dissemination of antimicrobial resistance traits in the hospital environment. The objectives of this study were: (1) to isolate fecal enterococci from six resident cats and to determine their concentration, diversity, clonality, and phenotypic antimicrobial resistance and virulence profiles; (2) to compare the clonality of feline enterococcal isolates to that of isolates from cage doors, thermometers, and stethoscopes in the corresponding hospitals (KuKanich et al., 2012).

MATERIALS AND METHODS

SAMPLE COLLECTION, ISOLATION, AND IDENTIFICATION OF ENTEROCOCCI

Ten privately owned SAVHs (hospitals 1–10) were enrolled in a previous surveillance study that was conducted to evaluate bacterial contamination on surfaces of cage doors, stethoscopes, thermometers, and mouth gags (KuKanich et al., 2012). As a follow-up of the surface study, fecal material was collected from six resident cats (6/10 SAVHs had resident pets) from individual SAVHs. Each cat was considered healthy by its primary veterinarian. Most cats had lived at the hospital premise since they were kittens; four of them had never received any antimicrobial treatment, and the remaining two were not exposed to antimicrobials for at least 1 year prior to our sampling. Detailed information regarding the enrolled cats and their diets is in **Table A1** in Appendix. This study was given exempt status from the institutional animal care and use committee because it did not involve any direct contact with animals.

Fresh feces were collected from the litter box and one gram of the feces was re-suspended in 10 ml of phosphate buffered saline (PBS, pH 7.2; MP Biomedicals, Solon, OH, USA). A 100 µl of the suspension was plated in triplicate on mEnterococcus agar (Difco, BD Diagnostic Systems, Sparks, MD, USA), and presumptive enterococcal colonies were selected and confirmed at the genus level as described previously (Macovei and Zurek, 2007). Identification at the species level was carried out for randomly selected 30 isolates per fecal sample using species-specific multiplex PCR (Kariyama et al., 2000) or sequencing of the manganese-dependent superoxide dismutase gene (*sodA*) (Poyaert et al., 2000). The concentration of enterococci for each sample was calculated in CFU per gram of feces. In order to assess whether the cat diets are the source of MDR enterococci, three out of six commercially available cat diets fed to individual cats were sampled and tested. Two grams of dry food were re-suspended in 20 ml PBS and 100 µl of the suspension was plated on mEnterococcus agar and incubated for 48 h in 37°C.

ANTIMICROBIAL SUSCEPTIBILITY TESTING AND HORIZONTAL GENE TRANSFER

The identified isolates were tested for their susceptibility to antimicrobials commonly used in veterinary medicine as well as those targeting human enterococcal infections. Antimicrobial susceptibility was determined by the disk diffusion method on Mueller-Hinton agar (Difco, BD Diagnostic Systems, Sparks, MD, USA) using 10 antimicrobial agents (µg disk^{-1}): ampicillin (10), tetracycline (30), doxycycline (30), gentamicin (120), erythromycin (15), enrofloxacin (5), vancomycin (30), quinupristin/dalfopristin (15), nitrofurantoin (300), and tigecycline (15). Agar dilution technique was carried out to determine resistance to the following antimicrobials (µg ml^{-1}): streptomycin (2,000) on brain heart infusion agar (BBL, BD Diagnostic Systems, Sparks, MD, USA) and chloramphenicol (16), rifampicin (24), and linezolid (8) on Mueller-Hinton agar. Minimum inhibitory concentration (MIC) for vancomycin (8–256 µg ml^{-1}, in double fold increments) and teicoplanin (0.25–8 µg ml^{-1}, in double fold increments) was determined for vancomycin resistant isolates by broth microdilution technique using Mueller-Hinton broth (BBL, BD Diagnostic Systems, Sparks, MD, USA; CLSI, 2008, 2010). Routine quality control of antimicrobial disks was performed using control strains of *Enterococcus faecalis* ATCC 19433 and *E. faecium* ATCC 19434. Diameters of zones of inhibition were interpreted in accordance with guidelines of the Clinical and Laboratory Standards Institute for *Enterococcus* spp. (CLSI, 2008, 2010). The breakpoint for tigecycline is according to manufacturer specifications upon recommendation from the CLSI. Antimicrobial breakpoints for enterococcal species are not all veterinary approved and are therefore not validated in relation to clinical outcome in a veterinary application. Multi-drug resistance was defined as resistance to three or more antimicrobial agents, regardless of class. Linear regression analysis was performed to compare the antimicrobial resistance profiles of each enterococcal species from all animals and animals with no antimicrobial treatment. The antimicrobial resistance profiles of feline isolates were also compared to that of the isolates from hospital surfaces from our previous work (KuKanich et al., 2012) although in that study the isolates were not screened for resistance to tetracycline, streptomycin, linezolid, rifampicin, and chloramphenicol.

PCR amplification of *vanA* and *vanB* genes was conducted with primers described previously (Kariyama et al., 2000; Elsayed et al., 2001). Broth and filter conjugation assays were carried out as described by Ike et al. (1998) and Tendolkar et al. (2006), respectively to determine the mobility of the vancomycin resistance trait among *E. faecalis* strains. The recipients included the following strains with resistance markers: 41–31 (linezolid, MIC = 8 µg ml^{-1}; Qi et al., 2006), OG1SSp (spectinomycin, MIC = 250 µg ml^{-1}; Dunny et al., 1982), and JH2-2 (rifampicin, MIC = 30 µg ml^{-1}; Oancea et al., 2004). Both assays were performed with a donor and recipient ratio of 1:10. The transconjugants were detected on agar supplemented with vancomycin (16 µg ml^{-1}) and the respective antimicrobial markers of the recipient strains with the same antimicrobial concentrations as described above.

SCREENING FOR VIRULENCE TRAITS BY PHENOTYPE

Identified isolates were tested for the gelatinase on Todd Hewitt Agar (Difco, BD Diagnostic Systems, Sparks, MD, USA) with 1.5% skim milk, and for the cytolysin (β-hemolysis) on Columbia blood agar base (Difco, BD Diagnostic Systems, Sparks, MD, USA) supplemented with 5.0% human blood (Rockland Immunochemicals, Inc., Gilbertsville, PA, USA) as described previously (Macovei and Zurek, 2006).

GENOTYPING BY PULSED-FIELD GEL ELECTROPHORESIS

The feline enterococcal isolates (*E. faecalis*, $n = 15$; *E. faecium*, $n = 18$) for pulsed-field gel electrophoresis (PFGE) were selected based on their different antibiotic resistance profile. These were genotyped to assess their clonality within and between cats. Also, in this study, nine *E. faecalis* isolates from various hospital surfaces were genotyped and compared with the feline isolates while the feline *E. faecium* isolates were compared to the 42 *E. faecium* hospital isolates typed in our previous study (KuKanich et al., 2012). PFGE was carried out following the protocols of Murray et al. (1990) and Turabelidze et al. (2000). Briefly, agarose plugs were restriction digested with 20U of *Sma*I (New England Biolab, Ipswich, MA, USA) for 4 h at 25°C. Digested plugs were run on to a 1.0% SeaKem Gold Agarose (Lonza, Rockland, ME, USA) gel using CHEF Mapper (Bio-Rad Laboratories, Hercules, CA, USA) with initial pulse time for 1 s and final time for 20 s at 200 V for 21 h. Cluster analysis was performed with BioNumerics (Applied Maths, Inc., Austin, TX, USA) by using the band-based Dice correlation coefficient and the unweighted pair group mathematical average algorithm (UPGMA) with position tolerance setting of 1.0% for both optimization and band comparison. The cut off 95% was used to classify the strains as clonal.

RESULTS

ENTEROCOCCAL CONCENTRATION AND SPECIES DIVERSITY

The mean enterococcal concentration was $1.1 \pm 0.7 \times 10^6$ CFU g^{-1} of feces in five cats, with one outlying cat that had 6.0×10^8 CFU g^{-1} (**Table 1**). There was great variation in enterococcal composition at the species level among individual cats (**Table 1**). Altogether, of the 180 isolates, *E. hirae* (69/180, $38.3 \pm 18.6\%$) was the most common, followed by *E. faecium* (63/180, $35.0 \pm 14.3\%$), *E. faecalis* (43/180, $23.9 \pm 11.0\%$), and *E. avium* (5/180, $2.8 \pm 2.2\%$). No enterococcal colonies were detected in the cat diets.

ANTIMICROBIAL RESISTANCE AND VIRULENCE

Eighty-eight out of 180 enterococcal isolates (48.9%) from feces of the enrolled healthy cats showed multi-drug resistance (3–8 antimicrobials; **Table 2**). Overall, *E. faecium* exhibited high incidence of resistance to tetracycline, erythromycin, and enrofloxacin while resistance to rifampicin, quinupristin/dalfopristin, doxycycline, ampicillin, gentamicin, streptomycin, and nitrofurantoin was less frequent (**Figure 1**). *E. faecalis* was frequently resistant to a total of six different antimicrobials, namely rifampicin, erythromycin, gentamicin, tetracycline, doxycycline, and streptomycin whereas resistance to vancomycin, and chloramphenicol was less common (**Figure 1**). A considerable number of *E. hirae* were resistant to tetracycline, rifampicin, and erythromycin (**Figure 1**). Five isolates of *E. avium* showed resistance to rifampicin, erythromycin, and/or tetracycline (**Table A2** in Appendix). No resistance was detected to the newer generation drugs, linezolid and tigecycline, in any enterococcal isolates. The antimicrobial resistance profile for cats that had never been administered antimicrobial agents is depicted in **Figure A1** in Appendix. Based on statistical analysis, antimicrobial resistance profiles of enterococcal species from all cats and cats with no antimicrobial treatment were highly similar [$R = 0.9458$ (*E. faecalis*), $R = 0.9764$ (*E. faecium*), and $R = 0.9844$ (*E. hirae*)].

Isolates from feces of individual cats provided a unique profile of antimicrobial resistance. In cat-A, 8 out of 11 *E. faecium* were resistant to quinupristin/dalfopristin, and several were resistant to high level of streptomycin (4/11), doxycycline (3/11), and nitrofurantoin (3/11; **Table A2** in Appendix). *E. faecalis* strains (5/15) resistant to chloramphenicol and high level of gentamicin were also detected in the feces of this cat. In cat-D, the enterococcal population in the gut was almost equally shared by MDR strains of *E. faecalis* (11/30), *E. hirae* (10/30), and *E. faecium* (9/30). Among these, all *E. faecalis* were phenotypically resistant to six antimicrobials (tetracycline, doxycycline, erythromycin, rifampicin, gentamicin, and streptomycin) whereas one *E. faecium* strain was resistant to tetracycline, erythromycin, enrofloxacin, rifampicin, and streptomycin (**Table 2**; **Table A2** in Appendix). Cat-E was colonized with MDR *E. faecalis* (7/30)

Table 1 | Enterococcal concentration and species diversity in the feces of resident cats from small animal veterinary hospitals.

Sample ID	Hospital ID[†]	Concentration (CFU g^{-1})	Diversity (%)*			
			E. faecalis	*E. faecium*	*E. hirae*	*E. avium*
Cat-A	1	6.0×10^8	50.0	36.7	0	13.3
Cat-B	2	9.1×10^5	0	100	0	0
Cat-C	4	2.1×10^5	0	10.0	90.0	0
Cat-D	6	1.7×10^5	36.7	30.0	33.3	0
Cat-E	7	4.1×10^6	56.7	33.3	6.7	3.3
Cat-F	9	1.1×10^5	0	0	100	0

[†] *Hospital IDs consistent with previous surveillance study (KuKanich et al., 2012).*

**Based on 30 isolates from each fecal sample.*

Table 2 | Multiple (≥3) antimicrobial resistance profile among enterococcal isolates from the feces of resident cats from small animal veterinary hospitals.

Resistance profile	Number of strains (%)*			Origin
	E. faecalis (*n* = 43)	*E. faecium* (*n* = 63)	*E. hirae* (*n* = 69)	
TET, E, ENO		24 (38.1)		Cat-B
TET, E, Q/D		5 (7.9)		Cat-A
TET, E, RIF			10 (14.5)	Cat-D
TET, E, ENO, RIF		2 (3.2)		Cat-D
E, ENO, RIF		7 (11.1)		Cat-D
E, GM, VA	7 (16.3)			Cat-E
TET, E, GM, CM	3 (6.9)			Cat-A
TET, E, Q/D, NF		1 (1.6)		Cat-A
TET, E, RIF, GM, CM	2 (4.6)			Cat-A
TET, E, ENO, RIF, STR		1 (1.6)		Cat-D
TET, E, ENO, STR, AM		1 (1.6)		Cat-A
TET, E, Q/D, STR, D		2 (3.2)		Cat-A
TET, E, Q/D, D, NF		1 (1.6)		Cat-A
TET, E, RIF, GM, STR, D	11 (25.6)			Cat-D
TET, E, ENO, STR, AM, NF		1 (1.6)		Cat-A
TET, E, ENO, Q/D, RIF, GM, AM, D		10 (15.9)		Cat-E

AM, ampicillin; CM, chloramphenicol; TET, tetracycline; D, doxycycline; GM, gentamicin; STR, streptomycin; RIF, rifampicin; E, erythromycin; ENO, enrofloxacin; VA, vancomycin; Q/D, quinupristin/dalfopristin; NF, nitrofurantoin.

**E. avium are not included as they were not multi-drug resistant.*

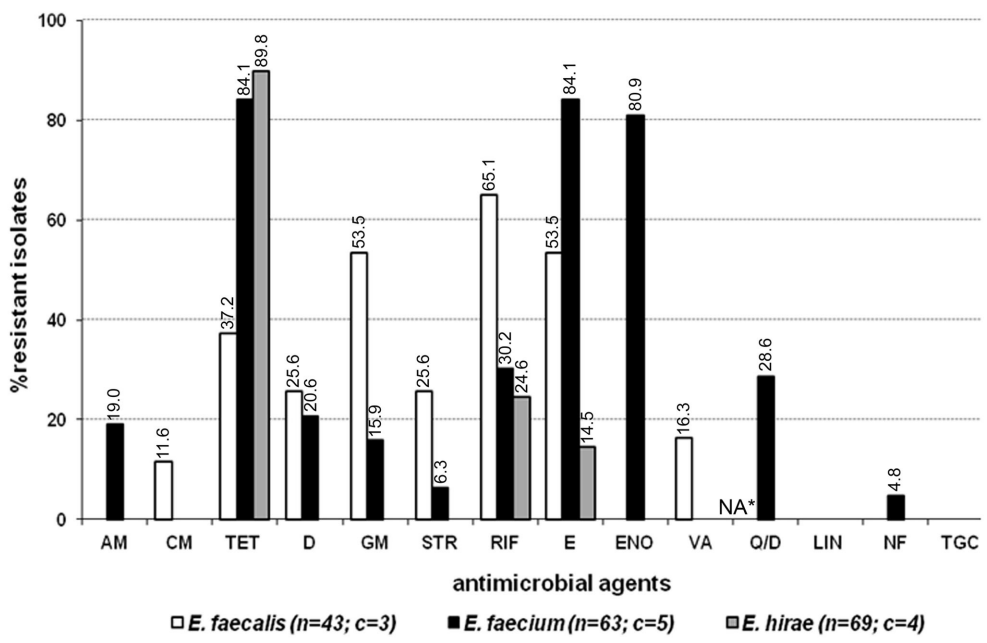


FIGURE 1 | Antimicrobial resistance profile of enterococci from the feces of six resident cats from small animal veterinary hospitals. AM, ampicillin; CM, chloramphenicol; TET, tetracycline; D, doxycycline; GM, gentamicin; STR, streptomycin; RIF, rifampicin; E, erythromycin; ENO, enrofloxacin; VA, vancomycin; Q/D, quinupristin/dalfopristin; LIN, linezolid; NF, nitrofurantoin; TGC, tigecycline; *c*, number of cats that contributed for the total number (*n*) of isolates. *Not applicable for *E. faecalis* isolates due to their intrinsic resistance.

and *E. faecium* (10/30), and all *E. faecalis* were vancomycin resistant with an MIC of 16–32 $\mu g\,ml^{-1}$, but susceptible to teicoplanin (MIC $\leq 1\,\mu g\,ml^{-1}$). These vancomycin resistant strains harbored the *vanB* gene but not *vanA*. Standard *in vitro* conjugation assays

failed to transfer *vanB* from the feline isolates to several *E. faecalis* recipient strains. Besides vancomycin, these *E. faecalis* were also resistant to high level of gentamicin and erythromycin. In addition, all *E. faecium* from cat-E displayed resistance to 8 antimicrobials (tetracycline, doxycycline, erythromycin, enrofloxacin, quinupristin/dalfopristin, rifampicin, gentamicin, and ampicillin; **Table 2**, **Table A2** in Appendix). Cat-B was colonized with *E. faecium* (24/30) resistant to enrofloxacin, erythromycin, and tetracycline. *E. hirae* from cat-C were resistant to tetracycline (27/30) while those from cat-F were resistant to tetracycline (25/30) and rifampicin (5/30) (**Table A2** in Appendix).

All feline isolates were screened for two enterococcal virulence factors, gelatinase and cytolysin. The majority of *E. faecalis* (38/43, 88.4%) showed strong gelatinase phenotype, and several (5/43, 11.6%) were β-hemolytic on human blood agar. *E. faecium* (58/63, 92.1%) and *E. hirae* (66/69, 95.6%) showed weak gelatinase phenotype; the remainder were negative for gelatinase activity and none were β-hemolytic.

GENOTYPING OF FELINE ENTEROCOCCAL ISOLATES

Pulsed-field gel electrophoresis analysis revealed that enterococcal isolates from individual cats were host-specific clonal groups (unique pulsotypes in individual cats; **Figures 2A,B**). The feline enterococcal isolates were also compared to the surface isolates from the corresponding hospitals (**Figure 2A**; KuKanich et al., 2012). *E. faecalis* from a cage door of Hospital-6 showed close genotypic similarity (96.9%) to several fecal isolates from the cat (cat-D) residing at the facility (**Figure 2A**). Furthermore, two *E. faecium* strain from feces of cat-A were clonally indistinguishable or closely related (96.9%) to isolates from the surfaces (a cage door, a stethoscope, and a thermometer) of Hospital-1 (KuKanich et al., 2012).

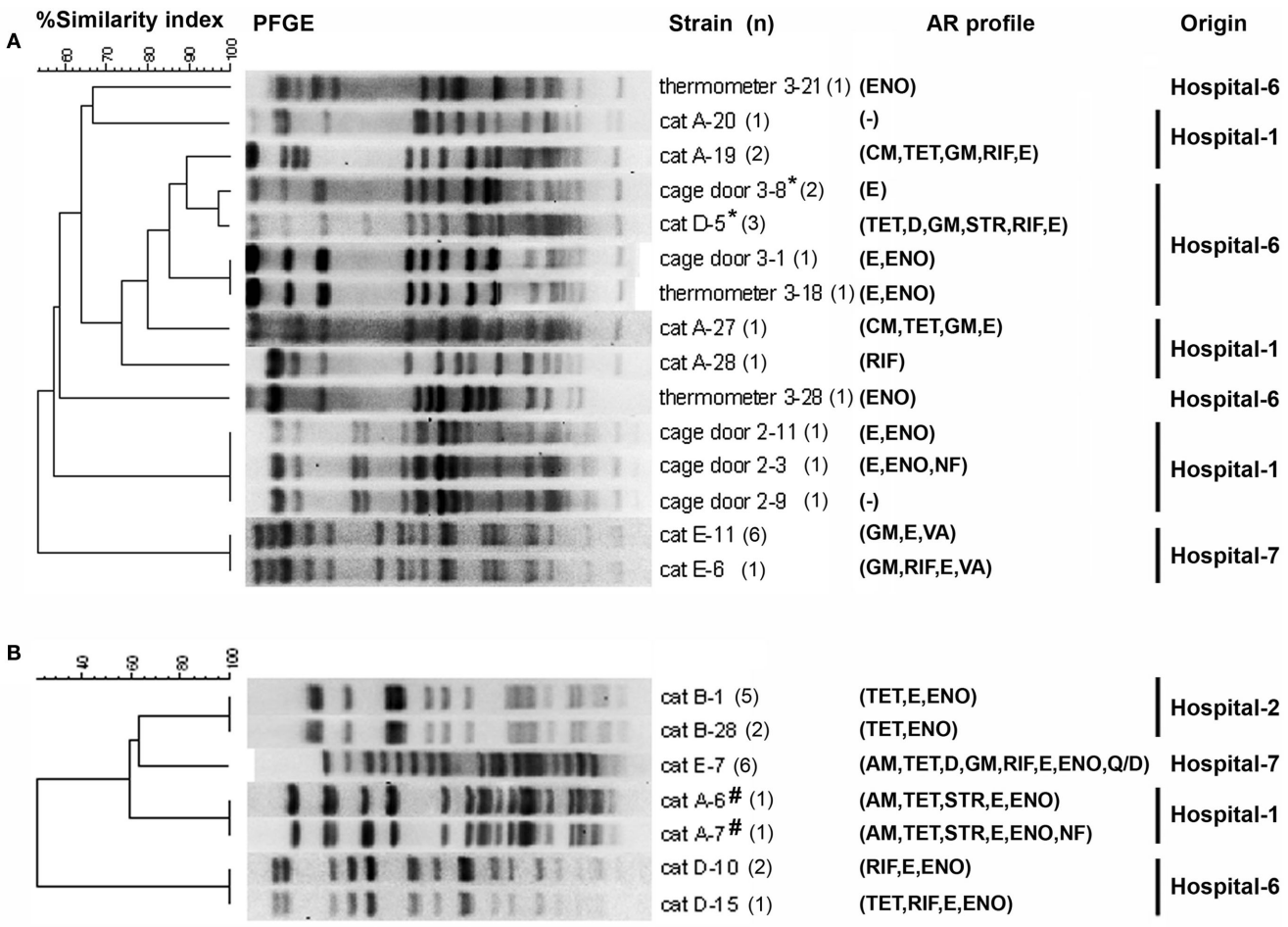


FIGURE 2 | Dendrograms based on SmaI restriction pattern resolved by pulsed-field gel electrophoresis (PFGE) depicting the relationships among representative isolates of (A) *E. faecalis* (feline, $n = 15$; surface, $n = 9$) and (B) *E. faecium* (feline, $n = 18$) in small animal veterinary hospitals. Strain numbers, number of strains typed (*n*), antimicrobial resistance (AR) profiles, and corresponding hospitals are included along each PFGE lane. "*" Indicates 96.9% similarity among *E. faecalis* isolated from the hospital surface and cat feces. "#" Indicates 100% similarity to *E. faecium* isolated from surface of a cage door and a stethoscope, and 96.9% similarity to *E. faecium* isolated from surface of a thermometer of the corresponding hospital (KuKanich et al., 2012); AM, ampicillin; CM, chloramphenicol; TET, tetracycline; D, doxycycline; GM, gentamicin; STR, streptomycin; RIF, rifampicin; E, erythromycin; ENO, enrofloxacin; VA, vancomycin; Q/D, quinupristin/dalfopristin; NF, nitrofurantoin. Hospital surface isolates were not screened for resistance to tetracycline, chloramphenicol, linezolid, streptomycin, and rifampicin.

DISCUSSION

With large numbers of pet animals in the USA, maintaining a healthy environment within SAVHs is highly desirable. Recent studies have reported surface contamination by MDR enterococci and other bacteria in SAVH environment (Aksoy et al., 2010; KuKanich et al., 2012). Our study was designed to assess healthy resident cats in SAVHs as carriers/reservoirs of MDR enterococci.

The mean value of enterococcal population size in resident cats was comparable to that reported from the feces of healthy pets (10^3–10^6 CFU g^{-1} of feces) (Damborg et al., 2009). Though the enterococcal species composition was diverse among individual cats, the overall diversity at the species level indicated a healthy feline gut microbiota and was in congruence with other studies on enterococcal population in healthy cats (Devriese et al., 1992; de Leener et al., 2005). Previously, Poeta et al. (2006) reported an intermediate prevalence of resistance in enterococci with few MDR strains isolated from healthy cats in Portugal. de Leener et al. (2005) and Moyaert et al. (2006) screened cats from diverse populations in Belgium and demonstrated that enterococcal strains in cats from hospitals and catteries had relatively higher prevalence of resistance to several antimicrobial agents (erythromycin, tylosin, lincomycin, tetracycline, chloramphenicol, and kanamycin) compared to that of healthy domestic cats. In the USA, Jackson et al. (2009a) reported antimicrobial resistant *E. faecium* and *E. faecalis* from a total of 116 healthy cats visiting veterinary clinics. They detected MDR *E. faecalis* and *E. faecium* (3–8 antimicrobials) but no vancomycin or quinupristin/dalfopristin resistant strains. Our results showed that four out of six healthy cats harbored a MDR enterococcal population with *E. faecium* occurring most frequently. To our knowledge, our study reported for the first time MDR (tetracycline, erythromycin, and rifampicin) *E. hirae* from a healthy companion animal. All of our enterococcal isolates were susceptible to the relatively new antimicrobial agents linezolid and tigecycline, and this is in agreement with the study of Jackson et al. (2009a). Importantly, the overall antimicrobial resistance profile of the isolates from our healthy resident cats was similar to that of cattery and hospitalized feline isolates rather than the isolates from healthy domestic cats.

Due to limited number of animals and bacterial isolates, we could not assess a correlation between the antimicrobials administered to resident cats and antimicrobial resistance profiles of the feline enterococcal isolates. Clearly, three out of four cats that have never been administered antimicrobials carried a MDR enterococcal population. Vice versa, feces of one of the two cats that received an antimicrobial treatment had the enterococcal community susceptible to all antibiotics tested with the exception of tetracycline. While enterococci are not commonly associated with infections in small animals, due to their ability to horizontally transfer resistance traits to other bacteria including *Staphylococcus aureus* (Noble et al., 1992; Clewell and Dunny, 2002; Weigel et al., 2003), the enterococcal resistance reported in this study is important especially that to doxycycline, enrofloxacin, and ampicillin. This is because tetracyclines, fluoroquinolones, and β-lactams are very commonly used in small animal veterinary medicine to treat various bacterial infections (Prescott et al., 2002). Due to close physical contact between pets and people, the likelihood of transfer of bacteria is very high and it has been demonstrated for different bacterial taxa including enterococci and staphylococci (Simjee et al., 2002; Guardabassi et al., 2004; Bramble et al., 2011; Chomel and Sun, 2011). Once established in the digestive tract, MDR enterococci can persist for months to years (Byers et al., 2002; Sørum et al., 2006). Detection of chloramphenicol resistant *E. faecalis* and quinupristin/dalfopristin resistant *E. faecium* requires attention since these antimicrobials are used to treat human infections caused by vancomycin resistant enterococci (VRE) (Lautenbach et al., 1998; Linden et al., 2001; Arias and Murray, 2008). We also report vancomycin resistant *E. faecalis* with the *vanB* gene not transferable *in vitro*; however, transfer of this trait in natural niches cannot be ruled out. van Belkum et al. (1996) reported that 4/24 (16%) of the rectal swabs from cats that attended a veterinary practice in the Netherlands were positive for vancomycin resistant *E. faecium* with *vanA*. Another study reported association of vancomycin resistant *E. faecium* in a cat with cholangiohepatitis (Pressel et al., 2005). To our knowledge, isolation of vancomycin resistant *E. faecalis* from a healthy cat in our study is the first of its kind. Interestingly, VRE were not detected in our previous surveillance study (KuKanich et al., 2012) and this antibiotic is also typically not used to treat animals at SAVHs (Weese, 2008). Although the source of VRE in this cat is unknown, it is likely of a human (pet owner, hospital staff) origin since VRE strains are rare in the USA outside of the human clinical environment (Sapkota et al., 2007; Roberts et al., 2009b). The VRE transfer from personnel in human hospitals to patients as well as between pet owners and pets has been suggested in several studies (Hayden, 2000; Guardabassi et al., 2004; Tacconelli and Cataldo, 2008) and it is therefore also possible in SAVHs. Resistance to ampicillin and high level of gentamicin in *E. faecium* is also a concern, as gentamicin in combination with β-lactams or glycopeptides is typically used to treat human enterococcal infections (Arias and Murray, 2008).

Gelatinase and cytolysin are among the virulence traits implicated in enhancement of enterococcal infections (Gilmore et al., 2002). Gulhan et al. (2006) demonstrated that prevalence of gelatinase and cytolytic activity among *E. faecalis* and *E. faecium* were negligible in healthy cats. In contrast, the presence of gelatinolytic as well as several β-hemolytic strains of *E. faecalis* isolated from the healthy resident cats in our study indicates these isolates have increased virulence potentially resulting in biofilm formation (Ghosh et al., 2011), proliferation in bloodstream, and direct tissue damage (Gilmore et al., 2002). Therefore, our data demonstrate that resident cats in SAVHs are carriers and possible reservoirs of antimicrobial resistant and potentially virulent enterococcal strains. Since fecal samples of these cats were not tested prior to their adoption as resident cats (as kittens), we cannot entirely exclude a rather remote possibility that the cats in this study acquired the enterococci from an outside source before their stay in SAVHs.

Sharing of methicillin-resistant *S. aureus* (MRSA) clones among hospital staff, surfaces, patients, and resident animals had been documented in a veterinary hospital (Loeffler et al., 2005) as well as in a human geriatric ward (Scott et al., 1988) indicating cross-contamination within the hospital environment. In our study, clonal matches based on PFGE clearly demonstrate cross-contamination between the resident cats and the hospital environment although the directionality of the bacterial transfer requires

further investigation. The PFGE dendrograms also revealed that feline enterococcal isolates in our study were host-specific (unique to individual cats) and not diverse although genotyping of more isolates is warranted to assess the diversity to a greater extent. These findings differ from the study by Jackson et al. (2009b) where they found that healthy domestic cats harbored MDR enterococcal strains of diverse clonal origin. We hypothesize that the antimicrobial use in the SAVHs leads to the selection of specific MDR clonal lineages in the patients that contaminate the hospital environment. Consequently, this contamination becomes the source of MDR enterococci for the resident cats that then become carriers/reservoirs of these bacteria and possibly re-contaminate the hospital environment. The cat diet is an unlikely source of the antimicrobial resistant enterococci as all resident cats enrolled in this study were fed commercial cat food (**Table A1** in Appendix) and our random screening of these diets failed to detect any enterococcal contamination. Other possible sources of antimicrobial resistant enterococci such as the hospital staff or pet owners cannot be ruled out.

In conclusion, we demonstrate that resident cats in SAVHs in the USA can carry and shed MDR enterococci and this may create risk to people and animals within veterinary hospitals. Further research is warranted to determine direction of enterococcal transmission between resident cats and environmental surfaces of the clinics, and also to evaluate the duration (transient or long-lasting) of such carriage. Nonetheless, proper disposal and handling of fecal material of resident cats, good overall hygiene, effective infection control, and restricted movement of these animals within the ward are recommended.

ACKNOWLEDGMENTS

Funding and support provided by NIH T35 RR007064, Department of Clinical Sciences Research Grant, College of Veterinary Medicine, Kansas State University, and the Veterinary Research Scholars Program. We thank Michael Haden for technical help. This is contribution no. 12-270-J of the Kansas Agricultural Experiment Station.

REFERENCES

Aksoy, E., Boag, A., Brodbelt, D., and Grierson, J. (2010). Evaluation of surface contamination with staphylococci in a veterinary hospital using a quantitative microbiological method. *J. Small Anim. Pract.* 51, 574–580.

APPA. (2009/2010). *The 2009/2010 APPA National Pet Owners Survey*, ed. American Pet Products Association. Greenwich: APPA Press.

Arias, C. A., and Murray, B. E. (2008). Emergence and management of drug-resistant enterococcal infections. *Expert Rev. Anti Infect. Ther.* 6, 637–655.

AVMA. (2007). *U.S. Pet Ownership and Demographics Sourcebook*, 2nd Edn. ed. American Veterinary Medical Association. Schaumburg: AVMA Press.

Benedict, K. M., Morley, P. S., and van Metre, D. C. (2008). Characteristics of biosecurity and infection control programs at veterinary teaching hospitals. *J. Am. Vet. Med. Assoc.* 233, 767–773.

Boerlin, P., Eugster, S., Gaschen, F., Straub, R., and Schawalder, P. (2001). Transmission of opportunistic pathogens in a veterinary teaching hospital. *Vet. Microbiol.* 82, 347–359.

Bramble, M., Morris, D., Tolomeo, P., and Lautenbach, E. (2011). Potential role of pet animals in household transmission of methicillin-resistant *Staphylococcus aureus*: a narrative review. *Vector Borne Zoonotic Dis.* 2010.0025.

Byers, K. E., Anglim, A. M., Anneski, C. J., and Farr, B. M. (2002). Duration of colonization with vancomycin-resistant *Enterococcus*. *Infect. Cont. Hosp. Epidemiol.* 23, 207–211.

Chomel, B. B., and Sun, B. (2011). Zoonoses in the bedroom. *Emerg. Infect. Dis.* 17, 167–172.

Clewell, D. B., and Dunny, G. M. (2002). "Conjugation and genetic exchange in enterococci" in *The Enterococci: Pathogenesis, Molecular Biology and Antibiotic Resistance*, eds M. S. Gilmore, D. B. Clewell, P. Courvalin, G. M. Dunny, B. E. Murray, and L. B. Rice (Washington, DC: ASM Press), 265–300.

CLSI. (2008). "Performance standards for antimicrobial disk and dilution susceptibility tests for bacteria isolated from animals," in *Approved Standard CLSI Document M31-A3*, Vol. 28, 3rd Edn. ed. Clinical and Laboratory Standards Institute (Wayne: CLSI Press), 1–99.

CLSI. (2010). "Performance standards for antimicrobial susceptibility testing; twentieth informational supplement," in *CLSI Document M100-S20*, Vol. 30. ed. Clinical and Laboratory Standards Institute (Wayne: CLSI Press), 76–79.

Damborg, P., Top, J., Hendrickx, A. P. A., Dawson, S., Willems, R. J. L., and Guardabassi, L. (2009). Dogs are a reservoir of ampicillin-resistant *E. faecium* lineages associated with human infections. *Appl. Environ. Microbiol.* 75, 2360–2365.

de Leener, E., Decostere, A., de Graef, E. M., Moyaert, H., and Haesebrouck, F. (2005). Presence and mechanism of antimicrobial resistance among enterococci from cats and dogs. *Microb. Drug Resist.* 11, 395–403.

Devriese, L. A., Colque, J. I. C., de Herdt, P., and Haesebrouck, F. (1992). Identification and composition of the tonsillar and anal enterococcal and streptococcal flora of dogs and cats. *J. Appl. Microbiol.* 73, 421–425.

Dunny, G., Yuhasz, M., and Ehrenfeld, E. (1982). Genetic and physiological analysis of conjugation in *Streptococcus faecalis*. *J. Bacteriol.* 151, 855–859.

Elsayed, S., Hamilton, N., Boyd, D., and Mulvey, M. (2001). Improved primer design for multiplex PCR analysis of vancomycin-resistant *Enterococcus* spp. *J. Clin. Microbiol.* 39, 2367–2368.

Ghosh, A., Dowd, S. E., and Zurek, L. (2011). Dogs leaving the ICU carry a very large multi-drug resistant enterococcal population with capacity for biofilm formation and horizontal gene transfer. *PLoS ONE* 6, e22451. doi:10.1371/journal.pone.0022451

Gilmore, M. S., Coburn, P. S., Nallapareddy, S. R., and Murray, B. E. (2002). "Enterococcal virulence" in *The Enterococci: Pathogenesis, Molecular Biology and Antibiotic Resistance*, eds M. S. Gilmore, D. B. Clewell, P. Courvalin, G. M. Dunny, B. E. Murray, and L. B. Rice (Washington, DC: ASM Press), 301–354.

Guardabassi, L., Schwarz, S., and Lloyd, D. H. (2004). Pet animals as reservoirs of antimicrobial-resistant bacteria. *J. Antimicrob. Chemother.* 54, 321–332.

Gulhan, I., Aksakal, A., and Ekunc, U. H. (2006). Virulence factors of *Enterococcus faecium* and *Enterococcus faecalis* strains isolated from humans and pets. *Turk. J. Vet. Anim. Sci.* 30, 477–482.

Hayden, M. K. (2000). Insights into the epidemiology and control of infection with vancomycin-resistant enterococci. *Clin. Infect. Dis.* 31, 1058–1065.

Helie, P., and Higgins, R. (1999). Diarrhea associated with *Enterococcus faecium* in an adult cat. *J. Vet. Diagn. Invest.* 11, 457–458.

Hota, B. (2004). Contamination, disinfection, and cross-colonization: are hospital surfaces reservoirs for nosocomial infection? *Clin. Infect. Dis.* 39, 1182–1189.

Ike, Y., Tanimoto, K., Tomita, H., Takeuchi, K., and Fujimoto, S. (1998). Efficient transfer of the pheromone-independent *Enterococcus faecium* plasmid pMG1 (Gmr) (65.1 kilobases) to *Enterococcus* strains during broth mating. *J. Bacteriol.* 180, 4886–4892.

Jackson, C. R., Fedorka-Cray, P. J., Davis, J. A., Barrett, J. B., and Frye, J. G. (2009a). Prevalence, species distribution and antimicrobial resistance of enterococci isolated from dogs and cats in the United States. *J. Appl. Microbiol.* 107, 1269–1278.

Jackson, C. R., Fedorka-Cray, P. J., Davis, J. A., Barrett, J. B., Brousse, J. H., Gustafson, J., and Kucher, M. (2009b). Mechanisms of antimicrobial resistance and genetic relatedness among enterococci isolated from dogs and cats in the United States. *J. Appl. Microbiol.* 108, 2171–2179.

Kariyama, R., Mitsuhata, R., Chow, J. W., Clewell, D. B., and Kumon, H. (2000). Simple and reliable multiplex PCR assay for surveillance isolates of vancomycin-resistant enterococci. *J. Clin. Microbiol.* 38, 3092–3095.

Kramer, A., Schwebke, I., and Kampf, G. (2006). How long do nosocomial pathogens persist on inanimate surfaces? A systematic review. *BMC Infect. Dis.* 6, 130. doi:10.1186/1471-2334-6-130

KuKanich, K., Ghosh, A., Skarbek, J., Lothamer, K., and Zurek, L. (2012). Surveillance of bacterial contamination in small animal veterinary hospitals with special focus on antimicrobial resistance and virulence traits of enterococci. *J. Am. Vet. Med. Assoc.* 240, 437–445.

Lautenbach, E., Schuster, M. G., Bilker, W. B., and Brennan, P. J. (1998). The role of chloramphenicol in the treatment of bloodstream infection due to vancomycin-resistant *Enterococcus. Clin. Infect. Dis.* 27, 1259–1265.

Linden, P. K., Moellering, R. C. Jr., Wood, C. A., Rehm, S. J., Flaherty, J., Bompart, F., and Talbot, G. H. (2001). Treatment of vancomycin-resistant *Enterococcus faecium* infections with quinupristin/dalfopristin. *Clin. Infect. Dis.* 33, 1816–1823.

Loeffler, A., Boag, A. K., Sung, J., Lindsay, J. A., Guardabassi, L., Dalsgaard, A., Smith, H., Stevens, K. B., and Lloyd, D. H. (2005). Prevalence of methicillin-resistant *Staphylococcus aureus* among staff and pets in a small animal referral hospital in the UK. *J. Antimicrob. Chemother.* 56, 692–697.

Macovei, L., and Zurek, L. (2006). Ecology of antibiotic resistance genes: characterization of enterococci from houseflies collected in food settings. *Appl. Environ. Microbiol.* 72, 4028–4035.

Macovei, L., and Zurek, L. (2007). Influx of enterococci and associated antibiotic resistance and virulence genes from ready-to-eat food to the human digestive tract. *Appl. Environ. Microbiol.* 73, 6740–6747.

Manson, J. M., Keis, S., Smith, J. M. B., and Cook, G. M. (2003). Characterization of a vancomycin-resistant *Enterococcus faecalis* (VREF) isolate from a dog with mastitis: further evidence of a clonal lineage of VREF in New Zealand. *J. Clin. Microbiol.* 41, 3331–3333.

Marsh-Ng, M. L., Burney, D. P., and Garcia, J. (2007). Surveillance of infections associated with intravenous catheters in dogs and cats in an intensive care unit. *J. Am. Anim. Hosp. Assoc.* 43, 13–20.

Moyaert, H., de Graef, E. M., Haesebrouck, F., and Decostere, A. (2006). Acquired antimicrobial resistance in the intestinal microbiota of diverse cat populations. *Res. Vet. Sci.* 81, 1–7.

Murray, B. E., Singh, K. V., Heath, J. D., Sharma, B. R., and Weinstock, G. M. (1990). Comparison of genomic DNAs of different enterococcal isolates using restriction endonucleases with infrequent recognition sites. *J. Clin. Microbiol.* 28, 2059–2063.

Noble, W. C., Virani, Z., and Cree, R. G. (1992). Co-transfer of vancomycin and other resistance genes from *Enterococcus faecalis* NCTC 12201 to *Staphylococcus aureus. FEMS Microbiol. Lett.* 72, 195–198.

Oancea, C., Klare, I., Witte, W., and Werner, G. (2004). Conjugative transfer of the virulence gene, esp, among isolates of *Enterococcus faecium* and *Enterococcus faecalis. J. Antimicrob. Chemother.* 54, 232–235.

Poeta, P., Costa, D., Rodrigues, J., and Torres, C. (2006). Antimicrobial resistance and the mechanisms implicated in faecal enterococci from healthy humans, poultry and pets in Portugal. *Int. J. Antimicrob. Agents* 27, 131–137.

Poyaert, C., Quesnes, G., and Trieu-Cuot, P. (2000). Sequencing the gene encoding manganese-dependent superoxide dismutase for rapid species identification of enterococci. *J. Clin. Microbiol.* 38, 415–418.

Prescott, J. F., Hanna, W. J. B., Reld-Smith, R., and Drost, K. (2002). Antimicrobial drug use and resistance in dogs. *Can. Vet. J. 43*, 107–116.

Pressel, M. A., Fox, L. E., Apley, M. D., and Simutis, F. J. (2005). Vancomycin for multi-drug resistant *Enterococcus faecium* cholangiohepatitis in a cat. *J. Feline Med. Surg.* 7, 317–321.

Qi, C., Zheng, X., Obias, A., Scheetz, M. H., Malczynski, M., and Warren, J. R. (2006). Comparison of testing methods for detection of decreased linezolid susceptibility due to G2576T mutation of the 23S rRNA gene in *Enterococcus faecium* and *Enterococcus faecalis. J. Clin. Microbiol.* 44, 1098–1100.

Roberts, R. R., Hota, B., Ahmad, I., Scott, R. D., Foster, S. D., Abbasi, F., Schabowski, S., Kampe, L. M., Ciavarella, G. G., Supino, M., Naples, J., Cordell, R., Levy, S. B., and Weinstein, R. A. (2009a). Hospital and societal costs of antimicrobial-resistant infections in a Chicago teaching hospital: implications for antibiotic stewardship. *Clin. Infect. Dis.* 49, 1175–1184.

Roberts, M. C., Soge, O. O., Giardino, M. A., Mazengia, E., Ma, G., and Meschke, J. S. (2009b). Vancomycin-resistant *Enterococcus* spp. in marine environments from the West Coast of the USA. *J. Appl. Microbiol.* 107, 300–307.

Sapkota, A. R., Curriero, F. C., Gibson, K. E., and Schwab, K. J. (2007). Antibiotic-resistant enterococci and fecal indicators in surface waters and groundwater impacted by a concentrated swine feeding operation. *Environ. Health Perspect.* 115, 1040–1045.

Scott, G. M., Thomson, R., Malone-Lee, J., and Ridgway, G. L. (1988). Cross-infection between animals and man: possible feline transmission of *Staphylococcus aureus* infection in humans? *J. Hosp. Infect.* 12, 29–34.

Simjee, S., White, D. G., McDermott, P. F., Wagner, D. D., Zervos, M. J., Donabedian, S. M., English, L. L., Hayes, J. R., and Walker, R. D. (2002). Characterization of Tn1546 in vancomycin-resistant *Enterococcus faecium* isolated from canine urinary tract infections: evidence of gene exchange between human and animal enterococci. *J. Clin. Microbiol.* 40, 4659–4665.

Sørum, M., Johnsen, P. J., Aasnes, B., Rosvoll, T., Kruse, H., Sundsfjord, A., and Simonsen, G. S. (2006). Prevalence, persistence, and molecular characterization of glycopeptide-resistant enterococci in Norwegian poultry and poultry farmers 3 to 8 years after the ban on avoparcin. *Appl. Environ. Microbiol.* 72, 516–521.

Tacconelli, E., and Cataldo, M. A. (2008). Vancomycin-resistant enterococci (VRE): transmission and control. *Int. J. Antimicrob. Agents* 31, 99–106.

Tendolkar, P. M., Baghdayan, A. S., and Shankar, N. (2006). Putative surface proteins encoded within a novel transferable locus confer a high-biofilm phenotype to *Enterococcus faecalis. J. Bacteriol.* 188, 2063–2072.

Turabelidze, D., Kotetishvili, M., Kreger, A., Morris, J. G. Jr., and Sulakvelidze, A. (2000). Improved pulsed-field gel electrophoresis for typing vancomycin-resistant enterococci. *J. Clin. Microbiol.* 38, 4242–4245.

van Belkum, A., van den Braak, N., Thomassen, R., Verbrugh, H., and Endtz, H. (1996). Vancomycin-resistant enterococci in cats and dogs. *Lancet 348, 1038*–1039.

Weese, J. S. (2008). Issues regarding the use of vancomycin in companion animals. *J. Am. Vet. Med. Assoc.* 233, 565–567.

Weigel, L. M., Clewell, D. B., Gill, S. R., Clark, N. C., McDougal, L. K., Flannagan, S. E., Kolonay, J. F., Shetty, J., Killgore, G. E., and Tenover, F. C. (2003). Genetic analysis of a high-level vancomycin-resistant isolate of *Staphylococcus aureus. Science* 302, 1569–1571.

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 17 November 2011; accepted: 05 February 2012; published online: 21 February 2012.

*Citation: Ghosh A, KuKanich K, Brown CE and Zurek L (2012) Resident cats in small animal veterinary hospitals carry multi-drug resistant enterococci and are likely involved in cross-contamination of the hospital environment. Front. Microbio. **3**:62. doi: 10.3389/fmicb.2012.00062 This article was submitted to Frontiers in Antimicrobials, Resistance and Chemotherapy, a specialty of Frontiers in Microbiology.*

APPENDIX

Table A1 | Information on resident cats from the small animal veterinary hospitals.

Sample ID	Age (year)/sex/breed	Stay at clinic (year)	Antibiotic(s) administration (year ago)	Diet
Cat-A	11/MC/DSH	10	Never	HSD c/d multicat dry
Cat-B	2/MC/DSH	2	Never	HSD sensitive stomach maintenance dry
Cat-C	10/FS/DSH	10	Penicillin and enrofloxacin (5), amoxicillin–clavulanic acid (4), erythromycin ophthalmic drops (1)	HSD w/d dry
Cat-D	3/FS/DSH	3	Never	Felidae maintenance dry
Cat-E	8/FS/DLH	8	Amoxicillin (3)	HSD maintenance dry
Cat-F	1.3/MC/DSH	1.3	Never	HSD hairball light maintenance dry

FS, female spayed; MC, male castrated; DSH, domestic short haired; DLH, domestic long haired; HSD, Hill's science diet.

Table A2 | Antimicrobial resistance profile of enterococcal isolates from the feces of individual resident cats from small animal veterinary hospitals.

Strain	ID	AM	CM	TET	D	GM	STR	RIF	E	ENO	VA	Q/D	LIN	NF	TGC
CAT-A															
1	Av			■					■						
2	Av			■					■						
3	Av			■					■						
15	Av			■					■						
4	Fm			■	■				■			■		■	
5	Fm			■	■		■		■			■			
6	Fm	■		■			■		■	■					
7	Fm	■		■			■		■	■				■	
8	Fm			■					■						
9	Fm			■					■			■			
10	Fm			■					■			■			
11	Fm			■					■			■		■	
12	Fm			■					■			■			
13	Fm			■					■			■			
14	Fm			■	■		■		■			■			
16	Fs											-			
17	Fs							■				-			
18	Fs											-			
19	Fs		■	■		■		■	■			-			
20	Fs											-			
21	Fs											-			
22	Fs		■	■		■		■	■			-			
23	Fs							■				-			
24	Fs											-			
25	Fs		■	■		■			■			-			
26	Fs		■	■		■			■			-			
27	Fs		■	■		■			■			-			
28	Fs							■				-			
29	Fs							■				-			
30	Fs											-			
CAT-B															
1	Fm			■					■	■					
2	Fm			■					■	■					
3	Fm			■					■	■					
4	Fm			■					■	■					
5	Fm			■					■	■					
6	Fm			■					■	■					
7	Fm			■					■	■					
8	Fm			■					■	■					
9	Fm			■					■	■					
10	Fm			■					■	■					
11	Fm			■					■	■					
12	Fm			■					■	■					
13	Fm			■					■	■					
14	Fm			■					■	■					
15	Fm			■					■	■					
16	Fm			■					■	■					
17	Fm			■					■	■					
18	Fm			■					■	■					

(Continued)

Table A2 | Continued

Strain	ID	AM	CM	TET	D	GM	STR	RIF	E	ENO	VA	Q/D	LIN	NF	TGC
19	Fm			■					■	■					
20	Fm			■						■					
21	Fm			■						■					
22	Fm			■					■	■					
23	Fm			■					■	■					
24	Fm			■						■					
25	Fm			■					■	■					
26	Fm			■					■	■					
27	Fm			■						■					
28	Fm			■						■					
29	Fm			■						■					
30	Fm			■					■	■					
CAT-C															
2	Fm														
6	Fm														
18	Fm														
1	Hr			■											
8	Hr			■											
3	Hr			■											
4	Hr			■											
5	Hr			■											
7	Hr			■											
9	Hr			■											
10	Hr			■											
11	Hr			■											
12	Hr			■											
13	Hr			■											
14	Hr			■											
15	Hr			■											
16	Hr			■											
17	Hr			■											
19	Hr			■											
20	Hr			■											
21	Hr			■											
22	Hr			■											
23	Hr			■											
24	Hr			■											
25	Hr			■											
26	Hr			■											
27	Hr			■											
28	Hr			■											
29	Hr			■											
30	Hr			■											
CAT-D															
7	Fm							■	■	■					
10	Fm							■	■	■					
13	Fm							■	■	■					
15	Fm			■				■	■	■					
24	Fm							■	■	■					
27	Fm			■				■		■					

(Continued)

Table A2 | Continued

Strain	ID	AM	CM	TET	D	GM	STR	RIF	E	ENO	VA	Q/D	LIN	NF	TGC
19	Fm							■	■	■					
20	Fm							■	■	■					
21	Fm							■	■	■					
1	Fs			■	■	■	■	■	■			-			
3	Fs			■	■	■	■	■	■			-			
5	Fs			■	■	■	■	■	■			-			
11	Fs			■	■	■	■	■	■			-			
14	Fs			■	■	■	■	■	■			-			
16	Fs			■	■	■	■	■	■			-			
18	Fs			■	■	■	■	■	■			-			
22	Fs			■	■	■	■	■	■			-			
25	Fs			■	■	■	■	■	■			-			
26	Fs			■	■	■	■	■	■			-			
30	Fs			■	■	■	■	■	■			-			
2	Hr			■				■	■						
4	Hr			■				■	■						
6	Hr			■				■	■						
8	Hr			■				■	■						
9	Hr			■				■	■						
12	Hr			■				■	■						
17	Hr			■				■	■						
23	Hr			■				■	■						
28	Hr			■				■	■						
29	Hr			■				■	■						
CAT-E															
18	Av							■							
24	Hr							■							
25	Hr							■							
6	Fs					■		■	■		■	-			
11	Fs					■			■		■	-			
12	Fs					■			■		■	-			
13	Fs					■			■		■	-			
15	Fs					■			■		■	-			
16	Fs							■				-			
17	Fs							■				-			
19	Fs							■				-			
20	Fs					■			■		■	-			
21	Fs							■				-			
22	Fs							■				-			
23	Fs					■			■		■	-			
26	Fs							■				-			
27	Fs							■				-			
28	Fs							■				-			
29	Fs							■				-			
30	Fs							■				-			
1	Fm	■		■	■	■		■	■	■		■			
2	Fm	■		■	■	■		■	■	■		■			
3	Fm	■		■	■	■		■	■	■		■			
4	Fm	■		■	■	■		■	■	■		■			
5	Fm	■		■	■	■		■	■	■		■			
7	Fm	■		■	■	■		■	■	■		■			

(Continued)

Table A2 | Continued

Strain	ID	AM	CM	TET	D	GM	STR	RIF	E	ENO	VA	Q/D	LIN	NF	TGC
8	Fm	■		■	■	■		■	■	■		■			
9	Fm	■		■	■	■		■	■	■		■			
10	Fm	■		■	■	■		■	■	■		■			
14	Fm	■		■	■	■		■	■	■		■			
CAT-F															
1	Hr			■											
2	Hr			■											
3	Hr			■											
4	Hr			■											
5	Hr			■											
6	Hr			■											
7	Hr			■											
8	Hr			■											
9	Hr			■											
10	Hr			■											
11	Hr			■											
12	Hr			■											
13	Hr							■							
14	Hr							■							
15	Hr			■											
16	Hr			■											
17	Hr			■											
18	Hr			■											
19	Hr							■							
20	Hr			■											
21	Hr							■							
22	Hr			■											
23	Hr			■											
24	Hr			■											
25	Hr			■											
26	Hr							■							
27	Hr			■											
28	Hr			■											
29	Hr			■											
30	Hr			■											

Black filled box = resistance, white filled box = susceptible.

AM, ampicillin; CM, chloramphenicol; TET, tetracycline; D, doxycycline; GM, gentamicin; STR, streptomycin; RIF, rifampicin; E, erythromycin; ENO, enrofloxacin; VA, vancomycin; Q/D, quinupristin/dalfopristin; LIN, linezolid; NF, nitrofurantoin; TGC, tigecycline; "–," not applicable for E. faecalis isolates due to their intrinsic resistance. Av, Enterococcus avium; Fm, E. faecium; Fs, E. faecalis; Hr, E. hirae.

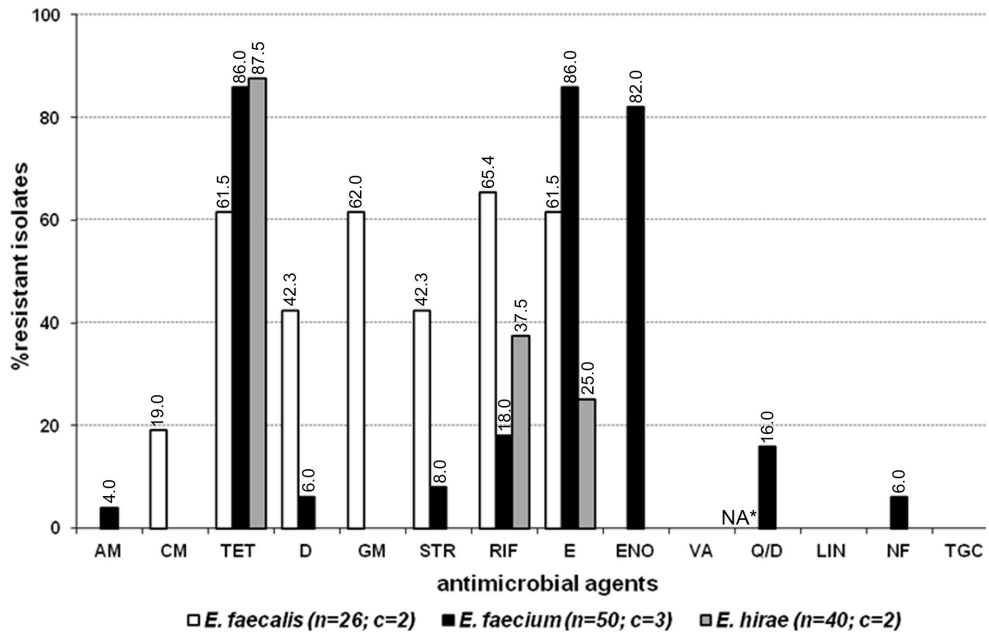


FIGURE A1 | Antimicrobial resistance profile of enterococcal isolates from the feces of four resident cats from small animal veterinary hospitals that had never been administered any antimicrobial agents. AM, ampicillin; CM, chloramphenicol; TET, tetracycline; D, doxycycline; GM, gentamicin; STR, streptomycin; RIF, rifampicin; E, erythromycin; ENO, enrofloxacin; VA, vancomycin; Q/D, quinupristin/dalfopristin; LIN, linezolid; NF, nitrofurantoin; TGC, tigecycline; *c*, number of cats that contributed for the total number (*n*) of isolates. *Not applicable for *E. faecalis* isolates due to their intrinsic resistance.

frontiers in
MICROBIOLOGY

ORIGINAL RESEARCH ARTICLE
published: 18 January 2012
doi: 10.3389/fmicb.2012.00002

IncP-1ε plasmids are important vectors of antibiotic resistance genes in agricultural systems: diversification driven by class 1 integron gene cassettes

Holger Heuer[1], Chu T. T. Binh[1†], Sven Jechalke[1], Christoph Kopmann[1], Ute Zimmerling[1], Ellen Krögerrecklenfort[1], Thomas Ledger[2], Bernardo González[2], Eva Top[3] and Kornelia Smalla[1]*

[1] *Federal Research Centre for Cultivated Plants, Institute for Epidemiology and Pathogen Diagnostics, Julius Kühn-Institut, Braunschweig, Germany*
[2] *Department de Ciencias Biológicas, Universidad Adolfo Ibañez, Santiago, Chile*
[3] *Department of Biological Sciences, Institute for Bioinformatics and Evolutionary Studies, University of Idaho, Moscow, ID, USA*

Edited by:
Rustam I. Aminov, University of Aberdeen, UK

Reviewed by:
Henning Sørum, Norwegian School of Veterinary Science, Norway
Yixin Shi, Arizona State University, USA
Peter Norberg, University of Gothenburg, Sweden

***Correspondence:**
Kornelia Smalla, Julius Kühn-Institut, Institute for Epidemiology and Pathogen Diagnostics, Messeweg 11-12, D-38104 Braunschweig, Germany.
e-mail: kornelia.smalla@jki.bund.de

†Present address:
Chu T. T. Binh, Department of Biology, Loyola University Chicago, 1032 West Sheridan Road, Chicago, IL 60660, USA.

The role of broad-host range IncP-1ε plasmids in the dissemination of antibiotic resistance in agricultural systems has not yet been investigated. These plasmids were detected in total DNA from all of 16 manure samples and in arable soil based on a novel 5′-nuclease assay for real-time PCR. A correlation between IncP-1ε plasmid abundance and antibiotic usage was revealed. In a soil microcosm experiment the abundance of IncP-1ε plasmids was significantly increased even 127 days after application of manure containing the antibiotic compound sulfadiazine, compared to soil receiving only manure, only sulfadiazine, or water. Fifty IncP-1ε plasmids that were captured in *E. coli* CV601gfp from bacterial communities of manure and arable soil were characterized by PCR and hybridization. All plasmids carried class 1 integrons with highly varying sizes of the gene cassette region and the *sul1* gene. Three IncP-1ε plasmids captured from soil bacteria and one from manure were completely sequenced. The backbones were nearly identical to that of the previously described IncP-1ε plasmid pKJK5. The plasmids differed mainly in the composition of a Tn*402*-like transposon carrying a class 1 integron with varying gene cassettes, IS*1326*, and in three of the plasmids the tetracycline resistance transposon Tn*1721* with various truncations. Diverse *Beta-* and *Gammaproteobacteria* were revealed as hosts of one of the IncP-1ε plasmids in soil microcosms. Our data suggest that IncP-1ε plasmids are important vectors for horizontal transfer of antibiotic resistance in agricultural systems.

Keywords: IncP-1ε plasmid, exogenous isolation, complete sequence, gene cassette, qPCR, arable soil, pig manure

INTRODUCTION

Spreading manure on agricultural soils was recently shown to promote spreading of transferable antibiotic resistances and residual veterinary medicines in agricultural soils (reviewed in Schauss et al., 2009; Heuer et al., 2011). Frequencies at which sulfadiazine (SDZ) resistance plasmids were captured from soil bacteria into *E. coli* were found to be higher for soils treated with manure than for soils that did not receive manure (Heuer and Smalla, 2007). Treatment of soil with manure spiked with SDZ resulted in significantly higher transfer frequencies compared to non-spiked manure (Heuer and Smalla, 2007). A survey of field-scale manure slurries used for soil fertilization revealed that antibiotic resistance plasmids could easily be captured into *E. coli* from the different manures (Binh et al., 2008). A large proportion of the plasmids could be assigned to known plasmid groups by DNA-hybridization and PCR. Remarkably, 13 of the plasmids captured gave a strong PCR product with primers targeting the *trfA* gene of IncP-1 plasmids (Götz et al., 1996) but did not hybridize with the probes derived from the two reference plasmids RK2 (IncP-1α) and R751 (IncP-1β). The *trfA* PCR products were cloned and sequenced and shown to be almost identical to the recently sequenced IncP-1ε plasmid pKJK5 (Binh et al., 2008).

Plasmids of the IncP-1 group are considered as one of the best studied plasmid groups. For decades, plasmids belonging to this incompatibility group have attracted the attention of molecular biologists and ecologists because of their efficient conjugative transfer to and their stable replication in a wide range of Gram-negative bacteria (Thomas, 2000). IncP-1 plasmids were originally designated as clinical plasmids because the prototype IncP-1α plasmid RK2 and the IncP-1β plasmid R751 were originally isolated from clinical strains (Pansegrau et al., 1994; Thorsted et al., 1998). The complete sequences of these two plasmids enabled the development of a PCR-based plasmid detection system (Götz et al., 1996) that virtually replaced the older hybridization method using incompatibility group-specific probes (Couturier et al., 1988). This greatly facilitated the detection of IncP-1 specific sequences not only in isolated strains and plasmids but also in microbial community DNA directly extracted from diverse environments. While earlier characterization of a few catabolic IncP-1 plasmids had already shown that IncP-1 plasmids occur in environmental as well as clinical isolates, only PCR-based detection in combination with Southern blot hybridization revealed that these plasmids were certainly not confined to the clinical environment but instead were frequently found in various environments such as soil, sediments,

sewage, or manure (Götz et al., 1996; Heuer et al., 2002). Often the abundance of populations carrying these plasmids seemed to be related to pollution (Smalla et al., 2000, 2006). Based on comparative genomics, the basic structure of the first set of described IncP-1 plasmids has been confirmed for many others by now (Schlüter et al., 2007; Sen et al., 2011). Besides their backbone functions for vegetative replication, stable maintenance, and transfer the accessory genes, that are typically found in between the blocks of backbone functions at up to three regions of insertion, confer resistances to nearly all clinically important classes of antimicrobial drugs, quaternary ammonium compounds, and mercury resistances or encode degradation of man-made compounds. However, the increasing number of completely sequenced IncP-1 plasmids also showed that there are additional groups of IncP-1 plasmids that clearly differ in their backbone from the IncP-1α and the IncP-1β plasmids. Thus novel subgroups often represented by one plasmid at the time have been proposed (Vedler et al., 2004; Haines et al., 2006; Bahl et al., 2007). Since these plasmids were too divergent in genome sequence from the IncP-1α and IncP-1β plasmids to be detected by means of the primer systems developed by Götz et al. (1996), new primer systems for detection of IncP-1 plasmids were published by Bahl et al. (2009) to encompass at least the known diversity of IncP-1 plasmids. The environmental distribution of the recently discovered IncP-1 subgroups is not well explored yet.

In the present study we aimed to explore the abundance of IncP-1ε plasmids and their role in dissemination of antibiotic resistance genes in the agro-ecosystem. A real-time PCR system was established to provide quantitative data on the abundance of populations carrying IncP-1ε plasmids in manure slurries and in agricultural soils, and how this correlates with selective pressure by antibiotics. We report on the characterization of 50 plasmids exogenously captured from manure, bulk, and rhizosphere soil samples of independent micro-, and mesocosms and field experiments that were assigned to the IncP-1ε group. The host range of one of the plasmids was determined in a soil microcosm experiment. The complete genome sequences of four IncP-1ε plasmids were analyzed and compared to the prototype pKJK5. As all four IncP-1ε plasmids contained class 1 integrons we hypothesized that antibiotic resistance gene cassettes might drive the diversification of IncP-1ε plasmids. To prove this hypothesis all exogenously captured plasmids assigned to the IncP-1ε group were analyzed for the presence of class 1 integron gene cassettes and the size of the gene cassettes integrated.

MATERIALS AND METHODS

SAMPLES AND PLASMID CAPTURE

Soil microcosm experiments were set up to investigate the effects of manure and SDZ on the abundance of antibiotic resistance plasmids. For each microcosm, 2 kg of top soil from an arable field near Kaldenkirchen, Germany [Gleyic Cambisol, 3.6% clay, 23.1% silt, 73.3% sand, pH ($CaCl_2$) 5.5, organic C 1.7%, maximal water holding capacity 27%] was mixed either with 80 g manure slurry or water (both either with or without addition of 16 mg SDZ and 16 mg acetyl-SDZ), and adjusted to 30% of the maximum water holding capacity. For each of these four treatments, four replicate microcosms per sampling time (29, 57, 127 days after treatment) were prepared and incubated at 10°C in the dark. The agricultural soils (silty sand; sandy loam; silt) that were not fertilized with manure for more than 10 years, originated from an experimental plot in Großbeeren (Germany).

The plasmids analyzed in the present study were obtained from manure (Heuer et al., 2002; Binh et al., 2008) and soil from an independent microcosm (Heuer and Smalla, 2007), mesocosm, and field experiments. Capture of plasmids from soil bacteria in the plasmid-free rifampicin resistant *E. coli* CV601 gfp recipient was done as previously described (Binh et al., 2007). Briefly, soil was shaken with glass beads for 2 h in 1:10 diluted Tryptic Soy Broth (BD Diagnostic Systems, Heidelberg, Germany) at 20°C, and mixed with *E. coli* cells. Coarse particles were settled out, cells from supernatants were pelleted and transferred to a membrane filter on Plate Count Agar (PCA; Merck, Darmstadt, Germany). After overnight incubation at 28°C, the suspended mating mixtures were spread plated on Mueller–Hinton agar NCCLS (Merck) supplemented with SDZ and rifampicin to select for transconjugants that captured a sulfonamide resistance plasmid. Plasmid pKS77 was obtained from pig manure as previously described (Heuer et al., 2002). Plasmid pKJK5 was kindly provided by the group of S. Sørensen, University of Copenhagen.

PLASMID ISOLATION AND CHARACTERIZATION BY PCR AND SOUTHERN BLOT ANALYSIS

Plasmid DNA was isolated from cell pellets harvested from colonies freshly grown on PCA using the Qiagen plasmid isolation kit. Restriction enzyme digestion of plasmid DNA, Southern blotting, and hybridization were done as described by Binh et al. (2008). The digoxigenin-labeled *trfA* probe was generated from PCR products obtained with the primers described by Bahl et al. (2009) from pKJK5. The *intI1* gene derived probes were generated by digoxigenin labeling of PCR products obtained from *Salmonella enterica* AM237806. Primers targeting *intI1*, and PCR conditions were as described by Moura et al. (2010). The *aadA* probe used was a mixed probe generated from *aadA1*, *aadA2*, *aadA9*, and *aadA13* (Binh et al., 2009). The plasmid DNA was analyzed for the presence of the *sul1* gene (Heuer and Smalla, 2007) and of IncP-1ε *trfA* (this study) by real-time PCR.

HOST RANGE STUDY

The host range of the IncP-1ε plasmids pHH3414 in rhizosphere soil was determined by introducing *E. coli* CV601gfp pHH3414 ($\sim 1 \times 10^6$ colony forming units per gram of soil) into soil microcosms planted with *Acacia caven*. The soil was previously treated with manure. After 4 weeks, serial 10-fold dilutions of rhizosphere and bulk soil in sterile saline were plated onto Mueller–Hinton agar NCCLS (Merck) supplemented with cycloheximide (100 mg l^{-1}), tetracycline (5 mg l^{-1}), SDZ (100 mg l^{-1}), and streptomycin (50 mg l^{-1}) for selection of putative transconjugants (recipients of plasmid pHH3414). Gfp negative colonies grown on selective media for transconjugants were picked, re-streaked, and cell lysates were screened by IncP-1ε PCR. The genomic and plasmid DNA extracted from transconjugants (IncP-1ε PCR positive colonies) were further characterized by BOX-PCR and *Sph*I

plasmid restriction digests, respectively, as previously described (Smalla et al., 2006).

TOTAL COMMUNITY DNA

The total DNA from manure was the same as used by Binh et al. (2008, 2009). Total DNA from soil samples (soils sieved through a 2 mm mesh size) was extracted using the FastPrep FP120 bead beating system for cell lysis in conjunction with the FastDNA SPIN Kit for Soil, and the GeneClean Spin Kit for purification of the extracted DNA (Qbiogene, Carlsbad, CA, USA).

QUANTITATIVE PCR TARGETING IncP-1ε PLASMIDS OR 16S rRNA GENES

Ribosomal gene targets in total DNA were quantified by 5′-nuclease assays in real-time PCR as previously described (Suzuki et al., 2000; Heuer and Smalla, 2007). Analogously, the *trfA* gene copies of IncP-1ε plasmids were determined, using primers trfAε941f (ACGAAGAAATGGTTGTCCTGTTC), trfAε1014r (CGTCAGCTTGCGGTACTTCTC), and the Taqman probe trfAε965tp (FAM-CCGGCGACCATTACAGCAAGTTCAT TT-TAMRA). Standard dilutions were generated from a gel-purified PCR product of the 281 bp *trfA* fragment amplified from plasmid pHH3408 using previously described primers (Bahl et al., 2009). Quantitative PCR was performed in a CFX96 real-time PCR detection system (Bio-Rad, Munich, Germany). PCR reactions contained standard or environmental DNA, 1.25 U TrueStart Taq DNA polymerase and buffer (Fermentas, St. Leon-Rot, Germany), 0.2 mM of each deoxynucleoside triphosphate, 2.5 mM $MgCl_2$, 0.1 mg ml^{-1} BSA, and 0.3 μM of primers and probe in 50 μl. Thermocycles were 5 min 95°C, and 40 cycles consisting of 15 s 95°C and 60 s 60°C. Effects of manure and SDZ on the relative abundance of IncP-1ε plasmids in soil were analyzed by ANOVA using the procedure MIXED for repeated-measures comparison included in the statistical software package SAS 9.2 (SAS Institute, Cary, NC, USA).

SEQUENCE ANALYSIS

Sequencing of shotgun libraries from the plasmids, sequence assembly, and gap closure by primer walking were performed by the U.S. Department of Energy Joint Genome Institute (Walnut Creek, CA, USA). Automatic annotation was carried out by the J. Craig Venter Institute Annotation Service[1] followed by manual annotation. Similarities of the plasmid sequences to other plasmids, transposons, IS elements, and integrons were found by BLASTN searches of GenBank[2]. Putative open reading frames in the complete nucleotide sequences were compared by BLASTN searches to GenBank sequences. Additional searches for genes, operons, promoters, and terminators were done using FGENESB, BPROM, and FindTerm at www.softberry.com (Softberry, Mount Kisco, NY, USA). The sequence data have been submitted to the DDBJ/EMBL/GenBank databases under accession numbers JQ004406–JQ004409.

RESULTS

ABUNDANCE OF IncP-1ε PLASMIDS IN BACTERIAL COMMUNITIES OF MANURE AND SOIL

To quantify IncP-1ε plasmids in total community DNA from manure and soil samples, a 5′-nuclease assay for real-time PCR specifically targeting the replication initiation gene *trfA* of these plasmids (*trfA*ε) was developed and applied. The abundance of the *trfA*ε gene in total DNA from manure relative to the *rrn* copy number varied from 10^{-1} to 10^{-5} (**Table 1**). A higher relative abundance of the *trfA*ε gene was observed in total DNA from manure obtained from pig producing facilities with large numbers of pigs, high-throughput piglet production, or documented usage of several antibiotic compounds (**Table 1**). In these farms, a high antibiotic usage is typical because of metaphylactic application in large herds and prophylactic application at weaning of

[1]http://www.jcvi.org/cms/research/projects/annotation-service/overview/
[2]http://ncbi.nlm.nih.gov

Table 1 | Abundance of IncP-1ε plasmids in field-scale manures from different pig production facilities.

No.	Farm size and type of pig production	Antibiotic usage	IncP-1ε plasmids log[copies *trfA*ε/*rrn*]
9	2000 pigs, 30–120 kg, 700 g/day increase, slatted floor	High (amoxicillin, doxycycline): large herd	−0.8
1	250 sows, 5250 piglets/year	High: frequent weaning	−1.3
8	300 sows, 6900 piglets/year, slatted floor	High: frequent weaning	−1.3
15	1800 pigs, 25–123 kg, 660 g/day increase, slatted floor	High (tylosin, penicillin): large herd	−1.4
3	80 sows, 1520 piglets/year	High (amoxicillin, penicillin, neomycin, tylosin, enrofloxacin, apramycin): weaning	−1.5
10	1300 pigs, 30–125 kg, 700 g/day increase, slatted floor	Medium (amoxicillin, enrofloxacin)	−2.1
7	600 pigs, 30–125 kg, 750 g/day increase, partly slatted floor	Medium (gentamicin, tylosin, tetracycline, lincomycin): prophylactic for new piglets	−2.2
6	1800 pigs, 8–140 kg, 800 g/day increase	Medium (amoxicillin, tetracycline): large herd, long life cycle	−2.6
12	Meat-production pigs, 30–120 kg, 650 g/day increase	Unknown	−2.8
4	80 sows, 1600 piglets/year	Medium (tulathromycin, streptomycin, tetracycline, enrofloxacin)	−3.2
5	800 pigs, 25–120 kg, 700 g/day increase	Low (tetracycline): small herd	−3.7
14	400 pigs 30–120 kg, 650 g/day increase, partly slatted floor	Low: small herd	−4.4
13	550 pigs, 32–110 kg, 550 g/day increase, partly slatted floor	No antibiotics used	−5.2

piglets. In contrast, manure from small pig producing facilities had up to four orders of magnitude lower abundances of IncP-1ε plasmids, especially farm no. 13 which did not apply any antibiotic compounds recently. The correlation of plasmid abundance with antibiotic usage suggested that these plasmids from manure typically carry accessory antibiotic resistance genes. We investigated whether manure application on agricultural soil could increase the abundance of resistance plasmids in the environment.

In a microcosm experiment, addition of antibiotic-free manure or the antibiotic SDZ to arable soil did not increase the level of IncP-1ε plasmids compared to untreated soil (**Figure 1**). However, when manure was added to soil that was spiked with SDZ at a concentration typical for manure from SDZ-treated pigs, the abundance of IncP-1ε plasmids was significantly increased compared to the other treatments (two-way ANOVA, $P = 0.025$). This indicated a synergistic effect of manure and SDZ causing an enrichment of bacteria that carry sulfonamide resistance conferring IncP-1ε plasmids within the soil community. An accumulation of these plasmids due to the repeated treatment was not observed. In three different agricultural soils from an experimental plot that had only received mineral fertilizer for more than 10 years, the abundance of the *trfA*ε gene relative to the *rrn* copy number varied around 10^{-5} (data not shown).

CAPTURING IncP-1ε PLASMIDS FROM MANURE TREATED SOIL

Fifty conjugative plasmids that were exogenously captured from manure, bulk soil, and rhizosphere bacteria into *E. coli* CV601gfp based on the acquired SDZ resistance were assigned to IncP-1ε based on DNA-hybridization with a pKJK5 derived *trfA* probe or by means of the IncP-1ε specific real-time PCR. The isolation of IncP-1ε plasmids from independent microcosms, mesocosms, and field experiments indicated a widespread dissemination of IncP-1ε plasmids in agricultural soils. In all IncP-1ε plasmids the SDZ resistance gene *sul1* and the integrase gene *intI1* of class 1 integrons were detected (**Table 2**). PCR amplification with primers targeting the regions flanking class 1 integrons revealed that three plasmids carried empty integrons (size of the fragment 300 bp) while all others carried gene cassettes of different sizes ranging from 500 to 4000 bp (**Table 2**). Southern blot hybridization of PCR amplified gene cassettes showed that 30 IncP-1ε carried the *aadA* gene. The complete sequence of four of the plasmids was determined.

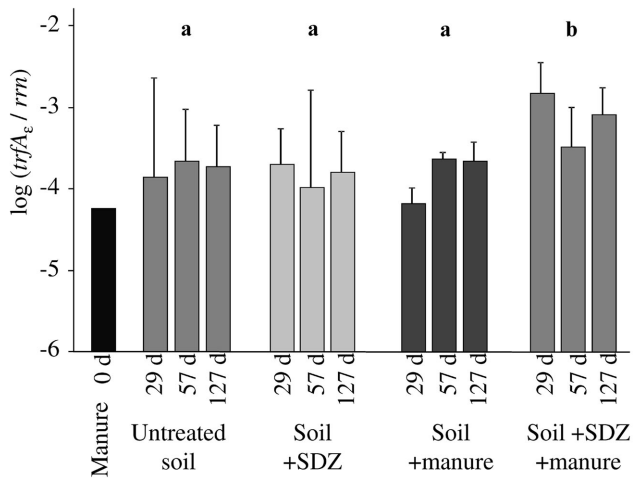


FIGURE 1 | Copy numbers of *trfA* (replication initiation gene) of IncP-1ε plasmids relative to 16S rRNA gene copies (*rrn*) in manure and in soil, which was treated either with the antibiotic compound sulfadiazine (SDZ), with manure without antibiotics, with manure containing SDZ, or with water (untreated). Sampling of the soil microcosms was repeated three times. Error bars indicate SD ($n = 4$). Differing letters show a significant effect of the treatment (ANOVA with repeated measures).

HOST RANGE

To investigate the host range of IncP-1ε plasmids, *E. coli* CV601gfp carrying pHH3414 was introduced into soil microcosms planted with *A. caven*. PCR screening of 30 *gfp* negative bacteria from the rhizosphere of *A. caven* grown on selective media for resistances conferred by pHH3414 resulted in six IncP-1ε positive isolates. BOX-PCR revealed that the transconjugants exhibited four different BOX patterns that were clearly distinct from BOX patterns of the donor *E. coli* CV601gfp pHH3414. The *Sph*I restriction patterns of plasmid DNA isolated from putative transconjugants were identical to the restriction patterns of plasmid pHH3414. Partial sequencing of the 16S rRNA gene of the transconjugants showed that they were affiliated to *Beta-* and *Gammaproteobacteria*. Three isolates displayed the highest sequence similarity to *Enterobacter amnigenus* (758/759). The 16S rRNA gene sequence of the other isolates had the highest sequence similarity to *Xanthomonas codiaei* (618/621), *Cupriavidus campinensis* (789/789), and *Alcaligenes* sp. (793/797).

COMPLETE SEQUENCE OF IncP-1ε PLASMIDS

The complete genome sequences of four IncP-1ε plasmids that conferred sulfonamide resistance (pKS77, pHH3414, pHH128, and pHH3408) were obtained and analyzed. Plasmids pHH128, pHH3408, and pHH3414 originated from an arable field soil near Kaldenkirchen (Germany), and were captured 8, 57, or 85 days after manure application, respectively. Plasmid pKS77 was exogenously isolated from pig manure. The backbone of all four plasmids was 99.9% identical to that of pKJK5, the first published complete sequence of the IncP-1ε plasmids (**Figure 2**). It comprised genetic modules for replication, partitioning and regulation, mating pair formation and conjugative transfer, and a region with genes of unknown function. In all four plasmids two accessory regions were inserted into the 5′ part of *parA*, which was partially deleted and may not be functional. The identity of backbones and insertion sites among the plasmids suggested a very recent spread from a common ancestor. One of the insertions in all the plasmids is similar to the IS-element ISPa*17* (**Figure 3**). The flanking 25 bp inverted repeats are the targets for the transposase *tnpA*, and the 6 bp direct repeats generated during transposition are still present in all the plasmids. The second insertion site in *parA* contains a Tn*402*-related transposon that carries the IS-element IS*1326* and a class 1 integron. The plasmids differ in the gene cassettes that were captured into the attachment site of the integron. They harbor *aadA* (pHH3414), *aadB* (pKS77), or *aadA1b*, *dfrA1b*, and two copies of *catB* (pHH128), or were devoid of any gene cassette (pHH3408). The *sul1* gene in the 3′ conserved segment of the integrons conferred sulfonamide resistance.

Table 2 | Characterization of exogenously isolated IncP-1ε plasmids from agro-ecosystems.

Plasmid	Source	PCR product with primers targeting 5′/3′CS of integron (kbp)	Hybridization with *aadA*
2-S2	Manure 2	1.0	+
2-S5	Manure 2	1.5	+
3-S1	Manure 3	1.0	+
4-T4	Manure 4	2.0	+
6-S1	Manure 6	1.0	–
7-S	Manure 7	1.0	+
9-T4	Manure 9	1.3	–
11-S2	Manure 11	1.0	+
1-83	Soil microcosm	1.0	+
1-91	Soil microcosm	5	–
1-111	Soil microcosm	1.6	+
1-115	Soil microcosm	3	–
1-127	Soil microcosm	1.3	–
1-131	Soil microcosm	1.7/2.3	+
1-135	Soil microcosm	5	–
1-146	Soil microcosm	5	–
1-153	Soil microcosm	5	–
1-163	Soil microcosm	2.0/4	+
1-167	Soil microcosm	5	–
1-168	Soil microcosm	5	–
2-238	Soil microcosm	5	–
3-385	Soil microcosm	5	–
3-407	Soil microcosm	3	–
3-409	Soil microcosm	1.0	+
3-420	Soil microcosm	2.1	+
3-422	Soil microcosm	2.1	+
3-423	Soil microcosm	2.1	+
3-425	Soil microcosm	2.1	+
3-426	Soil microcosm	2.1	+
3-427	Soil microcosm	2.1	+
3-428	Soil microcosm	2.1	+
C 66	Soil mesocosm	1.8	–
C 120	Soil mesocosm	2.1	+
C 126	Soil mesocosm	2.3	+
C 129	Soil mesocosm	5	–
C 131	Soil mesocosm	3.0/2.1	+
C 132	Soil mesocosm	2.1	+
C 159	Soil mesocosm	5	–
144	Field soil	1.4/2.9/3.5	+
253	Field soil	1.3	–
260	Field soil	5	–
263	Field soil	1.4/2.9/3.5	+
267	Field soil	2.1	+
268	Field soil	1.4/2.1/2.9	+
269	Field soil	5	–
858	Field soil	1.2	+
972	Field soil	1.1	+

The transposition modules *tniABQC* (Rådström et al., 1994) of the Tn*402*-like transposons were 3′ truncated to a different extent, and replaced by a more or less truncated derivative of the tetracycline resistance transposon Tn*1721*, except for plasmid pHH3408 (**Figure 3**). All Tn*402*-related transposons were flanked by inverted and direct repeats. Interestingly, a fragment of the IncP-1α *oriV* was found in plasmid pHH128 and pKS77 adjacent to IS*1326*, suggesting recombination between these incompatible plasmids.

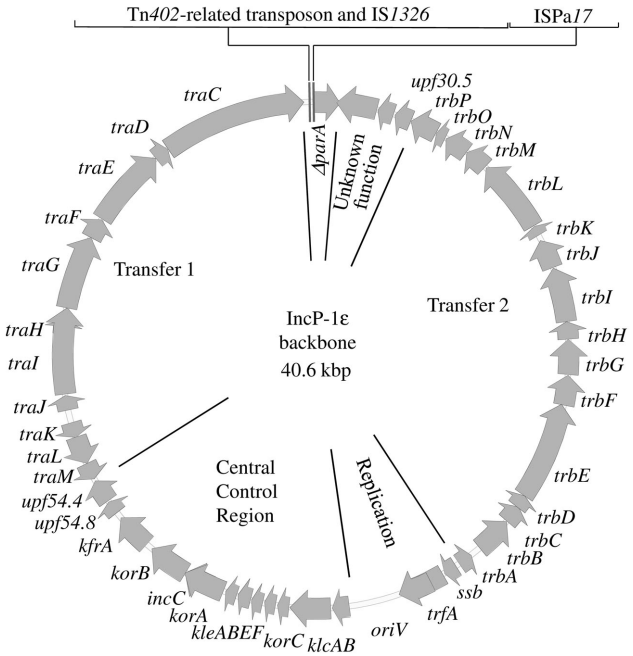


FIGURE 2 | The common plasmid backbone of the completely sequenced IncP-1ε plasmids pHH128, pKJK5, pKS77, pHH3414, and pHH3408. The two insertion sites of accessory elements within the gene *parA* are indicated.

DISCUSSION

Independent isolations of IncP-1ε plasmids and their detection in total community DNA by quantitative PCR showed that these plasmids are widely distributed in agricultural soils and pig manure. Previous attempts to detect IncP-1 plasmids in total community DNA from various environments must have missed this plasmid group as the *trfA* sequence of IncP-1ε plasmids shared less than 75% sequence identity in the region used for probes generated from RP4 or R751 (Götz et al., 1996). The present study adds 50 novel plasmids to this subgroup which was previously proposed by Bahl et al. (2009) based on two representatives. Very recently, three other IncP-1ε plasmids exogenously isolated from Norwegian agricultural soils were completely sequenced (Sen et al., 2011). In contrast to the completely sequenced plasmids of our study, the backbone genes of the Norwegian plasmids were considerably divergent from those of the reference plasmid pKJK5. They did not carry integrons, while on all exogenously isolated plasmids of the present study class 1 integron components (*intI1*, *sul1*, gene cassettes) were detected. With the exception of plasmid pKS77, all plasmids were captured based on the SDZ resistance conferred to

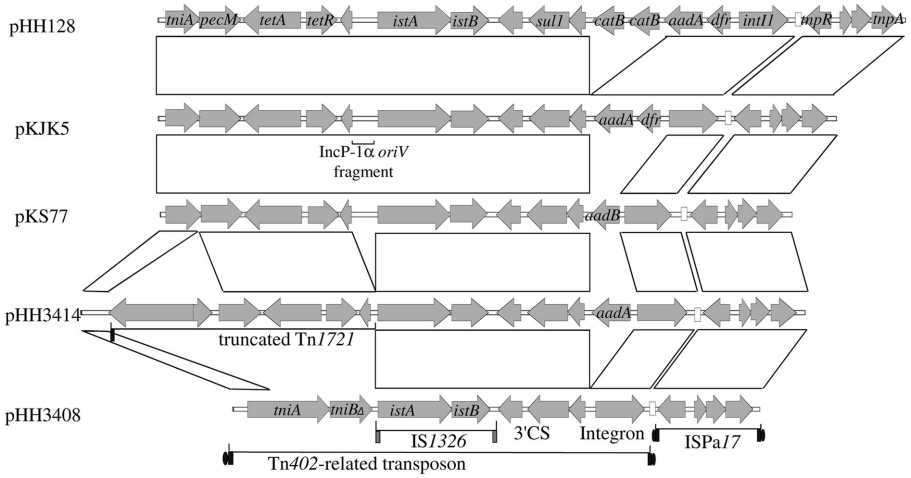


FIGURE 3 | Accessory regions of the completely sequenced IncP-1ε plasmids pHH128, pKJK5, pKS77, pHH3414, and pHH3408. Homologous regions are indicated by framed areas. Inverted repeats of the transposable elements are indicated by rectangles, target site duplications (direct repeats) are indicated by closed ovals.

E. coli CV601gfp. Therefore the finding that all IncP-1ε plasmids carried a class 1 integron might be not too surprising as these integrons often carry a *sul1* gene. In contrast, the Norwegian plasmids were captured based on the mercury resistance that they confer to the recipient (Sen et al., 2011).

Although restriction analysis of plasmid DNA indicated a remarkable diversity of the plasmids captured in our study, the complete sequence determined for four of the plasmids showed that the backbones comprising modules for replication, partitioning and regulation, mating pair formation and conjugative transfer, and a region of unknown function were almost identical with the backbone sequence of the reference plasmid pKJK5. The identity of the backbone and of insertion sites suggested a very recent spread from a common ancestor. As previously reported for pKJK5, a fragment of the IncP-1α *oriV* was found in plasmid pHH128 and pKS77 adjacent to IS*1326*, indicating recombination between incompatible plasmids. This finding is in agreement with other evidence in IncP-1 genome sequences that recombination between IncP-1 plasmids is occurring despite incompatibility (Schlüter et al., 2003; Norberg et al., 2011).

One of the drawbacks of exogenous isolation directly from soil bacteria is that the original hosts remain unknown. Therefore, *E. coli* CV601gfp carrying plasmid pHH3414 was introduced into soil planted with *A. caven*. The soil was amended with manure to stimulate plasmid transfer processes. Plasmid pHH3414 was chosen as the level of soil bacteria resistant toward tetracycline and SDZ was relatively low. After 4 weeks the numbers of the *gfp*-tagged *E. coli* significantly dropped and *gfp* negative colonies with Tc and SDZ phenotype could be picked. Although only cultivable hosts of IncP-1ε plasmids can be identified using this strategy stable replication is a prerequisite for detection. The host range determined in the rhizosphere of *A. caven* for pHH3414 was mainly confined to *Beta-* and *Gammaproteobacteria* and thus confirms the host range suggested for IncP-1 plasmids by analyzing the genomic signatures for host identification (Suzuki et al., 2010; Norberg et al., 2011). Several other strategies were previously used to determine the host range of plasmid pKJK5. Tagging of both the donor and the plasmids allowed a cultivation-independent quantification of donors and transconjugants (Mølbak et al., 2003). In another study by Mølbak et al. (2007) transconjugants mainly belonged to the family Enterobacteriaceae or the genus *Pseudomonas*. In order to determine the host range of pKJK5 in the rhizosphere of barley, Musovic et al. (2006) inoculated *Pseudomonas putida* harboring *lac*I_q with a *gfp*-tagged pKJK5. Transconjugants were obtained after sorting by flow cytometry. The identity of putative transconjugants was determined by cloning and sequencing, and for the first time conjugal transfer of IncP-1ε plasmids to Gram-positive bacteria was documented (*Arthrobacter*). As the transconjugants have not been cultured, the potential of IncP-1ε plasmids to replicate in *Arthrobacter* still needs to be confirmed. Differences in the spectrum of hosts reported for IncP-1ε plasmids might be explained by differences in bacterial community composition, but the detection methods are also assumed to have an effect. While homologous recombination might play an important role in the adaptation to different host backgrounds, class 1 integron gene cassettes seem to be important drivers of diversification to selective pressure posed by antibiotics. This assumption is further supported by the recent report by Guerin et al. (2009) on the induction of SOS response by antibiotic exposure, and a 340 times increase of gene cassette excision and integration might be of great importance. Several studies recently showed that class 1 integron gene cassettes were introduced via manure bacteria into soil (Binh et al., 2009; Byrne-Bailey et al., 2009, 2010). The localization of class 1 integrons on the broad-host range plasmids belonging to the IncP-1ε group further emphasizes their mobility potential. Interestingly, several of the gene cassettes previously reported from manure or manure treated soil, such as *aadA*, *aadB*, or *orfD* (Heuer and Smalla, 2007; Binh et al., 2009) were also found on IncP-1ε plasmids. But in contrast to the total community DNA analysis the size of the amplified gene cassettes was larger than 1.6 kb.

This might indicate a bias of the PCR-based amplification of class 1 integron gene cassettes from total community DNA approach toward smaller fragments.

Plasmids belonging to the IncP-1ε group were captured into *E. coli* from soil bacteria in independent experiments. Although the relative abundance of IncP-1ε plasmids as determined by real-time quantitative PCR is relatively low and thus would not be accessible by metagenomic approaches, the ability of these plasmids to efficiently transfer allowed their capture by exogenous plasmid isolation. Furthermore, the novel *trfA* based quantitative PCR system provided a tool to quantify IncP-1ε plasmid abundance in total community DNA which showed a correlation between plasmid abundance and antibiotic selective pressure. In piggery manures, the relative abundance of IncP-1ε was found to be high and seemed to be correlated with the size and antibiotic usage of the pig producing facility. Interestingly the lowest abundance of plasmids belonging to the IncP-1ε group was found in manure from a pig producing facility that never used antibiotics.

The remarkable diversity of antibiotic resistance gene cassettes reported in the present study, the ability to efficiently transfer under soil conditions and the wide host range of IncP-1ε plasmids strongly suggest that these plasmids are important vectors for spreading antibiotic resistances in the agro-ecosystem.

ACKNOWLEDGMENTS

This work was supported by the DFG, in the frame of the project "Veterinary Medicines in Soils" (Forschergruppe 566), and by grant EF-0627988 from the NSF Microbial Genome Sequencing Program to Eva Top. We are grateful to the US Department of Energy Joint Genome Institute for providing the draft plasmid genome sequences (with special thanks to Brian Foster, Alla Lapidus, and Kerry Barrie). Their work was supported by the Office of Science of the US Department of Energy under Contract No. DE-AC02-05CH11231. We also thank Celeste Brown for Bioinformatics support and Linda Rogers for technical assistance.

REFERENCES

Bahl, M. I., Burmolle, M., Meisner, A., Hansen, L. H., and Sorensen, S. J. (2009). All IncP-1 plasmid subgroups, including the novel epsilon subgroup, are prevalent in the influent of a Danish wastewater treatment plant. *Plasmid* 62, 134–139.

Bahl, M. I., Hansen, L. H., Goesmann, A., and Sorensen, S. J. (2007). The multiple antibiotic resistance IncP-1 plasmid pKJK5 isolated from a soil environment is phylogenetically divergent from members of the previously established alpha, beta and delta sub-groups. *Plasmid* 58, 31–43.

Binh, C. T. T., Heuer, H., Gomes, N. C. M., Kotzerke, A., Fulle, M., Wilke, B. M., Schloter, M., and Smalla, K. (2007). Short-term effects of amoxicillin on bacterial communities in manured soil. *FEMS Microbiol. Ecol.* 62, 290–302.

Binh, C. T. T., Heuer, H., Kaupenjohann, M., and Smalla, K. (2008). Piggery manure used for soil fertilization is a reservoir for transferable antibiotic resistance plasmids. *FEMS Microbiol. Ecol.* 66, 25–37.

Binh, C. T. T., Heuer, H., Kaupenjohann, M., and Smalla, K. (2009). Diverse *aadA* gene cassettes on class 1 integrons introduced into soil via spread manure. *Res. Microbiol.* 160, 427–433.

Byrne-Bailey, K. G., Gaze, W. H., Kay, P., Boxall, A. B., Hawkey, P. M., and Wellington, E. M. H. (2009). Prevalence of sulfonamide resistance genes in bacterial isolates from manured agricultural soils and pig slurry in the United Kingdom. *Antimicrob. Agents Chemother.* 53, 696–702.

Byrne-Bailey, K. G., Gaze, W. H., Zhang, L., Kay, P., Boxall, A. B., Hawkey, P. M., and Wellington, E. M. H. (2010). Integron prevalence and diversity in manured soil. *Appl. Environ. Microbiol.* 77, 684–687.

Couturier, M., Bex, F., Bergquist, P. L., and Maas, W. K. (1988). Identification and classification of bacterial plasmids. *Microbiol. Rev.* 52, 375–395.

Götz, A., Pukall, R., Smit, E., Tietze, E., Prager, R., Tschäpe, H., van Elsas, J. D., and Smalla, K. (1996). Detection and characterization of broad-host-range plasmids in environmental bacteria by PCR. *Appl. Environ. Microbiol.* 62, 2621–2628.

Guerin, É., Cambray, G., Sanchez-Alberola, N., Campoy, S., Erill, I., Da Re, S., Gonzalez-Zorn, B., Barbé, J., Ploy, M. C., and Mazel, D. (2009). The SOS response controls integron recombination. *Science* 324, 1034.

Haines, A. S., Akhtar, P., Stephens, E. R., Jones, K., Thomas, C. M., Perkins, C. D., Williams, J. R., Day, M. J., and Fry, J. C. (2006). Plasmids from freshwater environments capable of IncQ retrotransfer are diverse and include pQKH54, a new IncP-1 subgroup archetype. *Microbiology* 152, 2689–2701.

Heuer, H., Krögerrecklenfort, E., Wellington, E. M. H., Egan, S., van Elsas, J. D., van Overbeek, L. S., Collard, J. M., Guillaume, G., Karagouni, A. D., Nikolakopoulou, T. L., and Smalla, K. (2002). Gentamicin resistance genes in environmental bacteria: prevalence and transfer. *FEMS Microbiol. Ecol.* 42, 289–302.

Heuer, H., Schmitt, H., and Smalla, K. (2011). Antibiotic resistance gene spread due to manure application on agricultural fields. *Curr. Opin. Microbiol.* 14, 236–243.

Heuer, H., and Smalla, K. (2007). Manure and sulfadiazine synergistically increased bacterial antibiotic resistance in soil over at least two months. *Environ. Microbiol.* 9, 657–666.

Mølbak, L., Licht, T. R., Kvist, T., Kroer, N., and Andersen, S. R. (2003). Plasmid transfer from *Pseudomonas putida* to the indigenous bacteria on alfalfa sprouts: characterization, direct quantification, and in situ location of transconjugant cells. *Appl. Environ. Microbiol.* 69, 5536–5542.

Mølbak, L., Molin, S., and Kroer, N. (2007). Root growth and exudate production define the frequency of horizontal plasmid transfer in the rhizosphere. *FEMS Microbiol. Ecol.* 59, 167–176.

Moura, A., Henriques, I., Smalla, K., and Correia, A. (2010). Wastewater bacterial communities bring together broad-host range plasmids, integrons and a wide diversity of uncharacterized gene cassettes. *Res. Microbiol.* 161, 58–66.

Musovic, S., Oregaard, G., Kroer, N., and Sorensen, S. J. (2006). Cultivation-independent examination of horizontal transfer and host range of an IncP-1 plasmid among Gram-positive and Gram-negative bacteria indigenous to the barley rhizosphere. *Appl. Environ. Microbiol.* 72, 6687–6692.

Norberg, P., Bergström, M., Jethava, V., Dubhashi, D., and Hermansson, M. (2011). The IncP-1 plasmid backbone adapts to different host bacterial species and evolves through homologous recombination. *Nat. Commun.* 2, 268.

Pansegrau, W., Lanka, E., Barth, P. T., Figurski, D. H., Guiney, D. G., Haas, D., Helinski, D. R., Schwab, H., Stanisich, V. A., and Thomas, C. M. (1994). Complete nucleotide sequence of Birmingham IncP-alpha plasmids – compilation and comparative analysis. *J. Mol. Biol.* 239, 623–663.

Rådström, P., Sköld, O., Swedberg, G., Flensburg, J., Roy, P. H., and Sundström, L. (1994). Transposon Tn*5090* of plasmid R751, which carries an integron, is related to Tn7, Mu, and the retroelements. *J. Bacteriol.* 176, 3257–3268.

Schauss, K., Focks, A., Heuer, H., Kotzerke, A., Schmitt, H., Thiele-Bruhn, S., Smalla, K., Wilke, B. M., Matthies, M., Amelung, W., Klasmeier, J., and Schloter, M. (2009). Analysis, fate and effects of the antibiotic sulfadiazine in soil ecosystems. *Trends Anal. Chem.* 28, 612–618.

Schlüter, A., Heuer, H., Szczepanowski, R., Forney, L. J., Thomas, C. M., Pühler, A., and Top, E. M. (2003). The 64508 bp IncP-1β antibiotic multiresistance plasmid pB10 isolated from a waste-water treatment plant provides evidence for recombination between members of different branches of the IncP-1β group. *Microbiology* 149, 3139–3153.

Schlüter, A., Szczepanowski, R., Pühler, A., and Top, E. M. (2007). Genomics of IncP-1 antibiotic resistance plasmids isolated from wastewater treatment plants provides evidence for a widely accessible drug resistance gene pool. *FEMS Microbiol. Rev.* 31, 449–477.

Sen, D., Van der Auwera, G. A., Rogers, L. M., Thomas, C. M., Brown, C. J., and Top, E. M. (2011). Broad-host-range plasmids from agricultural soils have IncP-1 backbones with diverse accessory genes. *Appl. Environ. Microbiol.* 77, 7975–7983.

Smalla, K., Haines, A. S., Jones, K., Krögerrecklenfort, E., Heuer, H., Schloter, M., and Thomas, C. M. (2006). Increased abundance of IncP-1β plasmids and mercury resistance genes in mercury-polluted river sediments: first discovery of IncP-1β plasmids with a complex *mer* transposon as the sole accessory element. *Appl. Environ. Microbiol.* 72, 7253–7259.

Smalla, K., Krögerrecklenfort, E., Heuer, H., Dejonghe, W., Top, E., Osborn, M., Niewint, J., Tebbe, C., Barr, M., Bailey, M., Greated, A., Thomas, C., Turner, S., Young, P., Nikolakopoulou, D., Karagouni, A., Wolters, A., van Elsas, J. D., Dronen, K., Sandaa, R., Borin, S., Brabhu, J., Grohmann, E., and Sobecky, P. (2000). PCR-based detection of mobile genetic elements in total community DNA. *Microbiology* 146, 1256–1257.

Suzuki, H., Yano, H., Brown, C. J., and Top, E. M. (2010). Predicting plasmid promiscuity based on genomic signature. *J. Bacteriol.* 192, 6045–6055.

Suzuki, M. T., Taylor, L. T., and Delong, E. F. (2000). Quantitative analysis of small-subunit rRNA genes in mixed microbial populations via 5′-nuclease assays. *Appl. Environ. Microbiol.* 66, 4605–4614.

Thomas, C. M. (2000). Paradigms of plasmid organization. *Mol. Microbiol.* 37, 485–491.

Thorsted, P. A., Macartney, D. P., Akhtar, P., Haines, A. S., Ali, N., Davidson, P., Stafford, T., Pocklington, M. J., Pansegrau, W., Wilkins, B. M., Lanka, E., and Thomas, C. N. (1998). Complete sequence of the IncP beta plasmid R751: implications for evolution and organisation of the IncP backbone. *J. Mol. Biol.* 282, 969–990.

Vedler, E., Vahter, M., and Heinaru, A. (2004). The completely sequenced plasmid pEST4011 contains a novel IncP1 backbone and a catabolic transposon harboring *tfd* genes for 2,4-dichlorophenoxyacetic acid degradation. *J. Bacteriol.* 186, 7161–7174.

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 15 November 2011; paper pending published: 18 December 2011; accepted: 02 January 2012; published online: 18 January 2012.

Citation: Heuer H, Binh CTT, Jechalke S, Kopmann C, Zimmerling U, Krögerrecklenfort E, Ledger T, González B, Top E and Smalla K (2012) IncP-1ε plasmids are important vectors of antibiotic resistance genes in agricultural systems: diversification driven by class 1 integron gene cassettes. Front. Microbio. ***3****:2. doi: 10.3389/fmicb.2012.00002*

This article was submitted to Frontiers in Antimicrobials, Resistance and Chemotherapy, a specialty of Frontiers in Microbiology.

frontiers in
MICROBIOLOGY

REVIEW ARTICLE
published: 09 April 2012
doi: 10.3389/fmicb.2012.00119

Integron involvement in environmental spread of antibiotic resistance

Thibault Stalder[1,2,3], Olivier Barraud[1,2], Magali Casellas[3], Christophe Dagot[3] and Marie-Cécile Ploy[1,2]*

[1] INSERM, U1092, Limoges, France
[2] Université de Limoges, UMR-S1092, Limoges, France
[3] Université de Limoges, GRESE EA4330, ENSIL, Limoges, France

Edited by:
Rustam I. Aminov, University of Aberdeen, UK

Reviewed by:
Kornelia Smalla, Julius Kühn-Institut – Federal Research Centre for Cultivated Plants, Germany
Vincent Burrus, Université de Sherbrooke, Canada
Laurent Poirel, French Institute of Health, France
Henning Sørum, Norwegian School of Veterinary Science, Norway
Holger Heuer, Julius Kühn-Institut – Federal Research Centre for Cultivated Plants, Germany

***Correspondence:**
Marie-Cécile Ploy, UMR Inserm 1092, Faculté de Médecine, 2 rue du docteur Marcland, Limoges, France.
e-mail: marie-cecile.ploy@unilim.fr

The spread of antibiotic-resistant bacteria is a growing problem and a public health issue. In recent decades, various genetic mechanisms involved in the spread of resistance genes among bacteria have been identified. Integrons – genetic elements that acquire, exchange, and express genes embedded within gene cassettes (GC) – are one of these mechanisms. Integrons are widely distributed, especially in Gram-negative bacteria; they are carried by mobile genetic elements, plasmids, and transposons, which promote their spread within bacterial communities. Initially studied mainly in the clinical setting for their involvement in antibiotic resistance, their role in the environment is now an increasing focus of attention. The aim of this review is to provide an in-depth analysis of recent studies of antibiotic-resistance integrons in the environment, highlighting their potential involvement in antibiotic-resistance outside the clinical context. We will focus particularly on the impact of human activities (agriculture, industries, wastewater treatment, etc.).

Keywords: integron, antibiotic resistance, soil, aquatic ecosystems, wastewater, agriculture, water

Bacterial evolution has largely been shaped by the high plasticity of bacterial genomes, leading to their adaptation to most ecosystems. This ability to exchange and rearrange genomic sequences to gain new traits has been extensively demonstrated with the bacterial resistance to antibiotics. Today, the increasing rate of antibiotic resistant bacteria is a major public health issue (Davies and Davies, 2010). During the last decade, several studies have underlined the environmental resistome as a source of resistance genes of clinical interest (D'Costa et al., 2006; Aminov and Mackie, 2007; Martínez, 2008; Wright, 2010). While mutation events contribute to the bacterial adaptation, horizontal gene transfer seems to be the main cause of the rapid proliferation of antibiotic-resistance genes across a wide diversity of bacteria. Much of this horizontal gene transfers have been shown to occur in the environment (Aminov, 2011). Nevertheless, the diversity of mobile genetic elements currently described (Wozniak and Waldor, 2010; Bertels and Rainey, 2011), shows that beyond horizontal gene transfer, the loss and acquisition of functional modules are an important part in the processes of rapid bacterial adaptation and development of resistance. Integrons are one of the genetic elements involved in the adaptation of bacteria. We address the question of the involvement of integrons in the environmental spread of antibiotic resistance. More specifically, the anthropogenic impacts, which have been shown to be involved in the antibiotic-resistance spread in the environment, and the role of integrons in this process.

INTEGRONS: GENERALITIES

Integrons are bacterial genetic elements able to promote acquisition and expression of genes embedded within gene cassettes (GCs; Stokes and Hall, 1989). The definition of an integron is based on a functional platform (also called 5′ conserved segment, 5′CS), composed of three key elements: the *intI* gene, a specific recombination site *attI*, and a promoter, Pc (Hall and Collis, 1995; Boucher et al., 2007; **Figure 1**). The *intI* gene encodes an integrase protein IntI, which belongs to the family of tyrosine recombinases (Nunes-Düby et al., 1998).

The GCs are non-replicative mobile elements, which generally couple an open reading frame (ORF) with an *attC* site. GCs are integrated or excised from the functional platform by a site-specific recombination mechanism catalyzed by the IntI integrase. Two types of recombination can occur (**Figure 1**): (i) between *attI* and *attC* sites, resulting in the insertion of GCs at the *attI* site, and (ii) between two *attC* sites, leading to excision of the GCs (Mazel, 2006). The GCs can be found either as a linear form, included in an integron, or as a covalently closed circular free intermediate (Collis and Hall, 1992). GCs are usually promoterless and require the Pc promoter for their expression as in an operon. The consequence of this system is that the last integrated cassette is the closest to the Pc promoter (Collis et al., 1993; Collis and Hall, 2004), leading to the highest level of expression in the integron.

Two major groups of integrons have been described: "chromosomal integrons" (CIs), and "mobile integrons" (MIs). CIs are

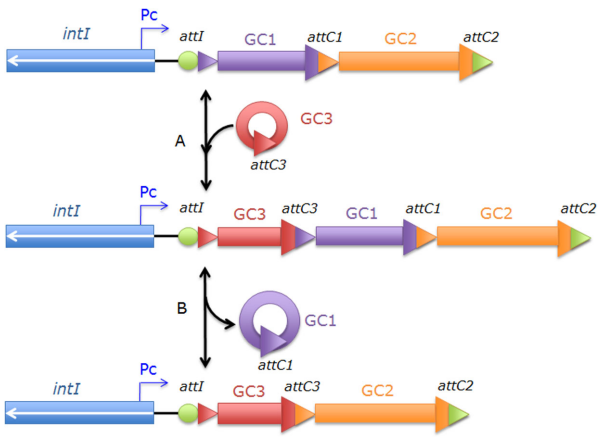


FIGURE 1 | General organization of an integron and gene cassette (GC) recombination mechanism. The IntI1 protein catalyzes the insertion **(A)** and excision **(B)** of the GC in the integron, with GC integration occurring at the *attI* recombination site. In example **(A)**, the circularized GC3 is integrated in linear form inside the integron platform *via* a specific recombination mechanism between the *attI* site and the *attC3* site of the GC3. GC excision preferentially occurs between two *attC* sites. In example **(B)**, the GC1 is excised following the recombination between the two *attC1* and *attC3* sites. Pc: gene cassette promoter; *attI*: integron recombination site; *attC1*, *attC2*, and *attC3*: *attC* GC recombination sites; *intI*: the integrase gene; GC1, GC2, GC3 are the gene cassettes, and arrows indicate the direction of coding sequences (Barraud and Ploy, 2011).

located on the chromosome of hundreds of bacterial species; *in silico* analysis showed that 17% of sequenced bacterial genomes exhibited such genetic arrangements (Cambray et al., 2010). CIs are often described in bacteria from marine or terrestrial ecosystems, such as *Vibrio* spp. and *Xanthomonas* spp., CIs have also been termed "super-integrons" (SIs) as they can carry up to 200 cassettes that mainly encode proteins with unknown functions. CIs may also carry cassettes without functional reading frames. MIs are not self-transposable elements but are located on mobile genetic elements such as transposons and plasmids, which promote their dissemination among bacteria. MIs contain a limited number of GCs (less than 10 GCs; Naas et al., 2001; GenBank DQ112222). The GCs described to date in these MIs usually encode antibiotic-resistance determinants. MIs are therefore sometimes also called "resistant integrons" (RIs) or "multidrug resistance integrons" (MRIs).

In this review, we will focus on MIs.

CLASSES OF MIs

Most MIs have been described in a wide range of Gram-negative bacteria, and only sporadically in Gram-positive bacteria (Martin et al., 1990; Nesvera et al., 1998; Nandi et al., 2004; Shi et al., 2006; Xu et al., 2010; Barraud et al., 2011). Based on the amino-acid sequence of the IntI protein, five classes of MIs have been described (Cambray et al., 2010). Classes 1, 2, and 3 are the most commonly detected. Classes 4 and 5 have only been detected once (Hochhut et al., 2001; GenBank AJ277063).

Class 1 MIs have been extensively studied due to their broad distribution among Gram-negative bacteria of clinical interest and are the most reported in human and animals. They have been described to be mainly associated with functional and non-functional transposons derived from *Tn*402. The non-functional type is the main common structural organization described in clinical isolates, and led some authors to call these class 1 MIs, "clinical integrons" (Gillings et al., 2008c). In addition, these structures are frequently embedded in plasmids or larger transposons, such as those of the *Tn*3 family (*Tn*21, Tn1696) allowing their dispersion (Labbate et al., 2009). The *intI1* gene sequence is highly conserved among MIs found in clinical isolates, while it shows variability in MIs-containing environmental isolates (Gillings et al., 2008b). Furthermore, many class 1 MIs exhibit a 3′ region usually called 3′-conserved segment (3′CS). However, some authors consider using this 3′CS to detect MIs could create a bias in detection, since some MIs lack this sequence (Betteridge et al., 2011). The 3′CS is composed of a *qacE*Δ*1* gene, a functional deletion of the *qacE* gene still conferring resistance to quaternary ammonium compounds (QACs; Paulsen et al., 1993), followed by a *sul1* gene conferring resistance to sulfonamides, and *orf5* encoding a protein of unknown function.

Class 2 MIs are the second most described group. In most class 2 MIs, the *intI2* gene is interrupted by a stop codon, resulting in a truncated and non-functional protein. This results in a stable GCs array, mainly composed of the GC *dfrA1* (involved in the resistance to trimethoprim), *sat2* (involved in the resistance to streptothricin), *aadA1* (involved in the resistance to streptomycin and spectinomycin), and *orfX* (unknown function; Hansson et al., 2002). However, some class 2 MIs with a different GCs array have been described, probably resulting from the ability of the integrase of class 1 MIs to catalyze recombination at the *attI2* site (Biskri and Mazel, 2003; Ahmed et al., 2005; Ramírez et al., 2005, 2010; Gassama Sow et al., 2010). Class 2 MIs are almost always associated with the *Tn*7 transposon and their derivatives, hence promoting their dissemination. Two class 2 MIs have been described recently with a functional integrase, one containing nine GCs encoding unknown function and the second one harbored the *dfrA14* GC followed by a second novel GC in which a lipoprotein signal peptidase gene has been predicted (Barlow and Gobius, 2006; Márquez et al., 2008).

Only few class 3 MIs have been described. Although their role in clinical antimicrobial resistance is less important, environmental ecosystems could harbor an important pool of these elements (see below).

MIs DISSEMINATION AND THEIR INVOLVEMENT IN ANTIBIOTIC RESISTANCE

Antibiotic pressure has probably played an important role in the MIs selection and dissemination in bacteria. More than 130 GCs conferring resistance to antibiotics and more than 60 GCs of unknown functions have been described in MIs (Partridge et al., 2009). Genes involved in resistance to almost all antibiotic families are embedded in GCs, including beta-lactams, aminoglycosides, trimethoprim, chloramphenicol, fosfomycin, macrolides, lincosamides, rifampicin, and quinolones. In addition, the *qac* GCs, encoding resistance determinants to antiseptics of the QACs family, are commonly found in MIs. Studies have suggested that MIs were more prevalent in bacterial communities subjected to direct or indirect antibiotic pressure in clinical, agricultural, and

environmental settings (Skurnik et al., 2005; Daikos et al., 2007; Barlow et al., 2009; Luo et al., 2010; Kristiansson et al., 2011). Other factors, such as QACs or heavy metals have also been shown to be involved in the MIs dissemination, and thus probably playing a role in their spread before the antibiotic era (see below). More generally, it has been shown, when studying animal fecal *E. coli*, that human activity in the near vicinity increased the prevalence of MIs in these bacteria (Skurnik et al., 2006). Concerning the role of the antibiotic selective pressure, no published studies have demonstrated the direct *in vivo* selection of resistance through the acquisition of an integron. One study has demonstrated the *in vivo* selection of resistance through a rearrangement of the GCs array within a class 1 MI under antibiotic selective pressure (Hocquet et al., 2011).

Recent *in vitro* studies have shown that antibiotics are able to induce integrase transcription, both in CIs and MIs, *via* the SOS response. The SOS response is a global regulatory network controlled by the transcriptional repressor LexA and induced by stress leading to direct or indirect DNA damage, such as damage resulting from exposure to some widely used antibiotics (fluoroquinolones, beta-lactams, trimethoprim, aminoglycosides; Guerin et al., 2009; Baharoglu et al., 2010; Cambray et al., 2011). The activation of the SOS response in bacteria results in integrase overexpression, which leads to the raise of GCs recombination events.

Clinical, veterinary, and environmental surveys have shown that bacteria harboring MIs are often associated with multidrug-resistant (MDR) phenotypes (Bass et al., 1999; Leverstein-van Hall et al., 2003; Biyela et al., 2004; Nijssen et al., 2005; Laroche et al., 2009). However, the MDR profile could not be linked only to the antibiotic-resistance GCs carried by the MIs, but also to other resistance genes located on MIs-containing plasmids and transposons. This way, MIs could be co-selected with the plasmid- and/or transposon-associated antibiotic-resistance genes (Laroche et al., 2009; Li et al., 2010). For example, co-selection of class 1 MIs on plasmids harboring a *tet* gene (involved in the tetracycline resistance) in oxytetracycline-contaminated environments has been reported (Li et al., 2010).

The link between MIs and antibiotic resistance is still controversial since several studies present divergent conclusions (Hoyle et al., 2006; Smith et al., 2007). Furthermore many data have to be interpreted with caution. Indeed, biases in the study of links between MIs and antibiotic resistance could be generated by the selective choice of antibiotic-specific resistant strains, leading to misinterpretation. Finally, this relationship between MIs and antibiotic resistance has mainly been studied in bacteria of clinical or veterinary interest, such as those within the family Enterobacteriaceae.

Otherwise, the environment contains a wide range of bacterial species and cultivation methods only permit the isolation of a small fraction (around 1%; Amann et al., 1995). Techniques based on the study of the metagenome have thus been developed to avoid this limitation. The combination of culturing and metagenomics approaches on environmental ecosystems has highlighted the roles of MIs in antibiotic-resistance dissemination. **Tables 1** and **2** present an extensive list of the studies that have quantified the occurrence of MIs in the environment, using either cultivation methods (**Table 1**) or cultivation independent methods (**Table 2**).

Genetic methods presented in this review quantify the abundance of integrase genes in the total DNA from different ecosystems. In order to normalize the quantity of gene to the total bacterial communities, most authors have used quantification of the ubiquitous bacterial 16S rRNA encoding genes. By dividing the abundance of integrase genes by the number of 16S rRNA genes, authors were able to demonstrate relative abundance. This ratio corresponds to the integrase genes proportion in the total bacterial communities. However, some authors have multiplying the ratio by the average number of copies of the 16S rRNA encoding genes per bacteria; which is approximately four (Klappenbach et al., 2001), and other authors present their results as percentages. In order to integrate all relative abundance data from diverse studies, results have been normalized to the same ratio for the purpose of this review and the relative abundance corresponds to the percentage of MIs per bacterial cell (**Table 2**).

MIs IN THE ENVIRONMENT

There is growing evidence that the environment plays a role in the spread of antibiotic resistance among pathogenic strains. Many questions have been raised concerning the impact of the release of antibiotics and antibiotic-resistant bacteria on the environment or on human and animal health (Aminov, 2010). The distribution of MIs, and especially the class 1 MIs, in the environment is a growing focus of attention, as illustrated by the recent publications presented in **Tables 1** and **2**.

MIs have been described in a wide range of natural ecosystems, both aquatic (e.g., lakes, rivers, estuaries) and terrestrial. However, their distribution has been investigated mainly in human-impacted environments such as amended soils and aquatic ecosystems influenced by urbanization, agriculture, aquaculture, industrial waste, and even in indoor and outdoor dust.

MI OCCURRENCE IN "NATURAL" ENVIRONMENTS

Different authors have investigated the occurrence of class 1 MIs in ecosystems considered to be untouched or barely affected by anthropogenic influence, these are often termed "reference sites" and correspond in **Tables 1** and **2** to the "clean area."

Only a few teams have studied MIs abundance in soils. Gaze et al. (2011) reported a class 1 MIs relative abundance of 0.00576% (**Table 2**) by a metagenomic approach in soils with no history of organic amendment, whereas the same authors previously found no class 1 MIs in the bacterial culturable fraction, which was composed of Bacillaceae, Paenibacillaceae, and Pseudomonadaceae (Gaze et al., 2005). In a study on forest soils, 11 out of 24 isolated Enterobacteriaceae strains (45%) were found to contain class 1 MIs, but these MIs harbored no GCs (Srinivasan et al., 2008).

In aquatics environments, Wright et al. (2008) and Hardwick et al. (2008) found, using metagenomics approaches, a relative class 1 MIs abundance recovery from 0.02 to 4%, in estuarine and stream water/sediments/biofilms, and 2.65% in creek sediments (**Table 2**). Using cultivation-dependent methods, class 1 MIs were found in lake sediments, with a prevalence of 1–4% (Stokes et al., 2006; Gillings et al., 2008a). Some studies investigated the GCs content of class 1 MIs. More often, one to three GCs were present, mainly encoding unknown function. GCs implied in the resistance to QACs (*qac* alleles) were also frequently described and

Table 1 | Bacterial prevalence of class 1 and 2 MIs in different ecosystems (results from cultivation-dependent studies).

	Ecosystem	Sample	Class 1 MIs% (*n*)	Class 2 MIs% (*n*)	Taxonomic affiliation	Reference
Clean area	Lake	Sediment	2.1 ($n=192$)	–	NS	Stokes et al. (2006)
			1–3 ($n=192$)	–		Gillings et al. (2008a)
	Soil/lake	Sediment	2–4 ($n=200$)	–	NS	Stokes et al. (2006)
	Soil	Forest soil	45.8 ($n=24$)	–	Enterobacteriaceae	Srinivasan et al. (2008)
		Agricultural land	0 ($n=262$)	–	NS and QACs^{R}	Gaze et al. (2005)
	Karst	Drinking water source	0 ($n=436$)		*E. coli*	Laroche et al. (2010)
Anthropogenic impacted***	River	US from the WWTP	0 ($n=75$) [a]	–	NS	Li et al. (2009)
			3 ($n=65$) [b]	–		Li et al. (2010)
			4.4 ($n=45$)	2.2 ($n=45$)	*E. coli*	Oberlé et al. (2012)
			6 ($n=301$) [c]			Koczura et al. (2012)
		DS from the WWTP	8 ($n=50$)	0 ($n=50$)		Oberlé et al. (2012)
			14 ($n=322$) [c]			Koczura et al. (2012)
			9.1 ($n=163$) [a]	–	NS	Li et al. (2009)
			86.2 ($n=87$) [b]	–		Li et al. (2010)
		Water	17.1 ($n=117$)	4.3 ($n=117$)	*E. coli***	Figueira et al. (2011)
			41 ($n=500$)	–	*E. coli*	Chen et al. (2011)
			58.1 ($n=43$)	–	MDR Enterobacteriaceae	Biyela et al. (2004)
			7.6 ($n=183$)	2.7 ($n=183$)	Enterobacteriaceae	Ozgumus et al. (2009)
			23 ($n=87$)	–	Enterobacteriaceae ^{R} and *Aeromonas* spp.^{R}	Guo et al. (2011)
			27.7 ($n=65$)	–		
		Water/sediment	13 ($n=32$)	3.1 ($n=32$)	MDR *E. coli*	Roe et al. (2003)
			44 ($n=313$)	–	*Aeromonas* sp.	Schmidt et al. (2001)
	Lake	Water	21 ($n=14$)	0	MDR *E. coli*	Dolejská et al. (2009)
	Estuaries	Water	8.9 ($n=279$)	1.4 ($n=279$)	*E. coli*	Laroche et al. (2009)
			29.6 ($n=54$)	7.4 ($n=54$)	amp^{R} Enterobacteriaceae**	Henriques et al. (2006)
			21 ($n=57$)	–	amp^{R} *Aeromonas* sp.**	
			3.6 ($n=3000$)	–	colif., *Pseudo.* And *Vibrio.**	Rosser and Young (1999)
	Hospital	wastewater	54.9 ($n=302$)	–	Enterobacteriaceae ^{R} and *Aeromonas* spp.^{R}	Guo et al. (2011)
			48.4 ($n=184$)			
			6 ($n=50$)	0 ($n=50$)	*E. coli*	Oberlé et al. (2012)
	Retirement home	wastewater	36 ($n=50$)	0 ($n=50$)	*E. coli*	Oberlé et al. (2012)
	WWTP	Raw effluent	15.1 ($n=643$) [c]		*E. coli*	Koczura et al. (2012)
		Treated effluent	11.5 ($n=174$) [c]			
		Activated sludge	3.7 ($n=378$) [c]			
		Raw effluent	10 ($n=61$)	8 ($n=61$)	Enterobacteriaceae and *Aeromonas* spp.**	Moura et al. (2007)
		Treated effluent	40 ($n=94$)	2 ($n=94$)		
		Activated sludge	61 ($n=35$)	6 ($n=35$)		
		Raw effluent	7.4 ($n=95$)	0 ($n=95$)	Enterobacteriaceae and *Aeromonas* spp.**	Moura et al. (2012)
		Treated effluent	4.6 ($n=131$)	0 ($n=131$)		
		Activated sludge	≈3 ($n=169$)	0.6 ($n=169$)		

(Continued)

Table 1 | Continued

	Ecosystem	Sample	Class 1 MIs% (*n*)	Class 2 MIs% (*n*)	Taxonomic affiliation	Reference
		Raw effluent	20.4 ($n=54$)	–	LF Enterobacteriaceae and *Aeromonas* spp.**	Ma et al. (2011a)
		Treated effluent	38.9 ($n=54$)	–		
		Activated sludge	30.9 ($n=81$)	–		
		Raw effluent	10	–	*E. coli***	Ferreira da Silva et al. (2007)
		Treated effluent	9.6	–		
		Raw effluent	19.1 ($n=204$)	4.9 ($n=204$)	*E. coli***	Figueira et al. (2011)
		Treated effluent	22.3 ($n=117$)	4.3 ($n=117$)		
		Raw effluent	16.4 ($n=49$)	0 ($n=49$)	*E. coli*	Oberlé et al. (2012)
		Treated effluent	8.5 ($n=49$)	2 ($n=49$)		
		Treated effluent[a]	14 ($n=179$)	–	NS	Li et al. (2009)
		Treated effluent[b]	97.4 ($n=189$)	–		Li et al. (2010)
		Activated sludge	33 ($n=109$)	–	LF Enterobacteriaceae**	Zhang et al. (2009b)
		Activated sludge	1 ($n=193$)	–	*E. coli* sul^R	Díaz-Mejía et al. (2008)
	Reed bed	Sediment	14.9 ($n=127$)	–	NS and QACs^R	Gaze et al. (2005)
	GWTP	AC biofilm	30 ($n=192$)	–	NS	Gillings et al. (2008a)
	Soil		6.6 ($n=500$)	10.2 ($n=500$)	NS + antibiotic^R	Byrne-Bailey et al. (2010)
			6.6 ($n=213$)	–	tet^R strains	Agerso and Sandvang (2005)
		Manured soil	89.3 ($n=56$)	–	Enterobacteriaceae	Srinivasan et al. (2008)
	Soil/pig slurry		6.2 ($n=531$)	9.6 ($n=531$)	NS + antibiotic^R	Byrne-Bailey et al. (2009)
	Compost		7.6 ($n=136$)	–	*E. coli* **	Heringa et al. (2010)
	Urban dust	Indoor	≈2 ($n=183$)	–	*E. coli* sul^R	Díaz-Mejía et al. (2008)
		outdoor	≈15 ($n=116$)	–		

*n, Number of isolated strains; LF, lactose fermenting; GWTP, ground water treatment plant; AC, activated carbon; NS, non-selective; US and DS, upstream (US) or downstream (DS) from the WWTP discharge in the receiving river; MDR, multidrug resistant; *coliform, Pseudomonas-like and Vibrio-like; **the taxonomic affiliation is based on 16S rRNA gene sequencing; ***impacted environment by urban and/or agricultural activities (sewage/industrial/WWTP/animal husbandaries facilities/fishponds/organic amendment); [a] the WWTP specifically treated effluents from a penicillin production facilities; [b] the WWTP specifically treated effluent from an oxytetracycline production facilities; [c] prevalence comprise both class 1 and 2 MIs; QACs^R, quaternary ammonium compounds resistant strains; Enterobacteriaceae and Aeromonas spp.^{R} refer to selected strains resistant to at least one antibiotic; amp^R, ampicillin resistant, sul^R, sulfonamide resistant, tet^R, tetracycline resistant; "≈": values have been extracted from graph.*

antibiotic-resistance GCs were rarely found (Gillings et al., 2008c, 2009a).

ENVIRONMENTAL SOURCE OF MIs

The class 1 MIs are ubiquitous elements naturally occurring in the environment, and different studies suggest that these elements emerged from ancestral environmental CIs (Rowe-Magnus et al., 2001; Mazel, 2006). Following the discovery of several class 1 MIs lacking resistance genes in environmental samples and located on the bacterial chromosome (Stokes et al., 2006; Gillings et al., 2008a), an evolutionary model was proposed and is now well documented (Gillings et al., 2008a; Labbate et al., 2009; Cambray et al., 2010; Stokes and Gillings, 2011). This model involves a succession of evolutionary recombination events, which facilitated the spread of class 1 MIs among pathogenic bacteria. These events led to the association of an "ancient" chromosomal class 1 MI with mobile functions of a *Tn*402-like transposon, and the acquisition of a *qacE* and *sul1* genes. During this evolution, deletions, insertions, and other rearrangements finally shaped the 3′CS of current class 1 MIs found in clinical isolates, as well as their inclusion in larger mobile platforms (plasmids and transposons), resulting in the spread of these elements among a broad range of bacteria, including pathogenic species (Gillings et al., 2008a; Labbate et al., 2009). Finally, it has been suggested that the class 1 MIs were probably widely distributed in Proteobacteria before the antibiotic era (Stokes and Gillings, 2011). These authors suggested that these class 1 MIs were unlikely to have GCs encoding antibiotic resistant determinants, and that they further evolved by acquisition of the 3′CS and antibiotic-resistance GCs. Nevertheless, a class 1 MI found in a *Pseudomonas* isolate recently recovered from 15,000- to 40,000-years-old Siberian permafrost with all the characteristics of a typical clinical class 1 MI, i.e., 5′CS and 3′CS, an antibiotic resistant GC (*aadA2* encoding resistance determinants to streptomycin and spectinomycin), localization on a mobile element (Tn5045 transposon), contradicts this hypothesis (Petrova et al., 2011).

ANTHROPOGENIC IMPACT ON MIs DISTRIBUTION

Rivers, seas, and lakes

Water is the main vector of pollutants in the environment and thus has received most attention. Furthermore, water bodies have been underlined as ideal vectors for the antibiotic-resistance

Table 2 | Concentration and relative abundance of class 1 MIs in total community DNA from different ecosystems.

		Samples	Characteristics	Class 1 MIs (*intI1*· L^{-1} or g^{-1})	Class 1 MIs relative abundance given by authors	Relative abundance recalculated (%)*	Reference
Clean area [a]	River/lake	Waters		$\approx 10^4$	≈0.01 [c]	4	Wright et al. (2008)
		Sediments		$\approx 10^3$–10^{4**}	≈0.001 [c]	0.4	
		Biofilm		$\approx 10^1$–10^{3**}	≈0.0001 [c]	0.04	
		Sediments		–	2.65 (max = 8) [d]	2.65 (max = 8)	Hardwick et al. (2008)
		Creek, GWTP, and pond biofilms		–	4.5 (min = 1/max = 9) [d]	4.5 (min = 1/max = 9)	Gillings et al. (2008c)
		River water		$< 10^6$	<0.0001 [c]	< 0.04	Lapara et al. (2011)
	Estuary	Waters		$\approx 10^3$–10^4	≈0.00005–0.0001 [c]	0.02–0.04	Wright et al. (2008)
		Sediments		$\approx 10^{4**}$	≈0.00005 [c]	0.02	
	Soil			–	0.0036 [e]	0.00576	Gaze et al. (2011)
Anthropogenic-impacted area [b]	River/lake	Waters	Industrial polluted	$\approx 10^3$–10^4	≈0.01 or 0.05 [c]	2 and 4	Wright et al. (2008)
		Sediments	Industrial polluted	$\approx 10^3$–10^{4**}	≈0.1 or 0.005 [c]	2 and 40	
		Biofilm	Industrial polluted	$\approx 10^2$–10^{4**}	≈0.0005 [c]	0.2	
		Waters microcosms	Initial (day 0)	$\approx 10^4$	≈0.0001 [c]	0.04	
		incubated at 23°C	Cadmium, 0.1 mM	$\approx 10^4$–10^5	≈0.001 [c]	0.4	
		during 7 days	Tetracycline, 30 mg L^{-1}	$\approx 10^3$–10^5	≈0.01 [c]	4	
			Control	$\approx 10^2$–10^3	≈0.0001 [c]	0.04	
		Sediments	Industrial area residential area DS a sewage output	–	≈1.5 (max = 4.294 for the sewage input) [f]	6 (max = 17)	Rosewarne et al. (2010)
			Agricultural/clean area	–	≈0.1 (mini = 0.02) [f]	0.4	
		Waters	Urban and agricultural influenced	$\approx 10^7$–10^8	≈0.000005–0.005 [c]	0.002–2	Luo et al. (2010)
		Sediments	Urban and agricultural influenced	$\approx 10^{11**}$			
		River Waters	Urban and industrial polluted	$\approx 10^5$	between 10^2 and 10^3 [g]	–	Zhang et al. (2009a)
		Lake Waters	Urban and industrial polluted	$\approx 10^6$	between 10^3 and 10^4 [g]	–	
		Lake Sediments	Urban and industrial polluted	$\approx 10^{11}$	between 10^3 and 10^4 [g]	–	
		River Water	DS of a sewage output	≈ 1–6×10^6	≈0.0005–0.005[c]	0.2–2	Lapara et al. (2011)
		River sediment	DS of a sewage output	2.4–$2.5 \times 10^{6**}$	–	0.05	
		Lake water	Far DS of a sewage output	ND	ND	ND	
		Lake sediment		4.9–$7.7 \times 10^{5**}$	–	0.02	
	Estuary	Waters	Industrial polluted	$\approx 10^4$	≈0.001 [c]	0.4	Wright et al. (2008)

	Sediments	Industrial polluted	$\approx 10^{4**}$	≈ 0.0001 [c]	0.04	
WWTP	Raw effluent	CAS	2.04×10^{10}	1.46×10^{5} [g]	–	Zhang et al. (2009a)
	Treated effluent	CAS	1.20×10^{9}	1.48×10^{5} [g]	–	
	Activated sludge	CAS	2.49×10^{12}	1.17×10^{5} [g]	–	
	Raw effluent	CAS	$\approx 10^{11}$ and 10^{12}	$\approx 10^{6}$ [g]	–	Zhang et al. (2009b)
	Treated effluent	CAS	$\approx 10^{9}$	$\approx 10^{5}$ and 10^{6} [g]	–	
			$\approx 10^{7}$	$\approx 10^{4}$ [g]	–	
		After disinfection step	$\approx 10^{6}$	$\approx 10^{3}$ [g]	–	
	Activated sludge		$\approx 10^{9}$	$\approx 10^{2}$–10^{5} [g]	–	
	Digested sludge		$\approx 10^{8}$ and 10^{11}	$\approx 10^{1}$ and 10^{2} [g]	–	
	Treated effluent		$\approx 1.8 \times 10^{7}$	0.009 [c]	3.6	Lapara et al. (2011)
	Sludges		$5.13 \times 10^{9**}$	≈ 0.01 [c]	4	Ma et al. (2011b)
			–	≈ 0.0004–0.0015 [c]	0.16–0.6	Ghosh et al. (2009)
			1.0 and 1.3×10^{12}	≈ 0.01–0.1 [c]	4–40	Diehl and Lapara (2010)
	Digested sludge	QACs + ATB polluted	–	1.01 [e]	1.616	Gaze et al. (2011)
	Treated sludges	limed and dewatered	–	0.56 [e]	0.896	
	Reed bed cores	QACs polluted	–	0.65 [e]	1.04	
GWTP	Biofilter	Raw influent	8.0 and 9.28×10^{4}	287.0 and 309.3 [g]	–	Zhang et al. (2009a)
		Treated effluent	1.29 and 1.39×10^{4}	194.9 and 177.4 [g]	–	
		Biofilms	ND	856.9 and 823 [g]	–	
Soil	Pig slurry	Initial	–	0.0002 [e]	0.00032	Byrne-Bailey et al. (2010)
	amendment	1 day PA	–	0.01 [e]	0.016	
	experiments	21 day PA	–	0.008 [e]	0.0128	
		90 day PA	–	0.003 [e]	0.0048	
		289 day PA	–	0.004 [e]	0.0064	
		1 month PA	–	0.36 [e]	0.576	Gaze et al. (2011)
		12 month PA	–	0.02 [e]	0.032	
		24 month PA	–	0.01 [e]	0.016	
Animal waste	Pig slurry	Antibiotic (tylosin)	–	0.21 [e]	0.336	
		treated pig	–	0.21 [e]	0.336	Byrne-Bailey et al. (2010)

**The relative abundance was calculated using the formula: [(intI/16S) × 4 × 100], with four being the average number of copies of the gene encoding 16S rRNA per bacterial cell, according to the ribosomal RNA database (Klappenbach et al., 2001). **The results are expressed as copies·g^{-1}.*

[a] Represent natural environments without hospital proximity, WWTP, agriculture or animal husbandries facilities or no historical organic amendment practice; [b] impacted environment by urban and/or agricultural activities (sewage/industrial/WWTP/animal husbandries facilities/fishponds/organic amendment); [c] the relative abundance of integron was calculated per 16S rRNA encoding gene (intI/16S); [d] the relative abundance of integron was calculated per percent of bacterial cells [(intI/16S) × 4 × 100]; [e] the relative abundance of integron was calculated per percent of bacterial cells [(intI/16S) × 2.5 × 100]; [f] the relative abundance of integron was calculated per percent of 16S rRNA encoding gene [(intI/16S) × 100]; [g] the relative abundance was calculated by dividing the number of gene per the amount of total extracted DNA; PA, post-application; ND, not detected; GWTP, ground water treatment plant; QACs, quaternary ammonium compounds; ATB, antibiotic; DS, downstream; CAS, conventional activated sludge system; "≈": values have been extracted from graphs.

dissemination (Lupo et al., 2012). Indeed, compared to the "natural" waters previously described, the prevalence of class 1 MIs-containing strains is higher in known polluted waters (**Table 1**). The variation of results observed among studies may depend on many factors, such as the selected bacterial species, the applied culture method (selective or not selective), as well as the sample characteristics (e.g., sediment or water, occurrence of rain events before sampling, close location of a wastewater discharged site). Using metagenomic approaches, urban and agricultural activities were positively associated with class 1 MIs. High concentrations of class 1 MIs were found in a Chinese river located in an urban and agriculturally influenced region, with around 10^7–10^8 copies $\cdot L^{-1}$ and 10^{11} copies $\cdot g^{-1}$ of sediment (Luo et al., 2010), whereas in a clean area, concentrations of class 1 MIs were found to be around 10^4 copies $\cdot L^{-1}$ and 10^3–10^4 copies $\cdot g^{-1}$ of sediments (Wright et al., 2008). Zhang et al. (2009b) found a significant enrichment of class 1 MIs into the Yangtze river along its course through the Nanjing city, highlighting the impact of urban areas on rivers. Also, the relative abundance of class 1 MIs has been strongly correlated with the contribution of treated sewage output flow in the receiving river sediment (Rosewarne et al., 2010). This has been confirmed in a recent study carried out by Lapara et al. (2011), underlining the role of the wastewater treatment plant (WWTP) in the dissemination of class 1 MIs in the environment. Otherwise, fish farming has been shown to significantly elevate the prevalence of class 1 MIs in motile Aeromonads in river waters. The MIs identified contained *dfr* GCs encoding trimethoprim determinants, and their occurrence correlated with the administration of combined sulfonamide/trimethoprim drugs in freshwater fish farms (Schmidt et al., 2001). In polluted estuaries, the prevalence of class 1 MIs appears to be less important than in the aquatic ecosystems previously described, with values ranging between 2.7 and 14.7% (Laroche et al., 2009). Nevertheless, it has been observed that in anthropogenically impacted estuaries the relative abundance was around 10 times more than in an unpolluted reference estuary (Wright et al., 2008; **Table 2**). The authors did not show any influence of the tide, the relative MIs abundance being similar during ebb or flood tides.

Studies involving effluents of factories which produce antibiotics showed that antibiotic production could have an effect on the prevalence of MI-containing bacteria in the receiving river (Li et al., 2009, 2010). In these two studies, the impact differed according to the industry production, although the effluent treatment processes were equivalent in the two industries (anaerobic digestion following by activated sludge process without disinfection step). Indeed, the penicillin production effluents elevated the prevalence of class 1 MIs-harboring strains in the river, from 0% upstream of the discharge to 9.1% after the treated effluent was discharged whereas the oxytetracycline production effluents elevated the MIs prevalence in the river from 3% upstream of the discharge to 86.2% downstream (Li et al., 2009, 2010). Moreover, the authors suggested that some *Pseudomonas* sp. and *Bacillus* sp. isolates harbored simultaneously up to seven different class 1 MIs per bacteria, from the effluent of the oxytetracycline factory, as well as in the receiving river. In comparison, the bacteria from upstream of the WWTP harbored only one class 1 MI. More recently in a metagenomics study, authors observed a 6.7-fold enrichment of class 1 MIs in river sediments downstream of a treated WWTP effluent discharge point from an antibiotic production complex (Kristiansson et al., 2011).

However, the impact of anthropogenic activities is not limited to antibiotic pressure alone, since similar observations have been made in environments without sources of antibiotics input. An Australian study has correlated the rise of the relative abundance of class 1 MIs with environmental parameters (Hardwick et al., 2008). When the environmental conditions were more stressful to the bacteria, the relative abundance of class 1 MIs was higher. Industrial activities (mainly resulting in heavy metal contamination) also have been shown specifically to contribute to the increase of class 1 MIs relative abundance (Wright et al., 2008; Rosewarne et al., 2010). It has been shown that adding tetracycline or cadmium to a water stream in microcosm experiments increased the MIs relative abundance by a factor of between 10- and 100-fold (Wright et al., 2008). The co-selection of resistance genes with heavy metal such as mercury resistance has been previously described (Aminov and Mackie, 2007). Class 1 MIs have been described on the *Tn*21 transposon which also contains a mercury resistance operon (Liebert et al., 1999). Antiseptic agents as QACs have also been shown to be associated with a higher prevalence of class 1 MIs (Gillings et al., 2008c). In QACs contaminated reed bed, it was shown that 95% of the isolated strains with class 1 MIs harbored a *qac* gene (Gaze et al., 2005). Heavy metals and QACs are thus probably involved in MIs dissemination and may have contributed to the MIs selection before the antibiotic era (Stokes and Gillings, 2011).

In anthropogenic-impacted waters, an important diversity of GCs has been recovered (Rosser and Young, 1999; Roe et al., 2003; Henriques et al., 2006; Taviani et al., 2008; Laroche et al., 2009; Li et al., 2009; Ozgumus et al., 2009; Verner-Jeffreys et al., 2009; Kumar et al., 2010; Rosewarne et al., 2010; Chen et al., 2011). Resistance to almost all families of antimicrobials has been recovered with various GCs: *aad*, *aac* (conferring resistance to aminoglycosides); bla_{CARB-2}, bla_{OXA}, bla_{P1} (conferring resistance to beta-lactams); *dfr* (conferring resistance to trimethoprim); *catB* (conferring resistance to chloramphenicol); *ereA* (conferring resistance to erythromycin); *arr* (conferring resistance to rifampicin); and *qac* (conferring resistance to QACs). Moreover, GCs with unknown function have been also commonly found. Several studies have characterized the total pool of integron GCs from environmental samples by using a PCR approach targeting only the GCs (*attC* sites) and not the integrase genes. They showed a huge GCs diversity mostly encoding unknown functions, and underlined the effect of both environmental and anthropogenic conditions on the GCs pool composition (Koenig et al., 2008, 2009, 2011; Huang et al., 2009; Elsaied et al., 2011). Anthropogenic activity thus increases the prevalence of class 1 MIs in microbial communities. These anthropogenic environmental changes result in an increase in transferable genetic elements potentially harboring resistance genes, and an ability to capture new resistance genes from autochthonous hosts. Antibiotic-resistance genes located in mobile genetic elements (plasmids, transposons, integrons) have been suggested to be "genetic pollutants" representative of human activities (Martinez, 2009a). Moreover, anthropogenic stresses has been suggested to facilitate the possible transfer of chromosomal

resistance genes to the mobile gene pool accelerating the evolution and the possible spread in human-pathogenic strains (Cattoir et al., 2008; Picão et al., 2008; Martinez, 2009a,b; Rahube and Yost, 2010).

Class 2 MIs are less prevalent than class 1 in polluted waters (0–7.4%), **Table 1**. In a culture-independent method survey, the low relative abundance rate of class 2 MIs from river has been underlined (Luo et al., 2010). These results suggest that their role in aquatic ecosystems is probably minor.

Sewage and wastewater treatment plants

Wastewater treatment plants are the interface between human waste and both the aquatic and soil environments (**Figure 2**). They collect effluents from diverse sources (such as hospital, private household, industries, animal husbandries), which contribute to the final ecosystem of the WWTP. These include the organics, chemicals, and microbiological wastes. Finally the WWTP ecosystem constitutes a "broth" where each element interacts with each other under a physical and chemical constraint resulting mainly in an organic degradation in the aqueous and solid phase. Microorganisms are key to the process resulting in organic and chemical degradation or transformation. The bacterial communities are organized in free biofilm entities (called bacterial flocs), which constitute the total biomass (the sludge). As suggested by many authors, the high bacterial density, due to the nutritional richness, indicates that WWTP are hot spots for horizontal gene transfer (Tennstedt et al., 2003). Moreover, the antibiotics potentially present in the WWTP could select antibiotic-resistant bacteria, as shown for erythromycin (Louvet et al., 2010), thereby enabling the persistence of antibiotic-resistance plasmids. It has been shown that sulfamethoxazole or amoxicillin at sub-inhibitory concentrations in activated sludge improved the stability of the pB10 plasmid in *E. coli* (Merlin et al., 2011). This co-existence of bacteria and antibiotics in WWTP increases the frequency of genetic variations (as recombination events) and the possible emergence of novel mechanisms of resistance (Baquero et al., 2008).

The highest concentrations of class 1 MIs ever described have been recovered from raw effluents with values comprised between

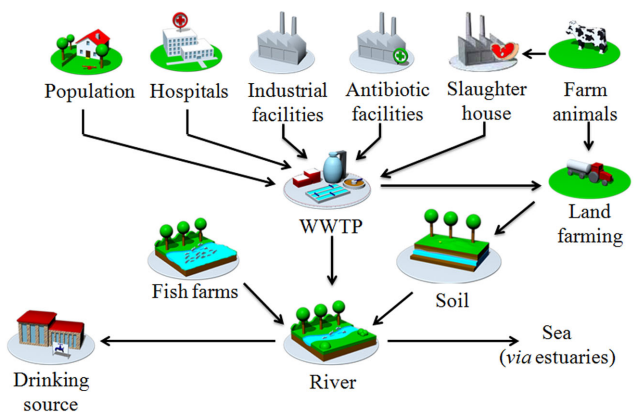


FIGURE 2 | Main route of MIs dissemination from anthropogenic sources to the environment (pictures derived from PILLS project: http://www.pills-project.eu).

10^{10} and 10^{12} copies $\cdot L^{-1}$ (**Table 2**). Class 1 MIs have been described at all stages of the WWTP process with variable prevalence or relative abundance (**Tables 1** and **2**), nevertheless their relative abundance in the final treated effluents highlights the inefficiency of the process to remove bacteria harboring these genetic elements (this will be described below in more details). In activated sludge, 3–61% of the isolated strains harbored a class 1 MI (**Table 1**). By metagenomics approach, the relative abundance of class 1 MIs varied (**Table 2**). These variations could be explained by the different methods of nucleic acids extraction as well as the primers used to detect the MIs. Nevertheless relative abundance up to 40% has been found suggesting that the activated sludge is a hot spot for class 1 MIs selection and/or dissemination. In addition, two studies showed that 12% of isolated plasmids from WWTP sludge carried MIs (Tennstedt et al., 2003; Moura et al., 2007), among which more than half were broad-host-range plasmids displaying very high transfer frequencies (Tennstedt et al., 2003).

As previously described in the aquatic ecosystems, the low prevalence of class 2 MIs in WWTP suggests that their role is probably minor. Although less than 10 publications have reported class 3 MIs, 2 of them have been described in *Delftia* sp. (*D. acidovorans* and *D. tsuruhatensis*) isolated from activated sludge (Xu et al., 2007). These class 3 MIs contained GCs of unknown function. Moreover, using molecular approach, class 3 MIs were detected in effluents from an urban WWTP and a slaughter house WWTP (Moura et al., 2010). These findings suggest that even if class 3 MIs play a minor role in clinical microbiology, their role in the environment is probably more extensive.

The analysis of GCs content from wastewater ecosystems showed a huge diversity of genes encoding antibiotic resistance: resistances to aminoglycosides with *aad*, *aacA* GCs; to beta-lactams with bla_{OXA}, bla_{VIM-2}, bla_{IMP}, bla_{P1}, bla_{GES-5}, and bla_{GES-7} GCs; to trimethoprim with *dfr* gene GCs; to chloramphenicol with *cat* and *cml* GCs; to erythromycin with *ereA* and *estX* GCs; to rifampicin with *arr* GCs; and to quinolones with *qnrVC4* GC (Tennstedt et al., 2003; Ferreira da Silva et al., 2007; Moura et al., 2007, 2012; Taviani et al., 2008; Li et al., 2009, 2010; Pellegrini et al., 2009, 2011; Zhang et al., 2009b; Xia et al., 2010; Girlich et al., 2011; Guo et al., 2011; Ma et al., 2011a; Scotta et al., 2011). A molecular approach describing the global pool of GCs in WWTP have shown a great diversity of GCs, mainly encoding for determinants implied in metabolic functions or unknown functions, suggesting the wide potential reservoir of GCs in WWTP (Moura et al., 2010).

Efficiency of WWTP process to remove MIs

While the WWTP reduced the bacterial load, it appears that the treatment is inefficient to remove both antibiotic resistant bacteria (Novo and Manaia, 2010; Luczkiewicz et al., 2010), and MIs-harboring bacteria.

As presented in **Tables 1** and **2**, the prevalence or relative abundance of MIs after the activated sludge process is not reduced, and is even often higher than in the raw effluent (Ferreira da Silva et al., 2007; Moura et al., 2007; Figueira et al., 2011; Ma et al., 2011a). These authors often concluded that activated process can remove bacteria, but do not reduce significantly the bacteria harboring class 1 MIs. When using abundance normalized to the total

DNA amount, same observation have been done (Zhang et al., 2009a), however in another study, these authors found that the effluent treatment process decreased the MIs rate (Zhang et al., 2009b). Nevertheless, normalization to DNA amount is critical as total community DNA usually contains DNA of non-bacterial origin. The removal of bacteria bearing antibiotic-resistance genetics elements by the WWTP is a new challenge for the future. Several studies have investigated the efficiency of different advanced processes such as UV treatment, membrane biological reactors, and chlorination, to remove bacteria carrying antibiotic-resistance genes (Auerbach et al., 2007; Garcia et al., 2007; Kim et al., 2010; Huang et al., 2011; Munir et al., 2011), but no studies have examined the effects on MIs. Recently, hospital effluents were shown to be potential sources of dissemination of MIs in the sewage network (Guo et al., 2011). Oberlé et al. (2012) noted a decrease of the prevalence of class 1 MIs in *E. coli* along the effluents treatment from the hospital to the WWTP and the receiving river continuum. As treatment of hospital effluents onsite is a growing and controversial question (Pauwels and Verstraete, 2006; Kümmerer, 2008; Ort et al., 2010; Escher et al., 2011), these data need confirmation by further studies in order to assess the impact of these specific effluents on the release of MIs in the WWTP.

Activated sludges are often used in agriculture as organic amendment (**Figure 2**). However, before their use, treatments in order to reduce their volume and improve stability are applied. Several studies have specifically investigated the potential of aerobic and anaerobic treatments to reduce class 1 MIs in activated sludges, demonstrating a better performance of anaerobic thermophilic process (50–60°C) to decrease the relative abundance of class 1 MIs (Ghosh et al., 2009; Diehl and Lapara, 2010). However, dissimilar results have been obtained in same conditions by Ma et al. (2011b), suggesting that other factors may influence the MIs occurrence during the sludge digestion. Evidence of horizontal gene transfers in WWTP sludge has been shown by Merlin et al. (2011). They have shown that, horizontal transfer of pB10 plasmid occurred in sludge from the anaerobic digesters or from fixed biofilm reactors, with higher efficiency in fixed biofilm conditions.

Soil ecosystem and the animal wastes as sources of MIs

While the soil "resistome" is a vast original reservoir of resistance genes (D'Costa et al., 2006; Allen et al., 2010), manure has been shown to significantly increase the mobile genetic resistance elements pool (Heuer et al., 2011). Recent studies have highlighted the role of the amendment practice on the input of MIs in soil (Heuer and Smalla, 2007; Binh et al., 2009; Byrne-Bailey et al., 2010; Gaze et al., 2011), see **Table 2**. Moreover, studies on sewage sludge and pig slurry amendment showed that even if the prevalence of class 1 MIs decreased after the particular amendment (2 years and 10 months, respectively), the prevalence was still higher than in control soils without amendment (around 100 times more; Byrne-Bailey et al., 2010; Gaze et al., 2011). Some authors studied the GCs array of class 1 MIs introduced in soil *via* manure amendment and mainly found streptomycin and spectinomycin resistance *aadA* GCs (Heuer and Smalla, 2007; Binh et al., 2009). Class 2 MIs have been also identified from amended soils with relative high rates (Byrne-Bailey et al., 2009, 2010; Rodríguez-Minguela et al., 2009). The high antibiotics consumption in some animal husbandries, and their systemic application as food additives in the past, had probably significantly contributed to MIs dissemination in amended soils. Tschäpe (1994) showed that the streptothricin usage as food additive contributed to the dissemination of *sat* genes in amended soils *via* mobile genetic structures, such as the *Tn7* transposon carrying a class 2 MI usually bearing a streptothricin-resistant *sat2* GC.

Animal wastes (e.g., manure, poultry litter, and slurry) are the main vectors of MIs dissemination in soil. As recently reviewed by Heuer et al. (2011), only a few studies have investigated the reduction of some resistance genes following different processes, such as storage, composting, and anaerobic digestion (Chen et al., 2007, 2010; Heuer et al., 2008). Only composting was efficient in reducing the prevalence and absolute amount of erythromycin resistance genes (Chen et al., 2007). Concerning the MIs, one study reported that after 57 days of storage of manure at 20°C, the class 1 MIs GCs array electrophoresis gel profiles were almost identical to that at the beginning of the experiment; however the GCs contents was not investigated (Heuer et al., 2008).

ROLE OF THE FOOD CHAIN

The food chain probably also takes place in the transit of MIs from the environments to the human. Indeed, bacteria harboring MIs have been recovered from a variety of aquatic living organisms, such as in prawns, with an *Enterobacter cloacae* harboring a class 1 MI (Gillings et al., 2009b); or in *Corbicula* with a class 1 MIs relative abundance of 4% (Wright et al., 2008); and in oysters where the uncommon class 3 MIs prevailed (Barkovskii et al., 2010). Transfers of MIs between animals and human occur and have been well reviewed by Stokes and Gillings (2011). Class 1 MIs have been also reported from biofilms of drinking water supplies (**Tables 1** and **2**; Gillings et al., 2008a; Zhang et al., 2009a). All these results underline the link, *via* the food chain, between the environmental MIs and the human or animal MIs.

CONCLUSION

As described in this review, MIs are efficient tools for bacterial adaptation and play a significant role in antibiotic resistance. Environmental studies demonstrated that anthropogenic impact lead to enrichment of class 1 MIs. More specifically, all factors leading to bacterial stress, such as antibiotics, QACs, or high concentrations of heavy metals resulted in the selection of class 1 MIs-harboring bacteria. Several hot spots of class 1 MIs dissemination have been identified, as agricultural manure amendment, WWTP, or industrial effluents. While these wastes are treated in varying degrees before their discharge, it appears that the current processes are inefficient to reduce MIs dissemination. This uncontrolled dissemination of MIs in the environment could represent a risk for human health.

ACKNOWLEDGMENTS

This work was supported by the regional council of Limousin. The authors wish to thank Colin Hunter and William Rawlinson for their help in critical reading of the manuscript.

REFERENCES

Agerso, Y., and Sandvang, D. (2005). Class 1 integrons and tetracycline resistance genes in *Alcaligenes*, *Arthrobacter*, and *Pseudomonas* spp. isolated from pigsties and manured soil. *Appl. Environ. Microbiol.* 71, 7941–7947.

Ahmed, A. M., Nakano, H., and Shimamoto, T. (2005). Molecular characterization of integrons in nontyphoid *Salmonella* serovars isolated in Japan: description of an unusual class 2 integron. *J. Antimicrob. Chemother.* 55, 371–374.

Allen, H. K., Donato, J., Wang, H. H., Cloud-Hansen, K. A., Davies, J., and Handelsman, J. (2010). Call of the wild: antibiotic resistance genes in natural environments. *Nat. Rev. Microbiol.* 8, 251–259.

Amann, R. I., Ludwig, W., and Schleifer, K. H. (1995). Phylogenetic identification and in situ detection of individual microbial cells without cultivation. *Microbiol. Rev.* 59, 143–169.

Aminov, R. I. (2010). A brief history of the antibiotic era: lessons learned and challenges for the future. *Front. Microbiol.* 1:134. doi:10.3389/fmicb.2010.00134

Aminov, R. I. (2011). Horizontal gene exchange in environmental microbiota. *Front. Microbiol.* 2:158. doi:10.3389/fmicb.2011.00158

Aminov, R. I., and Mackie, R. I. (2007). Evolution and ecology of antibiotic resistance genes. *FEMS Microbiol. Lett.* 271, 147–161.

Auerbach, E. A., Seyfried, E. E., and McMahon, K. D. (2007). Tetracycline resistance genes in activated sludge wastewater treatment plants. *Water Res.* 41, 1143–1151.

Baharoglu, Z., Bikard, D., and Mazel, D. (2010). Conjugative DNA transfer induces the bacterial SOS response and promotes antibiotic resistance development through integron activation. *PLoS Genet.* 6, e1001165. doi:10.1371/journal.pgen.1001165

Baquero, F., Martínez, J. L., and Cantón, R. (2008). Antibiotics and antibiotic resistance in water environments. *Curr. Opin. Biotechnol.* 19, 260–265.

Barkovskii, A. L., Green, C., and Hurley, D. (2010). The occurrence, spatial and temporal distribution, and environmental routes of tetracycline resistance and integrase genes in *Crassostrea virginica* beds. *Mar. Pollut. Bull.* 60, 2215–2224.

Barlow, R. S., Fegan, N., and Gobius, K. S. (2009). Integron-containing bacteria in faeces of cattle from different production systems at slaughter. *J. Appl. Microbiol.* 107, 540–545.

Barlow, R. S., and Gobius, K. S. (2006). Diverse class 2 integrons in bacteria from beef cattle sources. *J. Antimicrob. Chemother.* 58, 1133–1138.

Barraud, O., Badell, E., Denis, F., Guiso, N., and Ploy, M.-C. (2011). Antimicrobial drug resistance in *Corynebacterium diphtheriae* mitis. *Emerging Infect. Dis.* 17, 2078–2080.

Barraud, O., and Ploy, M.-C. (2011). Actualités sur les intégrons de résistance aux antibiotiques: mise au point. *J. Anti-infectieux* 13, 133–144.

Bass, L., Liebert, C. A., Lee, M. D., Summers, A. O., White, D. G., Thayer, S. G., and Maurer, J. J. (1999). Incidence and characterization of integrons, genetic elements mediating multiple-drug resistance, in avian Escherichia coli. *Antimicrob. Agents Chemother.* 43, 2925–2929.

Bertels, F., and Rainey, P. B. (2011). Within-genome evolution of REPINs: a new family of miniature mobile DNA in bacteria. *PLoS Genet.* 7, e1002132. doi:10.1371/journal.pgen.1002132

Betteridge, T., Partridge, S. R., Iredell, J. R., and Stokes, H. W. (2011). Genetic context and structural diversity of class 1 integrons from human commensal bacteria in a hospital intensive care unit. *Antimicrob. Agents Chemother.* 55, 3939–3943.

Binh, C. T. T., Heuer, H., Kaupenjohann, M., and Smalla, K. (2009). Diverse aadA gene cassettes on class 1 integrons introduced into soil via spread manure. *Res. Microbiol.* 160, 427–433.

Biskri, L., and Mazel, D. (2003). Erythromycin esterase gene ere(A) is located in a functional gene cassette in an unusual class 2 integron. *Antimicrob. Agents Chemother.* 47, 3326–3331.

Biyela, P. T., Lin, J., and Bezuidenhout, C. C. (2004). The role of aquatic ecosystems as reservoirs of antibiotic resistant bacteria and antibiotic resistance genes. *Water Sci. Technol.* 50, 45–50.

Boucher, Y., Labbate, M., Koenig, J. E., and Stokes, H. W. (2007). Integrons: mobilizable platforms that promote genetic diversity in bacteria. *Trends Microbiol.* 15, 301–309.

Byrne-Bailey, K. G., Gaze, W. H., Kay, P., Boxall, A. B. A., Hawkey, P. M., and Wellington, E. M. H. (2009). Prevalence of sulfonamide resistance genes in bacterial isolates from manured agricultural soils and pig slurry in the United Kingdom. *Antimicrob. Agents Chemother.* 53, 696–702.

Byrne-Bailey, K. G., Gaze, W. H., Zhang, L., Kay, P., Boxall, A., Hawkey, P. M., and Wellington, E. M. H. (2010). Integron prevalence and diversity in manured soil. *Appl. Environ. Microbiol.* 77, 684–687.

Cambray, G., Guerout, A.-M., and Mazel, D. (2010). Integrons. *Annu. Rev. Genet.* 44, 141–166.

Cambray, G., Sanchez-Alberola, N., Campoy, S., Guerin, E., Da Re, S., Gonzalez-Zorn, B., Ploy, M.-C., Barbe, J., Mazel, D., and Erill, I. (2011). Prevalence of SOS-mediated control of integron integrase expression as an adaptive trait of chromosomal and mobile integrons. *Mob. DNA* 2, 6.

Cattoir, V., Poirel, L., Aubert, C., Soussy, C.-J., and Nordmann, P. (2008). Unexpected occurrence of plasmid-mediated quinolone resistance determinants in environmental *Aeromonas* spp. *Emerging Infect. Dis.* 14, 231–237.

Chen, B., Zheng, W., Yu, Y., Huang, W., Zheng, S., Zhang, Y., Guan, X., Zhuang, Y., Chen, N., and Topp, E. (2011). Class 1 integrons, selected virulence genes, and antibiotic resistance in *Escherichia coli* isolates from the Minjiang River, Fujian Province, China. *Appl. Environ. Microbiol.* 77, 148–155.

Chen, J., Michel, F. C. Jr, Sreevatsan, S., Morrison, M., and Yu, Z. (2010). Occurrence and persistence of erythromycin resistance genes (erm) and tetracycline resistance genes (tet) in waste treatment systems on swine farms. *Microb. Ecol.* 60, 479–486.

Chen, J., Yu, Z., Michel, F. C., Jr, Wittum, T., and Morrison, M. (2007). Development and application of real-time PCR assays for quantification of erm genes conferring resistance to macrolides-lincosamides-streptogramin B in livestock manure and manure management systems. *Appl. Environ. Microbiol.* 73, 4407–4416.

Collis, C. M., Grammaticopoulos, G., Briton, J., Stokes, H. W., and Hall, R. M. (1993). Site-specific insertion of gene cassettes into integrons. *Mol. Microbiol.* 9, 41–52.

Collis, C. M., and Hall, R. M. (1992). Gene cassettes from the insert region of integrons are excised as covalently closed circles. *Mol. Microbiol.* 6, 2875–2885.

Collis, C. M., and Hall, R. M. (2004). Comparison of the structure-activity relationships of the integron-associated recombination sites attI3 and attI1 reveals common features. *Microbiology* 150, 1591–1601.

Daikos, G. L., Kosmidis, C., Tassios, P. T., Petrikkos, G., Vasilakopoulou, A., Psychogiou, M., Stefanou, I., Avlami, A., and Katsilambros, N. (2007). Enterobacteriaceae Bloodstream infections: presence of integrons, risk factors, and outcome. *Antimicrob. Agents Chemother.* 51, 2366–2372.

Davies, J., and Davies, D. (2010). Origins and evolution of antibiotic resistance. *Microbiol. Mol. Biol. Rev.* 74, 417–433.

D'Costa, V. M., McGrann, K. M., Hughes, D. W., and Wright, G. D. (2006). Sampling the antibiotic resistome. *Science* 311, 374–377.

Díaz-Mejía, J. J., Amábile-Cuevas, C. F., Rosas, I., and Souza, V. (2008). An analysis of the evolutionary relationships of integron integrases, with emphasis on the prevalence of class 1 integrons in *Escherichia coli* isolates from clinical and environmental origins. *Microbiology* 154, 94–102.

Diehl, D. L., and Lapara, T. M. (2010). Effect of temperature on the fate of genes encoding tetracycline resistance and the integrase of class 1 integrons within anaerobic and aerobic digesters treating municipal wastewater solids. *Environ. Sci. Technol.* 44, 9128–9133.

Dolejská, M., Bierosová, B., Kohoutová, L., Literák, I., and Cízek, A. (2009). Antibiotic-resistant i and *Escherichia coli* isolates with integrons and extended-spectrum beta-lactamases in surface water and sympatric black-headed gulls. *J. Appl. Microbiol.* 106, 1941–1950.

Elsaied, H., Stokes, H. W., Kitamura, K., Kurusu, Y., Kamagata, Y., and Maruyama, A. (2011). Marine integrons containing novel integrase genes, attachment sites, attI, and associated gene cassettes in polluted sediments from Suez and Tokyo Bays. *ISME J.* 5, 1162–1177.

Escher, B. I., Baumgartner, R., Koller, M., Treyer, K., Lienert, J., and McArdell, C. S. (2011). Environmental toxicology and risk assessment of pharmaceuticals from hospital wastewater. *Water Res.* 45, 75–92.

Ferreira da Silva, M., Vaz-Moreira, I., Gonzalez-Pajuelo, M., Nunes, O. C., and Manaia, C. M. (2007). Antimicrobial resistance patterns in Enterobacteriaceae isolated from an urban wastewater treatment plant. *FEMS Microbiol. Ecol.* 60, 166–176.

Figueira, V., Serra, E., and Manaia, C. M. (2011). Differential patterns of antimicrobial resistance in population subsets of *Escherichia coli* isolated from waste- and surface waters. *Sci. Total Environ.* 409, 1017–1023.

Garcia, S., Wade, B., Bauer, C., Craig, C., Nakaoka, K., and Lorowitz, W. (2007). The effect of wastewater treatment on antibiotic resistance in *Escherichia coli* and *Enterococcus* sp. *Water Environ. Res.* 79, 2387–2395.

Gassama Sow, A., Aïdara-Kane, A., Barraud, O., Gatet, M., Denis, F., and Ploy, M. C. (2010). High prevalence of trimethoprim-resistance cassettes in class 1 and 2 integrons in Senegalese *Shigella* spp isolates. *J. Infect. Dev. Ctries.* 4, 207–212.

Gaze, W. H., Abdouslam, N., Hawkey, P. M., and Wellington, E. M. H. (2005). Incidence of class 1 integrons in a quaternary ammonium compound-polluted environment. *Antimicrob. Agents Chemother.* 49, 1802–1807.

Gaze, W. H., Zhang, L., Abdouslam, N. A., Hawkey, P. M., Calvo-Bado, L., Royle, J., Brown, H., Davis, S., Kay, P., Boxall, A. B. A., and Wellington, E. M. (2011). Impacts of anthropogenic activity on the ecology of class 1 integrons and integron-associated genes in the environment. *ISME J.* 5, 1253–1261.

Ghosh, S., Ramsden, S. J., and LaPara, T. M. (2009). The role of anaerobic digestion in controlling the release of tetracycline resistance genes and class 1 integrons from municipal wastewater treatment plants. *Appl. Microbiol. Biotechnol.* 84, 791–796.

Gillings, M. R., Boucher, Y., Labbate, M., Holmes, A., Krishnan, S., Holley, M., and Stokes, H. W. (2008a). The evolution of class 1 integrons and the rise of antibiotic resistance. *J. Bacteriol.* 190, 5095–5100.

Gillings, M. R., Krishnan, S., Worden, P. J., and Hardwick, S. A. (2008b). Recovery of diverse genes for class 1 integron-integrases from environmental DNA samples. *FEMS Microbiol. Lett.* 287, 56–62.

Gillings, M. R., Xuejun, D., Hardwick, S. A., Holley, M. P., and Stokes, H. W. (2008c). Gene cassettes encoding resistance to quaternary ammonium compounds: a role in the origin of clinical class 1 integrons? *ISME J.* 3, 209–215.

Gillings, M. R., Holley, M. P., and Stokes, H. W. (2009a). Evidence for dynamic exchange of qac gene cassettes between class 1 integrons and other integrons in freshwater biofilms. *FEMS Microbiol. Lett.* 296, 282–288.

Gillings, M. R., Labbate, M., Sajjad, A., Giguère, N. J., Holley, M. P., and Stokes, H. W. (2009b). Mobilization of a Tn402-like class 1 integron with a novel cassette array via flanking miniature inverted-repeat transposable element-like structures. *Appl. Environ. Microbiol.* 75, 6002–6004.

Girlich, D., Poirel, L., Szczepanowski, R., Schlüter, A., and Nordmann, P. (2011). Carbapenem-hydrolyzing GES-5-encoding gene on different plasmid types from a sewage treatment plant. *Appl. Environ. Microbiol.* 78, 1292–1295.

Guerin, E., Cambray, G., Sanchez-Alberola, N., Campoy, S., Erill, I., Da Re, S., Gonzalez-Zorn, B., Barbé, J., Ploy, M.-C., and Mazel, D. (2009). The SOS response controls integron recombination. *Science* 324, 1034.

Guo, X., Xia, R., Han, N., and Xu, H. (2011). Genetic diversity analyses of class 1 integrons and their associated antimicrobial resistance genes in Enterobacteriaceae strains recovered from aquatic habitats in China. *Lett. Appl. Microbiol.* 52, 667–675.

Hall, R. M., and Collis, C. M. (1995). Mobile gene cassettes and integrons: capture and spread of genes by site-specific recombination. *Mol. Microbiol.* 15, 593–600.

Hansson, K., Sundström, L., Pelletier, A., and Roy, P. H. (2002). IntI2 integron integrase in Tn7. *J. Bacteriol.* 184, 1712–1721.

Hardwick, S. A., Stokes, H. W., Findlay, S., Taylor, M., and Gillings, M. R. (2008). Quantification of class 1 integron abundance in natural environments using real-time quantitative PCR. *FEMS Microbiol. Lett.* 278, 207–212.

Henriques, I. S., Fonseca, F., Alves, A., Saavedra, M. J., and Correia, A. (2006). Occurrence and diversity of integrons and beta-lactamase genes among ampicillin-resistant isolates from estuarine waters. *Res. Microbiol.* 157, 938–947.

Heringa, S., Kim, J., Shepherd, M. W. Jr, Singh, R., and Jiang, X. (2010). The presence of antibiotic resistance and integrons in Escherichia coli isolated from compost. *Foodborne Pathog. Dis.* 7, 1297–1304.

Heuer, H., Focks, A., Lamshöft, M., Smalla, K., Matthies, M., and Spiteller, M. (2008). Fate of sulfadiazine administered to pigs and its quantitative effect on the dynamics of bacterial resistance genes in manure and manured soil. *Soil Biol. Biochem.* 40, 1892–1900.

Heuer, H., Schmitt, H., and Smalla, K. (2011). Antibiotic resistance gene spread due to manure application on agricultural fields. *Curr. Opin. Microbiol.* 14, 236–243.

Heuer, H., and Smalla, K. (2007). Manure and sulfadiazine synergistically increased bacterial antibiotic resistance in soil over at least two months. *Environ. Microbiol.* 9, 657–666.

Hochhut, B., Lotfi, Y., Mazel, D., Faruque, S. M., Woodgate, R., and Waldor, M. K. (2001). Molecular analysis of antibiotic resistance gene clusters in *Vibrio cholera* O139 and O1 SXT constins. *Antimicrob. Agents Chemother.* 45, 2991–3000.

Hocquet, D., Llanes, C., Thouverez, M., Kulasekara, H., Legrand, F., Bertrand, X., Plésiat, P., Mazel, D., and Miller, S. (2011). "A *Pseudomonas aeruginosa* clinical isolate with antibiotic resistance promoted by the SOS response in a patient," in *ASM 111th General Meeting*, New Orleans, LA.

Hoyle, D. V., Davison, H. C., Knight, H. I., Yates, C. M., Dobay, O., Gunn, G. J., Amyes, S. G. B., and Woolhouse, M. E. J. (2006). Molecular characterisation of bovine faecal *Escherichia coli* shows persistence of defined ampicillin resistant strains and the presence of class 1 integrons on an organic beef farm. *Vet. Microbiol.* 115, 250–257.

Huang, J.-J., Hu, H.-Y., Tang, F., Li, Y., Lu, S.-Q., and Lu, Y. (2011). Inactivation and reactivation of antibiotic-resistant bacteria by chlorination in secondary effluents of a municipal wastewater treatment plant. *Water Res.* 45, 2775–2781.

Huang, L., Cagnon, C., Caumette, P., and Duran, R. (2009). First gene cassettes of integrons as targets in finding adaptive genes in metagenomes. *Appl. Environ. Microbiol.* 75, 3823–3825.

Kim, S., Park, H., and Chandran, K. (2010). Propensity of activated sludge to amplify or attenuate tetracycline resistance genes and tetracycline resistant bacteria: a mathematical modeling approach. *Chemosphere* 78, 1071–1077.

Koczura, R., Mokracka, J., Jabłońska, L., Gozdecka, E., Kubek, M., and Kaznowski, A. (2012). Antimicrobial resistance of integron-harboring *Escherichia coli* isolates from clinical samples, wastewater treatment plant and river water. *Sci. Total Environ.* 414, 680–685.

Klappenbach, J. A., Saxman, P. R., Cole, J. R., and Schmidt, T. M. (2001). rrndb: the Ribosomal RNA operon copy number database. *Nucleic Acids Res.* 29, 181–184.

Koenig, J. E., Boucher, Y., Charlebois, R. L., Nesbø, C., Zhaxybayeva, O., Bapteste, E., Spencer, M., Joss, M. J., Stokes, H. W., and Doolittle, W. F. (2008). Integron-associated gene cassettes in Halifax Harbour: assessment of a mobile gene pool in marine sediments. *Environ. Microbiol.* 10, 1024–1038.

Koenig, J. E., Bourne, D. G., Curtis, B., Dlutek, M., Stokes, H. W., Doolittle, W. F., and Boucher, Y. (2011). Coral-mucus-associated *Vibrio* integrons in the Great Barrier Reef: genomic hotspots for environmental adaptation. *ISME J.* 5, 962–972.

Koenig, J. E., Sharp, C., Dlutek, M., Curtis, B., Joss, M., Boucher, Y., and Doolittle, W. F. (2009). Integron gene cassettes and degradation of compounds associated with industrial waste: the case of the Sydney Tar Ponds. *PLoS ONE* 4, e5276. doi:10.1371/journal.pone.0005276

Kristiansson, E., Fick, J., Janzon, A., Grabic, R., Rutgersson, C., Weijdegård, B., Söderström, H., and Larsson, D. G. J. (2011). Pyrosequencing of antibiotic-contaminated river sediments reveals high levels of resistance and gene transfer elements. *PLoS ONE* 6, e17038. doi:10.1371/journal.pone.0017038

Kumar, A., Mukherjee, S., and Chakraborty, R. (2010). Characterization of a novel trimethoprim resistance gene, dfrA28, in class 1 integron of an oligotrophic *Acinetobacter johnsonii* strain, MB52, isolated from River Mahananda, India. *Microb. Drug Resist.* 16, 29–37.

Kümmerer, K. (2008). *Pharmaceuticals in the Environment Sources, Fate, Effects, and Risks*, 3rd Edn. Heidelberg: Springer.

Labbate, M., Case, R. J., and Stokes, H. W. (2009). The integron/gene cassette system: an active player in bacterial adaptation. *Methods Mol. Biol.* 532, 103–125.

Lapara, T. M., Burch, T. R., McNamara, P. J., Tan, D. T., Yan, M., and Eichmiller, J. J. (2011). Tertiary-treated municipal wastewater is a significant point source of antibiotic resistance genes into Duluth-Superior Harbor. *Environ. Sci. Technol.* 45, 9543–9549.

Laroche, E., Pawlak, B., Berthe, T., Skurnik, D., and Petit, F. (2009). Occurrence of antibiotic resistance and class 1, 2 and 3 integrons in *Escherichia coli* isolated from a densely populated estuary (Seine, France). *FEMS Microbiol. Ecol.* 68, 118–130.

Laroche, E., Petit, F., Fournier, M., and Pawlak, B. (2010). Transport of antibiotic-resistant *Escherichia coli* in a public rural karst water supply. *J. Hydrol.* 392, 12–21.

Leverstein-van Hall, M. A., Blok, H. E. M., Donders, A. R. T., Paauw, A.,

Fluit, A. C., and Verhoef, J. (2003). Multidrug resistance among Enterobacteriaceae is strongly associated with the presence of integrons and is independent of species or isolate origin. *J. Infect. Dis.* 187, 251–259.

Li, D., Yang, M., Hu, J., Zhang, J., Liu, R., Gu, X., Zhang, Y., and Wang, Z. (2009). Antibiotic-resistance profile in environmental bacteria isolated from penicillin production wastewater treatment plant and the receiving river. *Environ. Microbiol.* 11, 1506–1517.

Li, D., Yu, T., Zhang, Y., Yang, M., Li, Z., Liu, M., and Qi, R. (2010). Antibiotic resistance characteristics of environmental bacteria from an oxytetracycline production wastewater treatment plant and the receiving river. *Appl. Environ. Microbiol.* 76, 3444–3451.

Liebert, C. A., Hall, R. M., and Summers, A. O. (1999). Transposon Tn21, flagship of the floating genome. *Microbiol. Mol. Biol. Rev.* 63, 507–522.

Louvet, J. N., Giammarino, C., Potier, O., and Pons, M. N. (2010). Adverse effects of erythromycin on the structure and chemistry of activated sludge. *Environ. Pollut.* 158, 688–693.

Luczkiewicz, A., Jankowska, K., Fudala-Ksiazek, S., and Olanczuk-Neyman, K. (2010). Antimicrobial resistance of fecal indicators in municipal wastewater treatment plant. *Water Res.* 44, 5089–5097.

Luo, Y., Mao, D., Rysz, M., Zhou, Q., Zhang, H., Xu, L., and Alvarez, P. J. J. (2010). Trends in antibiotic resistance genes occurrence in the Haihe River, China. *Environ. Sci. Technol.* 44, 7220–7225.

Lupo, A., Coyne, S., and Berendonk, T. U. (2012). Origin and evolution of antibiotic resistance: the common mechanisms of emergence and spread in water bodies. *Front. Microbiol.* 3:18. doi:10.3389/fmicb.2012.00018

Ma, L., Zhang, X.-X., Cheng, S., Zhang, Z., Shi, P., Liu, B., Wu, B., and Zhang, Y. (2011a). Occurrence, abundance and elimination of class 1 integrons in one municipal sewage treatment plant. *Ecotoxicology* 20, 968–973.

Ma, Y., Wilson, C. A., Novak, J. T., Riffat, R., Aynur, S., Murthy, S., and Pruden, A. (2011b). Effect of various sludge digestion conditions on sulfonamide, macrolide, and tetracycline resistance genes and class I integrons. *Environ. Sci. Technol.* 45, 7855–7861.

Márquez, C., Labbate, M., Ingold, A. J., Roy Chowdhury, P., Ramírez, M. S., Centrón, D., Borthagaray, G., and Stokes, H. W. (2008). Recovery of a functional class 2 integron from an *Escherichia coli* strain mediating a urinary tract infection. *Antimicrob. Agents Chemother.* 52, 4153–4154.

Martin, C., Timm, J., Rauzier, J., Gomez-Lus, R., Davies, J., and Gicquel, B. (1990). Transposition of an antibiotic resistance element in mycobacteria. *Nature* 345, 739–743.

Martínez, J. L. (2008). Antibiotics and antibiotic resistance genes in natural environments. *Science* 321, 365–367.

Martinez, J. L. (2009a). Environmental pollution by antibiotics and by antibiotic resistance determinants. *Environ. Pollut.* 157, 2893–2902.

Martinez, J. L. (2009b). The role of natural environments in the evolution of resistance traits in pathogenic bacteria. *Proc. Biol. Sci.* 276, 2521–2530.

Mazel, D. (2006). Integrons: agents of bacterial evolution. *Nat. Rev. Microbiol.* 4, 608–620.

Merlin, C., Bonot, S., Courtois, S., and Block, J.-C. (2011). Persistence and dissemination of the multiple-antibiotic-resistance plasmid pB10 in the microbial communities of wastewater sludge microcosms. *Water Res.* 45, 2897–2905.

Moura, A., Henriques, I., Ribeiro, R., and Correia, A. (2007). Prevalence and characterization of integrons from bacteria isolated from a slaughterhouse wastewater treatment plant. *J. Antimicrob. Chemother.* 60, 1243–1250.

Moura, A., Henriques, I., Smalla, K., and Correia, A. (2010). Wastewater bacterial communities bring together broad-host range plasmids, integrons and a wide diversity of uncharacterized gene cassettes. *Res. Microbiol.* 161, 58–66.

Moura, A., Pereira, C., Henriques, I., and Correia, A. (2012). Novel gene cassettes and integrons in antibiotic-resistant bacteria isolated from urban wastewaters. *Res. Microbiol.* 163, 92–100.

Munir, M., Wong, K., and Xagoraraki, I. (2011). Release of antibiotic resistant bacteria and genes in the effluent and biosolids of five wastewater utilities in Michigan. *Water Res.* 45, 681–693.

Naas, T., Mikami, Y., Imai, T., Poirel, L., and Nordmann, P. (2001). Characterization of In53, a class 1 plasmid- and composite transposon-located integron of *Escherichia coli* which carries an unusual array of gene cassettes. *J. Bacteriol.* 183, 235–249.

Nandi, S., Maurer, J. J., Hofacre, C., and Summers, A. O. (2004). Gram-positive bacteria are a major reservoir of class 1 antibiotic resistance integrons in poultry litter. *Proc. Natl. Acad. Sci. U.S.A.* 101, 7118–7122.

Nesvera, J., Hochmannová, J., and Pátek, M. (1998). An integron of class 1 is present on the plasmid pCG4 from Gram-positive bacterium *Corynebacterium glutamicum*. *FEMS Microbiol. Lett.* 169, 391–395.

Nijssen, S., Florijn, A., Top, J., Willems, R., Fluit, A., and Bonten, M. (2005). Unnoticed spread of integron-carrying Enterobacteriaceae in intensive care units. *Clin. Infect. Dis.* 41, 1–9.

Novo, A., and Manaia, C. (2010). Factors influencing antibiotic resistance burden in municipal wastewater treatment plants. *Appl. Microbiol. Biotechnol.* 87, 1157–1166.

Nunes-Düby, S. E., Kwon, H. J., Tirumalai, R. S., Ellenberger, T., and Landy, A. (1998). Similarities and differences among 105 members of the Int family of site-specific recombinases. *Nucleic Acids Res.* 26, 391–406.

Oberlé, K., Capdeville, M.-J., Berthe, T., Budzinski, H., and Petit, F. (2012). Evidence for a complex relationship between antibiotics and antibiotic-resistant *Escherichia coli*: from medical center patients to a receiving environment. *Environ. Sci. Technol.* 46, 1859–1868.

Ort, C., Lawrence, M. G., Reungoat, J., Eaglesham, G., Carter, S., and Keller, J. (2010). Determining the fraction of pharmaceutical residues in wastewater originating from a hospital. *Water Res.* 44, 605–615.

Ozgumus, O. B., Sandalli, C., Sevim, A., Celik-Sevim, E., and Sivri, N. (2009). Class 1 and class 2 integrons and plasmid-mediated antibiotic resistance in coliforms isolated from ten rivers in northern Turkey. *J. Microbiol.* 47, 19–27.

Partridge, S. R., Tsafnat, G., Coiera, E., and Iredell, J. R. (2009). Gene cassettes and cassette arrays in mobile resistance integrons. *FEMS Microbiol. Rev.* 33, 757–784.

Paulsen, I. T., Littlejohn, T. G., Rådström, P., Sundström, L., Sköld, O., Swedberg, G., and Skurray, R. A. (1993). The 3′ conserved segment of integrons contains a gene associated with multidrug resistance to antiseptics and disinfectants. *Antimicrob. Agents Chemother.* 37, 761–768.

Pauwels, B., and Verstraete, W. (2006). The treatment of hospital wastewater: an appraisal. *J. Water Health* 4, 405–416.

Pellegrini, C., Celenza, G., Segatore, B., Bellio, P., Setacci, D., Amicosante, G., and Perilli, M. (2011). Occurrence of class 1 and 2 integrons in resistant Enterobacteriaceae collected from a urban wastewater treatment plant: first report from central Italy. *Microb. Drug Resist.* 17, 229–234.

Pellegrini, C., Mercuri, P. S., Celenza, G., Galleni, M., Segatore, B., Sacchetti, E., Volpe, R., Amicosante, G., and Perilli, M. (2009). Identification of bla(IMP-22) in *Pseudomonas* spp. in urban wastewater and nosocomial environments: biochemical characterization of a new IMP metallo-enzyme variant and its genetic location. *J. Antimicrob. Chemother.* 63, 901–908.

Petrova, M., Gorlenko, Z., and Mindlin, S. (2011). Tn5045, a novel integron-containing antibiotic and chromate resistance transposon isolated from a permafrost bacterium. *Res. Microbiol.* 162, 337–345.

Picão, R. C., Poirel, L., Demarta, A., Silva, C. S. F., Corvaglia, A. R., Petrini, O., and Nordmann, P. (2008). Plasmid-mediated quinolone resistance in *Aeromonas allosaccharophila* recovered from a Swiss lake. *J. Antimicrob. Chemother.* 62, 948–950.

Rahube, T. O., and Yost, C. K. (2010). Antibiotic resistance plasmids in wastewater treatment plants and their possible dissemination into the environment. *Afr. J. Biotechnol.* 9, 9183–9190.

Ramírez, M. S., Piñeiro, S., and Centrón, D. (2010). Novel insights about class 2 integrons from experimental and genomic epidemiology. *Antimicrob. Agents Chemother.* 54, 699–706.

Ramírez, M. S., Quiroga, C., and Centrón, D. (2005). Novel rearrangement of a class 2 integron in two non-epidemiologically related isolates of *Acinetobacter baumannii*. *Antimicrob. Agents Chemother.* 49, 5179–5181.

Rodríguez-Minguela, C. M., Apajalahti, J. H. A., Chai, B., Cole, J. R., and Tiedje, J. M. (2009). Worldwide prevalence of class 2 integrases outside the clinical setting is associated with human impact. *Appl. Environ. Microbiol.* 75, 5100–5110.

Roe, M. T., Vega, E., and Pillai, S. D. (2003). Antimicrobial resistance markers of class 1 and class 2 integron-bearing *Escherichia coli* from irrigation water and sediments. *Emerging Infect. Dis.* 9, 822–826.

Rosewarne, C. P., Pettigrove, V., Stokes, H. W., and Parsons, Y. M. (2010). Class 1 integrons in benthic bacterial

communities: abundance, association with Tn402-like transposition modules and evidence for coselection with heavy-metal resistance. *FEMS Microbiol. Ecol.* 72, 35–46.

Rosser, S. J., and Young, H. K. (1999). Identification and characterization of class 1 integrons in bacteria from an aquatic environment. *J. Antimicrob. Chemother.* 44, 11–18.

Rowe-Magnus, D. A., Guerout, A. M., Ploncard, P., Dychinco, B., Davies, J., and Mazel, D. (2001). The evolutionary history of chromosomal super-integrons provides an ancestry for multiresistant integrons. *Proc. Natl. Acad. Sci. U.S.A.* 98, 652–657.

Schmidt, A. S., Bruun, M. S., Dalsgaard, I., and Larsen, J. L. (2001). Incidence, distribution, and spread of tetracycline resistance determinants and integron-associated antibiotic resistance genes among motile aeromonads from a fish farming environment. *Appl. Environ. Microbiol.* 67, 5675–5682.

Scotta, C., Juan, C., Cabot, G., Oliver, A., Lalucat, J., Bennasar, A., and Albertí, S. (2011). Environmental microbiota represents a natural reservoir for dissemination of clinically relevant metallo-{beta}-lactamases. *Antimicrob. Agents Chemother.* 55, 5376–5379.

Shi, L., Zheng, M., Xiao, Z., Asakura, M., Su, J., Li, L., and Yamasaki, S. (2006). Unnoticed spread of class 1 integrons in Gram-positive clinical strains isolated in Guangzhou, China. *Microbiol. Immunol.* 50, 463–467.

Skurnik, D., Le Menac'h, A., Zurakowski, D., Mazel, D., Courvalin, P., Denamur, E., Andremont, A., and Ruimy, R. (2005). Integron-associated antibiotic resistance and phylogenetic grouping of *Escherichia coli* isolates from healthy subjects free of recent antibiotic exposure. *Antimicrob. Agents Chemother.* 49, 3062–3065.

Skurnik, D., Ruimy, R., Andremont, A., Amorin, C., Rouquet, P., Picard, B., and Denamur, E. (2006). Effect of human vicinity on antimicrobial resistance and integrons in animal faecal *Escherichia coli*. *J. Antimicrob. Chemother.* 57, 1215–1219.

Smith, J. L., Drum, D. J. V., Dai, Y., Kim, J. M., Sanchez, S., Maurer, J. J., Hofacre, C. L., and Lee, M. D. (2007). Impact of antimicrobial usage on antimicrobial resistance in commensal *Escherichia coli* strains colonizing broiler chickens. *Appl. Environ. Microbiol.* 73, 1404–1414.

Srinivasan, V., Nam, H.-M., Sawant, A. A., Headrick, S. I., Nguyen, L. T., and Oliver, S. P. (2008). Distribution of tetracycline and streptomycin resistance genes and class 1 integrons in Enterobacteriaceae isolated from dairy and nondairy farm soils. *Microb. Ecol.* 55, 184–193.

Stokes, H. W., and Gillings, M. R. (2011). Gene flow, mobile genetic elements and the recruitment of antibiotic resistance genes into Gram negative pathogens. *FEMS Microbiol. Rev.* 35, 790–819.

Stokes, H. W., and Hall, R. M. (1989). A novel family of potentially mobile DNA elements encoding site-specific gene-integration functions: integrons. *Mol. Microbiol.* 3, 1669–1683.

Stokes, H. W., Nesbø, C. L., Holley, M., Bahl, M. I., Gillings, M. R., and Boucher, Y. (2006). Class 1 integrons potentially predating the association with tn402-like transposition genes are present in a sediment microbial community. *J. Bacteriol.* 188, 5722–5730.

Taviani, E., Ceccarelli, D., Lazaro, N., Bani, S., Cappuccinelli, P., Colwell, R. R., and Colombo, M. M. (2008). Environmental *Vibrio* spp., isolated in Mozambique, contain a polymorphic group of integrative conjugative elements and class 1 integrons. *FEMS Microbiol. Ecol.* 64, 45–54.

Tennstedt, T., Szczepanowski, R., Braun, S., Pühler, A., and Schlüter, A. (2003). Occurrence of integron-associated resistance gene cassettes located on antibiotic resistance plasmids isolated from a wastewater treatment plant. *FEMS Microbiol. Ecol.* 45, 239–252.

Tschäpe, H. (1994). The spread of plasmids as a function of bacterial adaptability. *FEMS Microbiol. Ecol.* 15, 23–31.

Verner-Jeffreys, D. W., Welch, T. J., Schwarz, T., Pond, M. J., Woodward, M. J., Haig, S. J., Rimmer, G. S. E., Roberts, E., Morrison, V., and Baker-Austin, C. (2009). High prevalence of multidrug-tolerant bacteria and associated antimicrobial resistance genes isolated from ornamental fish and their carriage water. *PLoS ONE* 4, e8388. doi:10.1371/journal.pone.0008388

Wozniak, R. A. F., and Waldor, M. K. (2010). Integrative and conjugative elements: mosaic mobile genetic elements enabling dynamic lateral gene flow. *Nat. Rev. Microbiol.* 8, 552–563.

Wright, G. D. (2010). Antibiotic resistance in the environment: a link to the clinic? *Curr. Opin. Microbiol.* 13, 589–594.

Wright, M. S., Baker-Austin, C., Lindell, A. H., Stepanauskas, R., Stokes, H. W., and McArthur, J. V. (2008). Influence of industrial contamination on mobile genetic elements: class 1 integron abundance and gene cassette structure in aquatic bacterial communities. *ISME J.* 2, 417–428.

Xia, R., Guo, X., Zhang, Y., and Xu, H. (2010). qnrVC-like gene located in a novel complex class 1 integron harboring the ISCR1 element in an *Aeromonas punctata* strain from an aquatic environment in Shandong Province, China. *Antimicrob. Agents Chemother.* 54, 3471–3474.

Xu, H., Davies, J., and Miao, V. (2007). Molecular characterization of class 3 integrons from *Delftia* spp. *J. Bacteriol.* 189, 6276–6283.

Xu, Z., Li, L., Shirtliff, M. E., Peters, B. M., Peng, Y., Alam, M. J., Yamasaki, S., and Shi, L. (2010). First report of class 2 integron in clinical *Enterococcus faecalis* and class 1 integron in *Enterococcus faecium* in South China. *Diagn. Microbiol. Infect. Dis.* 68, 315–317.

Zhang, X., Wu, B., Zhang, Y., Zhang, T., Yang, L., Fang, H. H. P., Ford, T., and Cheng, S. (2009a). Class 1 integronase gene and tetracycline resistance genes tetA and tetC in different water environments of Jiangsu Province, China. *Ecotoxicology* 18, 652–660.

Zhang, X. X., Zhang, T., Zhang, M., Fang, H. H. P., and Cheng, S.-P. (2009b). Characterization and quantification of class 1 integrons and associated gene cassettes in sewage treatment plants. *Appl. Microbiol. Biotechnol.* 82, 1169–1177.

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 29 November 2011; accepted: 13 March 2012; published online: 09 April 2012.

Citation: Stalder T, Barraud O, Casellas M, Dagot C and Ploy M-C (2012) Integron involvement in environmental spread of antibiotic resistance. Front. Microbio. ***3****:119. doi: 10.3389/fmicb.2012.00119*

This article was submitted to Frontiers in Antimicrobials, Resistance and Chemotherapy, a specialty of Frontiers in Microbiology.

frontiers in
MICROBIOLOGY

REVIEW ARTICLE
published: 14 December 2012
doi: 10.3389/fmicb.2012.00399

Heavy metal driven co-selection of antibiotic resistance in soil and water bodies impacted by agriculture and aquaculture

Claudia Seiler* and Thomas U. Berendonk

Institute of Hydrobiology, Technische Universität Dresden, Dresden, Germany

Edited by:
Rustam I. Aminov, University of the West Indies, Jamaica

Reviewed by:
Henning Sørum, Norwegian School of Veterinary Science, Norway
Jan Siemens, University of Bonn, Germany

****Correspondence:***
Claudia Seiler, Institute of Hydrobiology, Technische Universität Dresden, Zellescher Weg 40, 01217 Dresden, Germany.
e-mail: claudia.seiler@mailbox.tu-dresden.de

The use of antibiotic agents as growth promoters was banned in animal husbandry to prevent the selection and spread of antibiotic resistance. However, in addition to antibiotic agents, heavy metals used in animal farming and aquaculture might promote the spread of antibiotic resistance via co-selection. To investigate which heavy metals are likely to co-select for antibiotic resistance in soil and water, the available data on heavy metal pollution, heavy metal toxicity, heavy metal tolerance, and co-selection mechanisms was reviewed. Additionally, the risk of metal driven co-selection of antibiotic resistance in the environment was assessed based on heavy metal concentrations that potentially induce this co-selection process. Analyses of the data indicate that agricultural and aquacultural practices represent major sources of soil and water contamination with moderately to highly toxic metals such as mercury (Hg), cadmium (Cd), copper (Cu), and zinc (Zn). If those metals reach the environment and accumulate to critical concentrations they can trigger co-selection of antibiotic resistance. Furthermore, co-selection mechanisms for these heavy metals and clinically as well as veterinary relevant antibiotics have been described. Therefore, studies investigating co-selection in environments impacted by agriculture and aquaculture should focus on Hg, Cd, Cu, and Zn as selecting heavy metals. Nevertheless, the respective environmental background has to be taken into account.

Keywords: co-selection, antibiotic resistance, heavy metal, agriculture, aquaculture, farming

INTRODUCTION

The number of infections caused by antibiotic resistant bacteria is rising worldwide (Levy and Marshall, 2004). Because of this critical development associated with the loss of the therapeutic potential of antibiotics, some reports are already describing the start of the post-antibiotic era with the consequence of rising difficulties in the treatment of infectious diseases (Alanis, 2005). The decrease of antibiotic use does not necessarily prevent the spread and maintenance of antibiotic resistance in clinical but also in natural environments (Salyers and Amábile-Cuevas, 1997). Therefore, we need to find additional ways to slow down this alarming development (Aminov, 2010). For this reason, it is essential to fully understand the mechanisms and especially the triggers of the evolution and dissemination of antibiotic resistance.

Since there is evidence for recent exchanges of antibiotic resistance genes between environmental and pathogenic bacteria (Poirel et al., 2002, 2005; Forsberg et al., 2012), research brought the natural environment into focus. It is likely that the spread and evolution of antibiotic resistance is triggered or catalysed by anthropogenic pollutants. It has been proved that antimicrobial agents different from antibiotics have the ability to promote a co-selection process, indirectly selecting for antibiotic resistance (Baker-Austin et al., 2006). Heavy metal contaminations are widely spread, whereby agriculture (Han et al., 2001) as well as aquaculture (Burridge et al., 2010) contributing to that environmental burden.

Additionally, due to agricultural and aquacultural practice antibiotics are transferred to soil and water environments, for example soil being fertilized with antibiotic containing manure and sewage sludge (Heuer et al., 2011). Once the antibiotics reach soil environments they may leach to water environments (Boxall et al., 2002). Although the use of antibiotics added to fish feed in aquacultures decreased significantly after the development of vaccinations (Sørum, 2006), the medication for fish with antibiotics as feed amendments is still in practice. The discharge of heavy metals together with antibiotics from agriculture and animal production-linked ecosystems to the environment may cause a combined effect of selection and co-selection toward antibiotic resistant bacteria. Therefore, soil and water bodies impacted by agriculture and aquaculture are hot spots of the evolution of antibiotic resistant bacteria and require special scientific consideration.

Here, we review the risk for metal driven co-selection of antibiotic resistance by addressing heavy metal sources as well as heavy metal toxicity, with regard to aquaculture and agriculture. Additionally, we will review co-selection mechanisms and identify heavy metal concentrations that potentially induce antibiotic resistance co-selection.

FARMING AND AQUACULTURE AS SOURCES OF HEAVY METALS

The anthropogenic contamination of the environment with heavy metals is a serious problem. Aquaculture (Burridge et al., 2010) and agricultural practices (Han et al., 2002; Nicholson et al., 2003) contribute to this world wide pollution due to diverse applications of metals in feed additives, organic and inorganic fertilizers, pesticides, and anti-fouling products.

Fish farmers frequently use pharmaceuticals (such as antibiotics) and metal containing products to prevent fouling, to feed and to treat fish in order to limit the spread of infections (Burridge et al., 2010). For instance, copper (Cu)-containing materials are applied as anti-fouling agents for farm cages and nets; some cages themselves are made from Cu alloys (Burridge et al., 2010). Therefore, bacterial communities of aquacultures are strongly exposed to the combination of heavy metals and antibiotics. The exposure to both antimicrobial substances may increase the likelihood of selection and co-selection of antibiotic resistance. Moreover, the high nutritional value and the relatively low cost of wastewater, excreta, and sewage sludge convert such heavy metal containing waste to valuable fish feed, especially in developing countries (WHO, 2006). The relevance of heavy metal contaminations in aquaculture has been illustrated by Choi and Cech (1998), who found unexpected high concentrations of mercury (Hg) in fish feed. The enrichment of aquaculture sediments with zinc (Zn) (Morrisey et al., 2000) and Cu (Smith et al., 2005; Burridge et al., 2010) as well as cadmium (Cd) (Dean et al., 2007) and lead (Pb) (Mendiguchía et al., 2006) was reported earlier and is presented in **Table 1**.

Most serious heavy metal contaminations in soils include Cu, Hg, Zn, Pb, and Cd (Han et al., 2002). Land application of metal containing fertilizers, sewage sludge, and liquid manure is common practice in agriculture not only in Europe but also in other regions of the world. Due to those applications heavy metals such as Pb, Hg, Cd, Cu, Zn, chromium (Cr), and nickel (Ni) are transferred to arable soil (**Table 1**). Because of its bactericidal and fungicidal properties, Cu-containing pesticides are applied in organic and conventional agriculture (Nemecek et al., 2011). Furthermore, metals such as iron (Fe), cobalt (Co), manganese (Mn), Cu, and Zn are applied as nutritional additives in animal feed for livestock farming and fish production in Europe (Commission Regulation 1831/2003/EC, 2003).

A pre-assessment of the environmental impact of Zn and Cu feed amendments in the European Union demonstrated the major role of aquaculture and agriculture as pollution sources of those metals. The applied models predicted that the no effect concentrations of Cu and Zn will be exceeded in some soil and water systems within the next 10–50 years (Monteiro et al., 2010). Moreover, agriculture was identified as the main source of Cu- and Zn-contamination of arable soil in England and Wales (Nicholson et al., 2003). In addition, 30% of the Cd input to the investigated agricultural soil originated from inorganic fertilizers.

HEAVY METAL TOXICITY AND RESISTANCE

Not all heavy metals are equally toxic to bacteria. Some are important trace metals involved in various physiological functions of the cell. For example Zn, Ni, Cr, Cu, and Co are metals of moderate to high physiological importance. They are essential micronutrients necessary for several cellular functions and components of DNA- and RNA-polymerases (Zn), urease (Ni), cytochrome (Cr) and cytochrom—c—oxidase (Cu). Pb, Cd, Hg, silver (Ag), and gold (Au) have reduced relevance as trace nutrients and they have limited physiological function. Cd and Hg are strong cellular toxins because of their ability to form harmful complexes (Nies, 1999). In contrast, the toxicity of trace metals such as Zn, Ni, Cu, Co, and Cr are strongly dependent on the concentration. As reviewed by Nies (1999), the elements Fe, Mn and molybdenum (Mo) were described as physiologically important with limited toxicity. Metals such as Zn, Ni, Cu, Co, Cr, vanadium (V), and tungsten (W) are toxic elements with metabolic relevance, while the elements Ag, Cd, Hg, Pb, antimony (Sb), and uranium (U) are strong toxins.

The toxicity of heavy metals in the environment strongly depends on the environmental conditions because these conditions influence the valence of the metal ions and therefore their bioavailability. Environmental Cr, for example mainly occurs in two different forms: as Cr^{3+} ion or as the hexavalent Cr associated with oxygen as chromate (for example CrO_4^{2-}). The Cr^{3+} ions are less toxic to bacteria than the chromate (Nies, 1999). Environmental conditions like the pH-value, the concentration of organic matter and the redox potential affect the concentrations and bioavailability of heavy metals in soil, sediment, and water. For instance, the oxygen level influences the redox potential and thereby affects the solubility of some metals. In some water bodies the decomposition of high concentrations of organic matter leads to a reduction of the oxygen level down to anaerobic conditions. Under such conditions the solubility of Cd and Zn is reduced (Schulz-Zunkel and Krueger, 2009). On the other hand, low pH-values increase the solubility of the metals Pb, Cd, and Zn. High contents of organic matter within the sediment act as a sink for some metals: for example Cr and Zn are known to bind to organic matter (Schulz-Zunkel and Krueger, 2009).

In general, the microbial toxicity of heavy metals is due to their chemical affinity to the thiol groups and macrobiomolecules but also depends on the solubility of the metal compound under physiological conditions (Nies, 1999). To avoid cellular damage caused by metal ions, bacteria evolved mechanisms of metal tolerance. There are three general mechanisms which result in heavy metal resistance: the first mechanism is the complex formation or sequestration of toxic metals (Silver and Phung, 1996). Upon metal binding, the concentration of the free toxic ions in the cytoplasm is minimized. Biosorption of toxic metals is known from cell membranes, cell walls and extracellular polymeric substance (EPS) of biofilms (Harrison et al., 2007). For example, the EPS matrix and the contained polysaccharides were reported to bind heavy metals (Teitzel and Parsek, 2003). Thus, the metal tolerance of the bacteria belonging to that biofilm was enhanced. The second mechanism of resistance to toxic metals is the detoxification through reduction of intracellular ions (Nies, 1999). A well understood example is the mercury reductase, encoded by the *merA* gene. This MerA protein reduces Hg^{2+} to the less toxic Hg^0 (Schiering et al., 1991). Hg^0 will then diffuse out of the cell, due to its low evaporation point (Nies, 1999). Finally, extrusion of toxic

Table 1 | Heavy metal concentrations of water, sediment, soil, sewage sludge, and manure.

Sample	Unit	Cd	Cr	Cu	Ni	Hg	Co	Pb	Zn	Influence	References
Water		**Dissolved heavy metal concentration**									
River Hwangryong (KP)	$\mu g\ L^{-1}$	0.27	0.38	1.31	0.52	–	0.39	0.21	7.93	Agriculture, rural, urban	Park et al., 2007
Asian rivers	$\mu g\ L^{-1}$	–	–	–	0–1.6	–	0–0.5	0–0.8	–	Agriculture, urban	Park et al., 2011
		Total heavy metal concentration									
Asian rivers	$\mu g\ L^{-1}$	–	–	–	0–5	–	0.3–2.5	0.8–9	–	Agriculture, urban	Park et al., 2011
River Elbe (DE)	$\mu g\ L^{-1}$	0.07–0.38	1.2–4.8	4.4–7.4	3.4–4.8	0.04–0.09	–	2.2–5.4	18–35	Agriculture, urban, industry	IKSE, 2006
River Thames (UK)	$\mu g\ L^{-1}$	0.03–0.4	–	1.5–30	2.5–15	–	–	2–35	0–70	Urban, agriculture	Power et al., 1999
Loch Craignish (UK)	$\mu g\ L^{-1}$	0.7	–	8	–	–	–	–	25	Marine aquaculture	Dean et al., 2007
Sediment		**Heavy metal concentration referring to fresh weight**									
River Seine (FR)	$mg\ kg^{-1}$	2	84	56	–	0.7	–	73	267	Agriculture, urban, industry	Le Cloarec et al., 2011
Chaohu Lake (CN)	$mg\ kg^{-1}$	0.15–0.50	50–90	15–35	15–40	–	–	10–30	50–150	Agriculture	Tang et al., 2010
		Heavy metal concentration referring to dry weight									
San Pedro River (ES)	$mg\ kg^{-1}$	–	–	15.49	–	–	–	11.32	45.6	Aquaculture	Mendiguchía et al., 2006
Bedfordshire (UK)	$mg\ kg^{-1}$	–	124.3–180.5	30.2–35	55.5–68.8	–	–	70.5–86.6	–	Agriculture	Quinton and Catt, 2007
River Seine (FR)	$mg\ kg^{-1}$	–	–	–	–	0.31	–	–	–	Agriculture, urban, industry	Ouddane et al., 2008
River Elbe (DE)	$mg\ kg^{-1}$	–	–	30–166	12–59	1–6	–	36–185	249–1358	Agriculture, urban, industry	Baborowski et al., 2012
River Medway (UK)	$mg\ kg^{-1}$	–	–	–	–	0.3	–	–	–	Agriculture, urban, industry	Ouddane et al., 2008
Stewart Island (NZ)	$mg\ kg^{-1}$	–	–	–	–	–	–	–	665	Marine aquaculture	Morrisey et al., 2000
Loch Craignish (UK)	$mg\ kg^{-1}$	1.3	–	100	–	–	–	–	450	Marine aquaculture	Dean et al., 2007
Soil		**Heavy metal concentration referring to dry weight**									
	$mg\ kg^{-1}$	0.1–0.4	37.7–59	1–4.3	13.4–17.8	0.1–0.4	–	11.9–21.6	2.7–10.8	Compost	Zhang et al., 2006
	$mg\ kg^{-1}$	0.2–0.4	33.4–50.2	0.8–2.9	12.7–15	0.1–0.4	–	12.3–16.6	3.5–11.4	Compost	Zhang et al., 2006
	$mg\ kg^{-1}$	0.1	36	0.6	12.8	0.3	–	11.1	0.7–0.8	NPKS fertilizer	Zhang et al., 2006
	$mg\ kg^{-1}$	0.2	30.3	0.8	12.1	0.1	–	11.1	2.9	NPKS fertilizer	Zhang et al., 2006
	$mg\ kg^{-1}$	0.1	35.3	0.6–0.7	13.0	0.2	–	11.0	0.7	PK fertilizer	Zhang et al., 2006
	$mg\ kg^{-1}$	0.2	30.5	0.6–0.7	12.0	0.1	–	14.1	3.1	PK fertilizer	Zhang et al., 2006
	$mg\ kg^{-1}$	0.75	–	–	–	–	–	–	–	Sewage sluge	Bergkvist et al., 2003
	$mg\ kg^{-1}$	–	54.2	130.5	34.2	–	–	–	179.5	Swine compost	Zhao et al., 2006
	$mg\ kg^{-1}$	–	54.5	108.2	33.2	–	–	–	106.2	N fertilizer	Zhao et al., 2006
	$mg\ kg^{-1}$	–	56.8	107.0	32.7	–	–	–	103.9	PK fertilizer	Zhao et al., 2006
	$mg\ kg^{-1}$	0.58–0.62	34.5–35	26.7–27.2	30.8–30.9	–	–	24.1–25.2	66.3–70.7	Biowaste-compost	Erhart et al., 2008
	$mg\ kg^{-1}$	0.59–0.63	34.7–35.6	25.8–26.8	30.5–32.4	–	–	23.9–24.4	64.8–67.3	NPK-fertilizer	Erhart et al., 2008
	$mg\ kg^{-1}$	–	58.1–72.5	7.2–9.3	19–21.8	–	–	24.9–28.3	–	Agriculture	Quinton and Catt, 2007
	$mg\ kg^{-1}$	–	–	149–421	–	–	–	–	–	Cu-pesticide	Scheffer and Schachtschabel, 2010

(Continued)

Table 1 | Continued

Sample	Unit	Cd	Cr	Cu	Ni	Hg	Co	Pb	Zn	References
Sewage sludge		**Heavy metal concentration referring to dry weight**								
	mg kg^{-1}	1.3–13	–	–	–	–	–	–	–	Bergkvist et al., 2003
	mg kg^{-1}	4	97	236	40	–	10	60	1640	Saviozzi et al., 1999
	mg kg^{-1}	1.3–2.3	42–71	184–330	30–43	–	–	61–152	1098–1550	Matamoros et al., 2012
	mg kg^{-1}	1.74	85.3	223	46.2	2.2	–	83.6	1025	Chen et al., 2012
	mg kg^{-1}	10	500	800	80	6	30	500	1700	Manara and Zabaniotou, 2012
	mg kg^{-1}	2.83	74.8	190	90.3	–	5.67	21.7	408	Xu et al., 2012
Limit value agricultural sludge application		**Heavy metal concentration referring to dry weight**								
	mg kg^{-1}	20–40	–	1000–1750	300–400	16–25	–	750–1200	2500–4000	Council Directive 86/278/EEC, 1986
Manure		**Heavy metal concentration referring to dry weight**								
Farmyard manure	mg kg^{-1}	6	9	66	14	–	4	60	340	Scheffer and Schachtschabel, 2010
Swine compost	mg kg^{-1}	–	8.5	221.0	11.6	–	–	–	691.9	Zhao et al., 2006
Biowaste compost	mg kg^{-1}	< 0.3–0.7	–	35–90	17–28	–	–	35–101	152–400	Bartl et al., 2002
	mg kg^{-1}	< 0.3–0.6	14–40	23–63	13–25	–	–	33–63	145–240	Erhart et al., 2008

–, *no data.*

ions by efflux systems is the third mechanism of heavy metal resistance (Nies and Silver, 1995). The cation/proton antiporter Czc, known for example from *Alcaligenes eutrophus*, mediates resistance to the metal ions Cd^{2+}, Zn^{2+}, and Co^{2+} by extrusion of metals from the cytoplasm though the inner and outer membrane to the surrounding environment (Silver and Phung, 1996). Population wide metal tolerance is increased by persister cells (Harrison et al., 2007). Persister cells mediate time dependent tolerance to toxic metal ions due to upregulation of resistance and stress response genes (Harrison et al., 2007).

Bacterial sensitivity can be quite complex, nevertheless, some generalizations seem to be possible. Gram positive bacteria are described to be more sensitive to toxic metals than gram negative bacteria (Sterritt and Lester, 1980). Moreover, two general microbial toxicity rankings were reported (Nies, 1999; Harrison et al., 2007). In these rankings bacterial susceptibility is described as a function of the particular metal sulfide dissociation constants (pK_{SP}) (Nies, 1999) and as a function of the standard reduction potentials (ΔE_0) (Harrison et al., 2007). Those two general toxicity rankings are shown in **Table 2**. Nevertheless, different types of bacteria show different sensitivities to toxic metals. Even the heavy metal susceptibility of bacteria belonging to the same genera can differ dramatically. As an example, while the growth of one *Aeromonas* isolate is inhibited by a concentration of 100 µg Zn ml^{-1}, another strain of the same genera isolated from the same sampling site has the ability to grow up to a concentration of 3200 µg Zn ml^{-1} (Matyar et al., 2010). Further examples showing bacteria and their susceptible to toxic metals are displayed in **Table 2**. The listed examples did not show the same pattern of toxicity as reported by Nies (1999) and Harrison et al. (2007). This demonstrates that environmental bacteria may adapt to their ecological conditions and may have been selected for certain metal tolerance mechanisms.

THE MACHINERY OF CO-SELECTION

Since the 1970's, there has been great concern about heavy metals selecting indirectly for antibiotic resistance by co-selection (Koditschek and Guyre, 1974). This indirect selection process is due to a coupling of the resistance mechanisms against antibiotics and heavy metals. Those mechanisms can be coupled physiologically (cross-resistance) and genetically (co-resistance). Cross-resistance describes mechanisms that provide tolerance to more than one antimicrobial agent such as antibiotics and heavy metals (Chapman, 2003). As an example, several multi drug efflux pumps are known to mediate decreased susceptibility toward antibiotics and heavy metals by rapid extrusion of the toxins out of the cell (Martinez et al., 2009). Further well-characterized cross-resistance mechanisms were reviewed by Baker-Austin et al. (2006). Co-resistance is defined as two or more genetically linked resistance genes, meaning that genes responsible for two or more resistances are located next to each other on one mobile genetic element (Chapman, 2003). As an example, Osman et al. (2010) isolated an aquatic bacterium harbouring a plasmid which contained genes conferring resistance to antibiotics and metals like Cr and Co. Due to the close arrangement of the genes it is likely that these genes are subject to a combined transmission in the case of a horizontal gene transfer. A genetic linkage of

Table 2 | Toxicity ranking of heavy metals in recent studies.

Testorganism	Metal sensitivity ranking	References
Escherichia coli	$Hg^{2+} > Ag^{+}/Au^{3+} > CrO_4^{2-} > Cd^{2+} > Co^{2+}/Ni^{2+}/Cu^{2+}/Zn^{2+} > Pb^{2+}/Cr^{3+} > Mn^{2+}$	Nies, 1999
Escherichia coli	$Hg^{2+} > Ag^{+} > Cu^{2+} > Cd^{2+} > Zn^{2+} > Ni^{2+} > Mn^{2+}$	Harrison et al., 2007
Pseudomonas spp. and *Aeromonas* spp.	$Cd^{2+} > Zn^{2+}/Co^{2+}/Cu^{2+}/Cr^{3+} > Pb^{2+} > Mn^{2+}$	Akinbowale et al., 2007
Pseudomonas aeruginosa	$Pb^{2+} > Cu^{2+} > Zn^{2+}$	Teitzel and Parsek, 2003
Pseudomonas spp.	$Hg^{2+} > Zn^{2+}/Cd^{2+}/Ni^{2+}/Pb^{2+}/Cr^{6+}/Cu^{2+}/Cr^{3+}$	Malik and Aleem, 2011
Escherichia coli	$Cr^{6+} > Cu^{2+}/Pb^{2+} > Ni^{2+}/Cr^{3+}/Co^{2+} > Zn^{2+} > Cd^{2+}$	Abskharon et al., 2008
Bacillus spp.	$Hg^{2+} > Cd^{2+} > Zn^{2+}$	Timoney et al., 1978

Compared are distributions of minimum inhibitory concentrations (MIC) of several metal ions.

Cu resistance encoded by the *tcrB* gene, macrolide [*erm*(B)] and glycopeptid resistance (*vanA*) was observed in *Enterococcus faecium* isolated from farm animals (Hasman and Aarestrup, 2002). Here co-resistance to Cu and antibiotics, all applied in farming practice was detected. Macrolides are commonly used in veterinary medicine (Grave et al., 2010) and glycopeptide antibiotics have been used as growth promoters for animal production in the past. Nowadays glycopeptide antibiotics, such as vancomycin, belong to the group of last resort antibiotics in human medicine. Thus, this genetic linkage found by Hasman and Aarestrup (2002) could be an example for a Cu-induced spread of resistance to antibiotics relevant in veterinary and human medicine. Furthermore, *Aeromonas salmonicida* subsp. *salmonicida* isolated from Atlantic salmon (*Salmo salar*) from aquaculture facilities was identified carrying Hg (*mer* operon) and multiple antibiotic resistance genes (*aadA7*, *sulI*, *sulII*, *floR*, *tetA*, *tetR*, *strA*, *strB*, and bla_{CMY-2}) on an IncA/C plasmid (McIntosh et al., 2008). This was the first finding of plasmid associated resistance to florfenicol (*floR*), an antibiotic usually used to treat furunculosis in aquacultures.

Integrons are genetic elements capable of acquiring and exchanging DNA fragments named gene cassettes. Furthermore, class 1 integrons are assumed to catalyse co-selection because they frequently contain gene cassettes that mediate resistance to antibiotics and they are frequently found in contaminated habitats. Presumably these integrons mediate a selective advantage to bacteria that occur under stressful environmental conditions, for example due to toxic metals. This assumption is supported by several studies which discovered elevated abundance of class 1 integrons in aquatic environments contaminated with heavy metals (Wright et al., 2008; Rosewarne et al., 2010). Further indications for a co-resistance mechanism in fresh water bacteria were given by Gillings et al. (2008) and Stokes et al. (2006). Both publications document class 1 integrons which are closely located to genes coding for the multi drug efflux pump czcA. This efflux pump is known to extrude the metal ions Zn^{2+}, Cd^{2+}, and Co^{2+}.

Moreover, environmental pollution by heavy metals not only triggers co-selection processes, but also increases the level of tolerance to antibiotics due to co-regulation of resistance genes. Heavy metal ions are known to co-regulate genes responsible for antibiotic resistance and decrease antibiotic susceptibility (Baker-Austin et al., 2006). For example, the soxS protein is a regulator for the AcrAB efflux system in *Escherichia coli*. Under oxidative stress, for instance caused by several metal ions like $Cr_2O_7^-$ and Cu^{2+}, *soxS* is upregulated (Harrison et al., 2009). The subsequently increased production of the AcrAB efflux system additionally mediates enhanced tolerance toward antibiotics such as chloramphenicol, tetracyclin, novobiocin, nafcillin, and oxacillin.

Studies investigating co-selection in the environment frequently show the correlation of increased heavy metal concentrations with increased phenotypic or genotypic antibiotic resistance (**Table 3**). However, some studies indicate that increasing heavy metal concentrations lead to a decrease of antibiotic resistance (Stepanauskas et al., 2005; Tuckfield and McArthur, 2008; Hölzel et al., 2012). These contradicting results were investigated by Hölzel et al. (2012). In consequence of the addition of mercury chloride ($HgCl_2$) to the antimicrobial test procedure the minimum inhibitory concentration (MIC) for a wide range of antibiotics decreased. This observation could be due to an interaction of Hg with enzymes or nucleic acids which cause antibiotic resistance. $HgCl_2$ could also have a co-toxic effect with antibiotics that interfere with ribosomes because the regeneration of the Hg-degraded enzyme would be inhibited. Furthermore, Hölzel et al. (2012) mentioned also a possible metal induced shift within the bacterial community toward Hg tolerant bacteria whereby the benefit of antibiotic resistance in the presence of antibiotics would be outcompeted. The increased antibiotic susceptibility in consequence of Hg exposure could also play a role in the observations of other field studies. For example, Tuckfield and McArthur (2008) observed decreasing microbial aminoglycoside resistance at sites with increased Hg concentrations.

RISK ASSESSMENT FOR METAL DRIVEN CO-SELECTION

To assess the risk for the co-selection of antibiotic and heavy metal resistance, two datasets of heavy metal concentrations were compared. One of the datasets is shown in **Table 3**, containing metal concentrations observed in studies that investigated co-selection in laboratory and field experiments. The second dataset is shown in **Table 1**, containing heavy metal concentrations that were measured in various environmental compartments which are impacted by agriculture and/or aquaculture. Additionally, we adapted a concept of the MIC originating from the antimicrobial susceptibility testing in clinical settings. The MIC is defined as the antibiotic concentration that

Table 3 | Heavy metal concentrations of studies investigating co-selection in laboratory and field experiments.

Sample	Study type	Unit	Cd	Cr	Cu	Ni	Hg	Co	Pb	Zn	References
Water			**Dissolved heavy metal concentration**								
	Field	$\mu g\ L^{-1}$	0.03–0.13	–	1.5–2.5	0.6–2.7	–	–	0.02–0.06[a]	18.7–26[a]	Wright et al., 2006
	Field	$\mu g\ L^{-1}$	0.08–1.12	–	1.27–12.71	0.29–11.74	–	0.05–3.54	0.15–0.2	19.61–98.07	Stepanauskas et al., 2005
	$MCC_{waterDC}$	$\mu g\ L^{-1}$	0.03	–	1.5	0.29	–	0.05	0.15	19.61	
			Total heavy metal concentration								
	Laboratory	$mg\ L^{-1}$	0–0.11	–	–	5.9–58.7	–	–	–	–	Stepanauskas et al., 2006
	$MCC_{waterTC}$	$mg\ L^{-1}$	0.11	–	–	58.7	–	–	–	–	
Sediment			**Heavy metal concentration referring to fresh weight**								
	Field	$mg\ kg^{-1}$	0.2–1.6[a]	58.4–197[a]	11.6–869	–	–	3.3–15.6	17.4–108	46.1–800	Graham et al., 2010
	MCC_{sedFW}	$mg\ kg^{-1}$	–	–	11.6	–	–	3.3	17.4	46.1	
			Heavy metal concentration referring to dry weight								
	Field	$mg\ kg^{-1}$	–	–	–	–	0.01–0.09	–	–	–	McArthur and Tuckfield, 2000
	Field	$mg\ kg^{-1}$	1–2.5	–	11.5–50	–	0.8–1.5	–	–	42.5–135.8	Timoney et al., 1978
	MCC_{sedDW}	$mg\ kg^{-1}$	1	–	11.5	–	0.01	–	–	42.5	
Soil			**Heavy metal concentration referring to fresh weight**								
	Field	$mg\ kg^{-1}$	–	–	116.7	–	–	–	–	–	Berg et al., 2005
	MCC_{soilFW}	$mg\ kg^{-1}$	–	–	116.7	–	–	–	–	–	
			Heavy metal concentration referring to dry weight								
	Field	$mg\ kg^{-1}$	–	–	3172	–	–	–	–	–	Berg et al., 2010
	Field	$mg\ kg^{-1}$	–	0–250	0–140	0–100	–	0–140[a]	10–1000[a]	0–38[a]	Knapp et al., 2011
Manure			**Heavy metal concentration referring to dry weight**								
	Field	$mg\ kg^{-1}$	5.3[a]	32.0[a]	11.79	97.3[a]	0.01[a]	–	2[a]	22.75	Hölzel et al., 2012
	$MCC_{manureDW}$	$mg\ kg^{-1}$	–	–	11.79	–	–	–	–	22.75	

–, no data.

[a]metal concentration without correlation or in negative correlation to antibiotic resistance.

$MCC_{waterDC}$, minimum co-selective concentration referring to dissolved metals in water.

$MCC_{waterTC}$, minimum co-selective concentration referring to total metals in water.

MCC_{sedFW}, minimum co-selective concentration referring to fresh weight of sediment.

MCC_{sedDW}, minimum co-selective concentration referring to dry weight of sediment.

MCC_{soilFW}, minimum co-selective concentration referring to fresh weight of soil.

$MCC_{manureDW}$, minimum co-selective concentration referring to dry weight of manure.

is needed to inhibit bacterial growth. If this MIC has been increased above an epidemiological cut-off value of a bacterial strain the strain will be defined as antibiotic (microbiological) resistant (URL: http://www.eucast.org). In this context the minimum heavy metal concentration which correlates with a detection of increased bacterial antibiotic resistance, was specified as the minimum co-selective concentration (MCC) of a metal (**Table 3**). Additionally, specific MCCs to every environmental compartment and the respective analytic detection method of the metals (for example for dissolved and total metal concentrations or metal content referring to dry or fresh weight of the solid samples) were defined (**Table 3**). Moreover, the MCCs for each metal were subsequently compared to the heavy metal levels found within the corresponding environmental compartments (**Table 4**). Environmental metal concentrations that exceeded the corresponding MCC were considered as potential drivers of co-selection of antibiotic resistance in the environment.

In the natural water environment (water and sediment) Cd, Cu, Ni, Hg, Co, Pb, and Zn frequently reach levels that exceed their respective MCC values (**Table 4**) and therefore, may drive co-selection. While there are several studies available investigating co-selection in the water environment, there are only a few publications considering soil environments (Berg et al., 2005, 2010; Knapp et al., 2011). Thus, the assessment of the risk for the co-selection of antibiotic and heavy metal resistance in soil is limited to Cr, Cu, and Ni (**Table 3**). Furthermore, the data of Knapp et al. (2011) does not allow extracting MCCs of metals because the lowest metal concentrations that may caused the increase in antibiotic resistance gene abundance are not shown. However, these results provide evidence for Cr, Cu, and Ni driven co-selection of antibiotic resistance in soil. Some Cu and Cr levels of the reviewed arable soil samples (**Table 1**) are similar or even higher than the levels observed by Knapp et al. (2011) (**Table 3**). Sewage sludge and manure are part of this risk assessment because those organic fertilizers themselves could facilitate metal driven co-selection of antibiotic resistance before entering soil environments and they might additionally transfer metals to arable soil. Cu and Zn concentration of sewage sludge and manure frequently exceeded the MCCs of manure (**Table 4**). Moreover, the limit values for heavy metal concentrations of sewage sludge for the use in agriculture (Council Directive 86/278/EEC, 1986) are much higher than the MCCs of manure (**Tables 1** and **3**). As mentioned earlier in this article, the use of Zn and Cu in animal farming and agriculture is common and those metals have been investigated in all considered environmental compartments. The concentrations for both metals exceed their MCCs for some water, sediment, sewage sludge, and manure samples. In soil Cu levels reach concentrations that are reported as potentially co-selective for antibiotic resistance genes (Knapp et al., 2011). In contrast, a Zn MCC for soil samples could not be evaluated because Knapp et al. (2011) did not detect increasing abundance of antibiotic resistance genes in correlation with elevated Zn concentrations. However, the Zn concentrations of soil samples investigated by Knapp et al. (2011) were relatively low compared to other soils (**Table 1**) and maybe within the no effect range. In summary, all considered heavy metals (frequently Cu and Zn) reach concentrations above their MCCs in the different environmental compartments. Therefore, the analysis of the data suggests that heavy metal concentrations in soil and water bodies occasionally reach levels that might drive a co-selection of antibiotic resistance.

This risk assessment of heavy metal driven co-selection is based on MCCs which are derived from positive correlations of increased metal concentrations with increased antibiotic resistance. This risk assessment provides a tool to estimate at which levels environmental metal concentrations may cause the dissemination of microbial antibiotic resistance due to co-selection. Ideally such a risk assessment would be conducted under laboratory conditions, as it is currently the case for the determination

Table 4 | Summary of all studies for which the MCCs were applied.

		Ratio (heavy metal concentration ≥ MCC/heavy metal concentration < MCC)							
Sample	**Applied MCC**	**Cd**	**Cr**	**Cu**	**Ni**	**Hg**	**Co**	**Pb**	**Zn**
Water	$MCC_{waterDC}$	1/0	–	0/1	2/0	–	2/0	2/0	–
	$MCC_{waterTC}$	0/3	–	–	0/3	–	–	–	–
Sediment	MCC_{sedFW}	–	–	2/0	–	–	–	–	–
	MCC_{sedDW}	1/0	–	4/0	–	3/0	–	–	4/0
Manure	$MCC_{manureDW}$	–	–	4/0	–	–	–	–	4/0
Sewage sludge	$MCC_{manureDW}$	–	–	5/0	–	–	–	–	5/0

–, no data.

$MCC_{waterDC}$, minimum co-selective concentration referring to dissolved metals in water.

$MCC_{waterTC}$, minimum co-selective concentration referring to total metals in water.

MCC_{sedFW}, minimum co-selective concentration referring to fresh weight of sediment.

MCC_{soilFW}, minimum co-selective concentration referring to fresh weight of soil.

MCC_{soilDW}, minimum co-selective concentration referring to dry weight of soil.

$MCC_{manureDW}$, minimum co-selective concentration referring to dry weight of manure.

Illustrated are ratios, which show the number of studies where the heavy metal concentrations were above the MCC versus the number of studies where the heavy metal concentrations were below the MCC.

of the MIC. For the purpose of this study this was not possible as we wanted to review existing studies and included laboratory and field data, in order to detect a first pattern or synthesis on heavy metal induced co-selection of antibiotic resistance in the field. The results of the MCC analysis for such data need to be carefully interpreted, mainly because positive correlations between metal levels and antibiotic resistance could also be false positive. This would be the case, if another selection pressure (for example by antibiotics) would be the trigger of the observed selection and not the co-occurring metal.

In order to better assess the risk of co-selection of antibiotic and heavy metal resistance more research is necessary. Only a limited number of studies is available that investigated the co-selection in water and soil environments and additionally measured heavy metal concentrations (**Table 3**). Especially for soil environments, there is only one multi metal study on co-selection and metal contamination (Knapp et al., 2011). The knowledge about the natural background of antibiotic resistance gene abundance (resistome) in the different environments is also limited. Thus, we cannot distinguish between the natural resistome and an elevated abundance of antibiotic resistance genes in different environmental samples. Therefore, it is difficult to detect an increase of antibiotic resistance genes in field studies. Further research should relate to the MCCs of heavy metals. Although we were able to derive the MCC values form recent studies, further research in field and laboratory experiments is urgently requested to broaden the database of co-selective concentrations. Stepanauskas et al. (2006) investigated such a selective concentration of Ni and Cd in a lab experiment. Nevertheless, the metal concentrations observed as minimum selective concentrations in those microcosms were much higher compared to the Ni and Cd levels observed in environmental water samples (**Tables 1** and **3**). This difference requires further investigation and may be an artifact of the growth in the laboratory since laboratory grown bacteria usually have better conditions than their environmental counterparts.

While our MCC approach provides a first step toward a unifying concept for analyzing co-selection of antibiotic resistance through heavy metals, there is an urgent need to extend this approach to a comprehensive risk assessment. The need of such a risk assessment is illustrated in our results, which show that in all considered environmental compartments (water, sediment, and soil) as well as sewage sludge and manure, one or more heavy metals reach concentrations that may lead to a metal driven co-selection of antibiotic resistance.

CONCLUSION

Concluding all these facts concerning the heavy metal driven co-selection of antibiotic resistance, metals such as Cd, Hg, Cu, and Zn are of great importance in water and soil environments that are influenced by agriculture and aquaculture. These metals are moderately to highly toxic to bacteria; they reach the environment and they accumulate to selective concentrations. Additionally, they can trigger co-selection of antibiotic resistance because responsible co-selection mechanisms that mediate resistance to these heavy metals and clinically as well as veterinary relevant antibiotics have already been described. Therefore, the elimination of antibiotics from the list of animal feed additives as growth promoters was a step in the right direction. Further steps need to be taken to reduce the alarming spread of antibiotic resistance genes. In addition to the avoidance of antibiotics in livestock farming and aquaculture, further antimicrobial agents such as heavy metals should be considered. These metals have the potential to act as a selective pressure that forces the proliferation and evolution of antibiotic and heavy metal resistance in the natural environment. With the exception of the above mentioned studies, investigations which explicitly test for the co-selection of heavy metals and antibiotics used in animal farms and aquaculture are still scarce. Future studies investigating heavy metal driven co-selection of antibiotic resistance in soil and water bodies impacted by agriculture and aquaculture should focus on Hg, Cd, Cu, and Zn as co-selecting factors for the evolution of antibiotic resistances. Nevertheless, the respective environmental background has to be taken into account.

ACKNOWLEDGMENTS

This work was kindly supported by Helmholtz Impulse and Networking Fund through Helmholtz Interdisciplinary Graduate School for Environmental Research (HIGRADE) and the funding of the BMBF programs "IWAS" and "ANTIRESIST". The authors also thank Anka Broßio and Shane Denecke for linguistic support.

REFERENCES

Abskharon, R. N. N., Hassan, S. H. A., Gad El-Rab, S. M. F., and Shoreit, A. A. M. (2008). Heavy metal resistant of *E.* coli isolated from wastewater sites in Assiut City, Egypt. *Bull. Environ. Contam. Toxicol.* 81, 309–315.

Akinbowale, O. L., Peng, H., Grant, P., and Barton, M. D. (2007). Antibiotic and heavy metal resistance in motile *Aeromonads* and *Pseudomonads* from Rainbow trout (*Oncorhynchus mykiss*) farms in Australia. *Int. J. Antimicrob. Agents* 30, 177–182.

Alanis, A. J. (2005). Resistance to antibiotics: are we in the post-antibiotic era? *Arch. Med. Res.* 36, 697–705.

Aminov, R. I. (2010). A brief history of the antibiotic era: lessons learned and challenges for the future. *Front. Microbio.* 1:134. doi: 10.3389/fmicb.2010.00134

Baborowski, M., Büttner, O., Morgenstern, P., Jancke, T., and Westrich, B. (2012). Spatial variability of metal pollution in groyne fields of the Middle Elbe—implications for sediment monitoring. *Environ. Pollut.* 167, 115–123.

Baker-Austin, C., Wright, M. S., Stepanauskas, R., and McArthur, J. V. (2006). Co-selection of antibiotic and metal resistance. *Trends Microbiol.* 14, 176–182.

Bartl, B., Hartl, W., and Horak, O. (2002). Long-term application of biowaste compost versus mineral fertilization: effects on the nutrient and heavy metal contents of soil and plants. *J. Plant Nutr. Soil Sci.* 165, 161–165.

Berg, J., Thorsen, M. K., Holm, P. E., Jensen, J., Nybroe, O., and Brandt, K. K. (2010). Cu exposure under field conditions coselects for antibiotic resistance as determined by a novel cultivation-independent bacterial community tolerance assay. *Environ. Sci. Technol.* 44, 8724–8728.

Berg, J., Tom-Petersen, A., and Nybroe, O. (2005). Copper amendment of agricultural soil selects for bacterial antibiotic resistance in the field. *Lett. Appl. Microbiol.* 40, 146–151.

Bergkvist, P., Jarvis, N., Berggren, D., and Carlgren, K. (2003). Long-term effects of sewage sludge applications on soil properties, cadmium availability and distribution in arable

soil. *Agric. Ecosyst. Environ.* 97, 167–179.

Boxall, A. B. A., Blackwell, P., Cavallo, R., Kay, P., and Tolls, J. (2002). The sorption and transport of a sulphonamide antibiotic in soil systems. *Toxicol. Lett.* 131, 19–28.

Burridge, L., Weis, J. S., Cabello, F., Pizarro, J., and Bostick, K. (2010). Chemical use in salmon aquaculture: a review of current practices and possible environmental effects. *Aquaculture* 306, 7–23.

Chapman, J. S. (2003). Disinfectant resistance mechanisms, cross-resistance, and co-resistance. *Int. Biodeterior. Biodegradation* 51, 271–276.

Chen, H., Yan, S. H., Ye, Z. L., Meng, H. J., and Zhu, Y. G. (2012). Utilization of urban sewage sludge: Chinese perspectives. *Environ. Sci. Pollut. Res.* 19, 1454–1463.

Choi, M. H., and Cech, J. J. (1998). Unexpectedly high mercury level in pelleted commercial fish feed. *Environ. Toxicol. Chem.* 17, 1979–1981.

Commission Regulation 1831/2003/EC. (2003). Regulation (EC) No 1831/2003 of the European Parliament and of the Council of 22 September 2003 on additives for use in animal nutrition. *Off. J. Eur. Union* L268, 29–43.

Council Directive 86/278/EEC. (1986). Council Directive 86/278/EEC of 12 June 1986 on the protection of the environment, and in particular of the soil, when sewage sludge is used in agriculture. *Off. J. Eur. Comm.* L181, 6–12.

Dean, R. J., Shimmield, T. M., and Black, K. D. (2007). Copper, zinc and cadmium in marine cage fish farm sediments: an extensive survey. *Environ. Pollut.* 145, 84–95.

Erhart, E., Hartl, W., and Putz, B. (2008). Total soil heavy-metal concentrations and mobile fractions after 10 years of biowaste-compost fertilization. *J. Plant Nutr. Soil Sci.* 171, 378–383.

Forsberg, K. J., Reyes, A., Wang, B., Selleck, E. M., Sommer, M. O. A., and Dantas, G. (2012). The shared antibiotic resistome of soil bacteria and human pathogens. *Science* 337, 1107–1111.

Gillings, M., Boucher, Y., Labbate, M., Holmes, A., Krishnan, S., Holley, M., et al. (2008). The evolution of class 1 integrons and the rise of antibiotic resistance. *J. Bacteriol.* 190, 5095–5100.

Graham, D., Olivares-Rieumont, S., Knapp, C., Lima, L., Werner, D., and Bowen, E. (2010). Antibiotic resistance gene abundances associated with waste discharges to the Almendares river near Havana, Cuba. *Environ. Sci. Technol.* 45, 418–424.

Grave, K., Torren-Edo, J., and Mackay, D. (2010). Comparison of the sales of veterinary antibacterial agents between 10 European countries. *J. Antimicrob. Chemother.* 65, 2037–2040.

Han, F., Kingery, W., and Selim, H. (2001). "Accumulation, redistribution, transport and bioavailability of heavy metals in wastea-mended soils," in *Trace Elements in Soil: Bioavailability, Fluxes and Transfer,* eds I. Iskander and M. Kirkham (Boca Raton, FL: CRC), 141–168.

Han, F. X., Banin, A., Su, Y., Monts, D. L., Plodinec, J. M., Kingery, W. L., et al. (2002). Industrial age anthropogenic inputs of heavy metals into the pedosphere. *Naturwissenschaften* 89, 497–504.

Harrison, J. J., Ceri, H., and Turner, R. J. (2007). Multimetal resistance and tolerance in microbial biofilms. *Nat. Rev. Microbiol.* 5, 928–938.

Harrison, J. J., Tremaroli, V., Stan, M. A., Chan, C. S., Vacchi-Suzzi, C., Heyne, B. J., et al. (2009). Chromosomal antioxidant genes have metal ion-specific roles as determinants of bacterial metal tolerance. *Environ. Microbiol.* 11, 2491–2509.

Hasman, H., and Aarestrup, F. M. (2002). *tcrB*, a gene conferring transferable copper resistance in *Enterococcus faecium*: occurrence, transferability, and linkage to macrolide and glycopeptide resistance. *Antimicrob. Agents Chemother.* 46, 1410–1416.

Heuer, H., Schmitt, H., and Smalla, K. (2011). Antibiotic resistance gene spread due to manure application on agricultural fields. *Curr. Opin. Microbiol.* 14, 236–243.

Hölzel, C. S., Müller, C., Harms, K. S., Mikolajewski, S., Schäfer, S., Schwaiger, K., et al. (2012). Heavy metals in liquid pig manure in light of bacterial antimicrobial resistance. *Environ. Res.* 113, 21–27.

IKSE. (2006). *Gewässergütebericht Elbe 2004–2005*. Magdeburg: IKSE.

Knapp, C. W., McCluskey, S. M., Singh, B. K., Campbell, C. D., Hudson, G., and Graham, D. W. (2011). Antibiotic resistance gene abundances correlate with metal and geochemical conditions in archived scottish soils. *PLoS ONE* 6:e27300. doi: 10.1371/journal.pone.0027300

Koditschek, L. K., and Guyre, P. (1974). Resistance transfer fecal coliforms isolated from the Whippany River. *Water Res.* 8, 747–752.

Le Cloarec, M. F., Bonte, P. H., Lestel, L., Lefèvre, I., and Ayrault, S. (2011). Sedimentary record of metal contamination in the Seine River during the last century. *Phys. Chem. Earth Parts A/B/C* 36, 515–529.

Levy, S. B., and Marshall, B. (2004). Antibacterial resistance worldwide: causes, challenges and responses. *Nat. Med.* 10, S122–S129.

Malik, A., and Aleem, A. (2011). Incidence of metal and antibiotic resistance in *Pseudomonas* spp. from the river water, agricultural soil irrigated with wastewater and groundwater. *Environ. Monit. Assess.* 178, 293–308.

Manara, P., and Zabaniotou, A. (2012). Towards sewage sludge based biofuels via thermochemical conversion –a review. *Renew. Sust. Energ. Rev.* 16, 2566–2582.

Martinez, J. L., Sánchez, M. B., Martínez-Solano, L., Hernandez, A., Garmendia, L., Fajardo, A., et al. (2009). Functional role of bacterial multidrug efflux pumps in microbial natural ecosystems. *FEMS Microbiol. Rev.* 33, 430–449.

Matamoros, V., Nguyen, L. X., Arias, C. A., Nielsen, S., Laugen, M. M., and Brix, H. (2012). Musk fragrances, DEHP and heavy metals in a 20 years old sludge treatment reed bed system. *Water Res.* 46, 3889–3896.

Matyar, F., Akkan, T., Uçak, Y., and Eraslan, B. (2010). *Aeromonas* and *Pseudomonas*: antibiotic and heavy metal resistance species from Iskenderun Bay, Turkey (northeast Mediterranean Sea). *Environ. Monit. Assess.* 167, 309–320.

McArthur, J. V., and Tuckfield, R. C. (2000). Spatial patterns in antibiotic resistance among stream bacteria: effects of industrial pollution. *Appl. Environ. Microbiol.* 66, 3722–3726.

McIntosh, D., Cunningham, M., Ji, B., Fekete, F. A., Parry, E. M., Clark, S. E., et al. (2008). Transferable, multiple antibiotic and mercury resistance in Atlantic Canadian isolates of *Aeromonas salmonicida* subsp *salmonicida* is associated with carriage of an IncA/C plasmid similar to the *Salmonella enterica* plasmid pSN254. *J. Antimicrob. Chemother.* 61, 1221–1228.

Mendiguchía, C., Moreno, C., Mánuel-Vez, M. P., and García-Vargas, M. (2006). Preliminary investigation on the enrichment of heavy metals in marine sediments originated from intensive aquaculture effluents. *Aquaculture* 254, 317–325.

Monteiro, S. C., Lofts, S., and Boxall, A. B. A. (2010). *Pre-Assessment of Environmental Impact of Zinc and Copper Used in Animal Nutrition*. Parma, Italy: European Food Safety Authority.

Morrisey, D. J., Gibbs, M. M., Pickmere, S. E., and Cole, R. G. (2000). Predicting impacts and recovery of marine-farm sites in Stewart Island, New Zealand, from the Findlay–Watling model. *Aquaculture* 185, 257–271.

Nemecek, T., Dubois, D., Huguenin-Elie, O., and Gaillard, G. (2011). Life cycle assessment of Swiss farming systems: I. *Integr. Org. Farm. Agric. Syst.* 104, 217–232.

Nicholson, F. A., Smith, S. R., Alloway, B. J., Carlton-Smith, C., and Chambers, B. J. (2003). An inventory of heavy metals inputs to agricultural soils in England and Wales. *Sci. Total Environ.* 311, 205–219.

Nies, D. H. (1999). Microbial heavy-metal resistance. *Appl. Microbiol. Biotechnol.* 51, 730–750.

Nies, D. H., and Silver, S. (1995). Ion efflux systems involved in bacterial metal resistances. *J. Ind. Microbiol.* 14, 186–199.

Osman, O., Tanguichi, H., Ikeda, K., Park, P., Tanabe-Hosoi, S., and Nagata, S. (2010). Copper-resistant halophilic bacterium isolated from the polluted Maruit Lake, Egypt. *J. Appl. Microbiol.* 108, 1459–1470.

Ouddane, B., Mikac, N., Cundy, A. B., Quillet, L., and Fischer, J. C. (2008). A comparative study of mercury distribution and methylation in mudflats from two macrotidal estuaries: the Seine (France) and the Medway (United Kingdom). *Appl. Geochem.* 23, 618–631.

Park, J.-H., Inam, E., Abdullah, M. H., Agustiyani, D., Duan, L., Hoang, T. T., et al. (2011). Implications of rainfall variability for seasonality and climate-induced risks concerning surface water quality in East Asia. *J. Hydrol.* 400, 323–332.

Park, J.-H., Lee, J.-H., Kang, S.-Y., and Kim, S.-Y. (2007). Hydroclimatic controls on dissolved organic matter (DOM) characteristics and implications for trace metal transport in Hwangryong River Watershed, Korea, during a summer monsoon period. *Hydrol. Process.* 21, 3025–3034.

Poirel, L., Kampfer, P., and Nordmann, P. (2002). Chromosome-encoded ambler class A beta-lactamase of Kluyvera georgiana, a probable progenitor of a subgroup of CTX-M extended-spectrum

beta-lactamases. *Antimicrob. Agents Chemother.* 46, 4038–4040.

Poirel, L., Rodriguez-Martinez, J.-M., Mammeri, H., Liard, A., and Nordmann, P. (2005). Origin of plasmid-mediated quinolone resistance determinant QnrA. *Antimicrob. Agents Chemother.* 49, 3523–3525.

Power, M., Attrill, M. J., and Thomas, R. M. (1999). Heavy metal concentration trends in the Thames estuary. *Water Res.* 33, 1672–1680.

Quinton, J. N., and Catt, J. A. (2007). Enrichment of heavy metals in sediment resulting from soil erosion on agricultural fields. *Environ. Sci. Technol.* 41, 3495–3500.

Rosewarne, C. P., Pettigrove, V., Stokes, H. W., and Parsons, Y. M. (2010). Class 1 integrons in benthic bacterial communities: abundance, association with Tn402-like transposition modules and evidence for coselection with heavy-metal resistance. *FEMS Microbiol. Ecol.* 72, 35–46.

Salyers, A. A., and Amábile-Cuevas, C. F. (1997). Why are antibiotic resistance genes so resistant to elimination? *Antimicrob. Agents Chemother.* 41, 2321–2325.

Saviozzi, A., Biasci, A., Riffaldi, R., and Levi-Minzi, R. (1999). Long-term effects of farmyard manure and sewage sludge on some soil biochemical characteristics. *Biol. Fertil. Soils* 30, 100–106.

Scheffer, F., and Schachtschabel, P. (2010). "Gefährdung der Bodenfunktionen," in *Lehrbuch der Bodenkunde*, eds H. Lieth and B. Markert (Heidelberg: Spektrum Akademischer Verlag), 449–519.

Schiering, N., Kabsch, W., Moore, M. J., Distefano, M. D., Walsh, C. T., and Pai, E. F. (1991). Structure of the detoxification catalyst mercuric ion reductase from *Bacillus* sp. strain-RC607. *Nature* 352, 168–172.

Schulz-Zunkel, C., and Krueger, F. (2009). Trace metal dynamics in floodplain soils of the River Elbe: a review. *J. Environ. Qual.* 38, 1349–1362.

Silver, S., and Phung, L. T. (1996). Bacterial heavy metal resistance: new surprises. *Annu. Rev. Microbiol.* 50, 753–789.

Smith, J. N., Yeats, P. A., and Milligan, T. G. (2005). "Sediment geochronologies for fish farm contaminants in Lime Kiln Bay, Bay of Fundy," in *Handbook of Environmental Chemistry*, ed B. T. Hargrave (Berlin: Springer), 221–238.

Sørum, H. (2006). "Antimicrobial drug resistance in fish pathogens," in *Antimicrobial Resistance in Bacteria of Animal Origin*, ed F. M. Aarestrup (Washington, DC: ASM Press), 213–238.

Stepanauskas, R., Glenn, T. C., Jagoe, C. H., Tuckfield, R. C., Lindell, A. H., King, C. J., et al. (2006). Coselection for microbial resistance to metals and antibiotics in freshwater microcosms. *Environ. Microbiol.* 8, 1510–1514.

Stepanauskas, R., Glenn, T. C., Jagoe, C. H., Tuckfield, R. C., Lindell, A. H., and McArthur, J. V. (2005). Elevated microbial tolerance to metals and antibiotics in metal-contaminated industrial environments. *Environ. Sci. Technol.* 39, 3671–3678.

Sterritt, R. M., and Lester, J. N. (1980). Interactions of heavy metals with bacteria. *Sci. Total Environ.* 14, 5–17.

Stokes, H. W., Nesbo, C. L., Holley, M., Bahl, M. I., Gillings, M. R., and Boucher, Y. (2006). Class 1 integrons potentially predating the association with Tn402-like transposition genes are present in a sediment microbial community. *J. Bacteriol.* 188, 5722–5730.

Tang, W., Shan, B., Zhang, H., and Mao, Z. (2010). Heavy metal sources and associated risk in response to agricultural intensification in the estuarine sediments of Chaohu Lake Valley, East China. *J. Hazard. Mater.* 176, 945–951.

Teitzel, G. M., and Parsek, M. R. (2003). Heavy metal resistance of biofilm and planktonic *Pseudomonas aeruginosa*. *Appl. Environ. Microbiol.* 69, 2313–2320.

Timoney, J. F., Port, J., Giles, J., and Spanier, J. (1978). Heavy-metal and antibiotic resistance in the bacterial flora of sediments of New York bight. *Appl. Environ. Microbiol.* 36, 465–472.

Tuckfield, R. C., and McArthur, J. V. (2008). Spatial analysis of antibiotic resistance along metal contaminated streams. *Microb. Ecol.* 55, 595–607.

WHO. (2006). *Guidelines for the Safe Use of Wastwater, Excreta and Greywater. Volume 3: Wastewater and Excreta Use in Aquaculture*. Geneva: World Health Organization.

Wright, M. S., Baker-Austin, C., Lindell, A. H., Stepanauskas, R., Stokes, H. W., and McArthur, J. V. (2008). Influence of industrial contamination on mobile genetic elements: class 1 integron abundance and gene cassette structure in aquatic bacterial communities. *ISME J.* 2, 417–428.

Wright, M. S., Peltier, G. L., Stepanauskas, R., and McArthur, J. V. (2006). Bacterial tolerances to metals and antibiotics in metal-contaminated and reference streams. *FEMS Microbiol. Ecol.* 58, 293–302.

Xu, J.-Q., Yu, R.-L., Dong, X.-Y., Hu, G.-R., Shang, X.-S., Wang, Q., et al. (2012). Effects of municipal sewage sludge stabilized by fly ash on the growth of Manilagrass and transfer of heavy metals. *J. Hazard. Mater.* 217–218, 58–66.

Zhang, M., Heaney, D., Henriquez, B., Solberg, E., and Bittner, E. (2006). A four-year study on influence of biosolids/MSW cocomposta application in less productive soils in Alberta: nutrient dynamics. *Compost Sci. Util.* 14, 68–80.

Zhao, B., Maeda, M., Zhang, J., Zhu, A., and Ozaki, Y. (2006). Accumulation and chemical fractionation of heavy metals in andisols after a different, 6-year fertilization management. *Environ. Sci. Pollut. Res.* 13, 90–97.

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 01 March 2012; accepted: 30 October 2012; published online: 14 December 2012.

Citation: Seiler C and Berendonk TU (2012) Heavy metal driven co-selection of antibiotic resistance in soil and water bodies impacted by agriculture and aquaculture. Front. Microbio. ***3****:399. doi: 10.3389/fmicb.2012.00399*

This article was submitted to Frontiers in Antimicrobials, Resistance and Chemotherapy, a specialty of Frontiers in Microbiology.

frontiers in
MICROBIOLOGY

ORIGINAL RESEARCH ARTICLE
published: 20 February 2012
doi: 10.3389/fmicb.2012.00027

Assessing the probability of detection of horizontal gene transfer events in bacterial populations

Jeffrey P. Townsend*[1], *Thomas Bøhn*[2,3] *and Kaare Magne Nielsen*[2,3]

[1] Department of Ecology and Evolutionary Biology, Yale University, New Haven, CT, USA
[2] GenØk-Centre for Biosafety, The Science Park, Tromsø, Norway
[3] Department of Pharmacy, Faculty of Health Sciences, University of Tromsø, Tromsø, Norway

Edited by:
Rustam I. Aminov, University of Aberdeen, UK

Reviewed by:
Morten Otto Alexander Sommer, Technical University of Denmark, Denmark
Lilia Macovei, The Forsyth Institute, USA
Douda Bensasson, University of Manchester, UK

***Correspondence:**
Kaare Magne Nielsen, Department of Pharmacy, Research Group in Microbiology, Molecular and Pharmaco-Epidemiology, Breivika, 9037 Tromso, Tromso, Norway
e-mail: kaare.nielsen@uit.no

Experimental approaches to identify horizontal gene transfer (HGT) events of non-mobile DNA in bacteria have typically relied on detection of the initial transformants or their immediate offspring. However, rare HGT events occurring in large and structured populations are unlikely to be detected in a short time frame. Population genetic modeling of the growth dynamics of bacterial genotypes is therefore necessary to account for natural selection and genetic drift during the time lag and to predict realistic time frames for detection with a given sampling design. Here we draw on statistical approaches to population genetic theory to construct a cohesive probabilistic framework for investigation of HGT of exogenous DNA into bacteria. In particular, the stochastic timing of rare HGT events is accounted for. Integrating over all possible event timings, we provide an equation for the probability of detection, given that HGT actually occurred. Furthermore, we identify the key variables determining the probability of detecting HGT events in four different case scenarios that are representative of bacterial populations in various environments. Our theoretical analysis provides insight into the temporal aspects of dissemination of genetic material, such as antibiotic resistance genes or transgenes present in genetically modified organisms. Due to the long time scales involved and the exponential growth of bacteria with differing fitness, quantitative analyses incorporating bacterial generation time, and levels of selection, such as the one presented here, will be a necessary component of any future experimental design and analysis of HGT as it occurs in natural settings.

Keywords: lateral or horizontal gene transfer, DNA uptake, modeling, monitoring, sampling, antibiotic resistance, GMO, biosafety

INTRODUCTION

Bacteria in natural populations are known to import and integrate exogenous genetic material of diverse, often unidentified, origins (Eisen, 2000; Ochman et al., 2000; Lawrence, 2002; Nakamura et al., 2004; Didelot and Maiden, 2010). Bacterial genomes can be exposed not only to the multitude of sources of exogenous DNA present in their natural environments (Levy-Booth et al., 2007; Nielsen et al., 2007; Pontiroli et al., 2007; Pietramellara et al., 2009; Rizzi et al., 2012), but also to introduced sources of novel DNA such as the fraction of recombinant DNA present in genetically modified organisms (GMOs). Such exposure can potentially lead to horizontal gene transfer (HGT) events of GMO recombinant DNA, dependent on the multitude of parameters that govern HGT processes in various environments (Bertolla and Simonet, 1999; Bensasson et al., 2004). However, for long-term persistence of infrequently acquired genetic material in new bacterial hosts, a conferred selective advantage is considered necessary (Feil and Spratt, 2001; Berg and Kurland, 2002; Johnsen et al., 2009; Kuo and Ochman, 2010). Experimental investigations have shown that most HGT events that integrate into the bacterial chromosome are deleterious (Elena et al., 1998; Remold and Lenski, 2004). Thus, in terms of the persistence of its signature and its effects on fitness, HGT processes resemble routine mutational processes that take place at similarly low frequencies in bacteria and that are eventually lost from the population (Kimura and Ohta, 1969; Jorgensen and Kurland, 1987; Lawrence et al., 2001; Mira et al., 2001; Johnsen et al., 2011). However, rare HGT events and mutations can be positively selected under particular conditions and are the sources of bacterial adaptation and evolution (Imhof and Schlötterer, 2001; Townsend et al., 2003; Orr, 2005; Barret et al., 2006). HGT is particularly well known for playing a central role in the evolution of resistance to antibacterial agents (Bergstrom et al., 2000; Heinemann and Traavik, 2004; Aminov and Mackie, 2007; Aminov, 2010, 2011).

The detection of HGT events in a given bacterial genome can be performed retrospectively through bioinformatics-based comparative analyses (Ochman et al., 2000; Spratt et al., 2001; Nakamura et al., 2004; Didelot and Maiden, 2010). Alternatively, events may be detected via focused experimental efforts on defined bacterial populations under controlled conditions in the laboratory or monitoring efforts on subsamples taken from bacterial populations present in various environments, e.g., from soil, water, wounds, or gastrointestinal tracts (GITs; Nielsen and Townsend, 2004; Thomas and Nielsen, 2005; Pontiroli et al., 2009; Aminov, 2011). The latter approach can enable the identification of HGT events as they occur in the context of complex interactions of

diverse bacterial communities. Its main limitation is sensitivity due to restricted sampling capacity of large bacterial populations, other methodological limitations, and cost of analysis. Representative analysis of HGT events in bacterial communities also depends on knowledge of the structure and population dynamics of the population and the sequence of the DNA transferred. Detection strategies frequently rely on hidden or implicit assumptions regarding the distribution and proportion of the individual cells in the sampled larger bacterial population that would carry the transferred DNA sequences (Keese, 2008; Heinemann et al., 2011).

Large-scale cultivation of genetically modified plants (GM-plants) result in multitudinous opportunities for bacterial exposure to recombinant DNA and therefore opportunities for unintended horizontal dissemination of transgenes (EFSA, 2004, 2009; Nielsen et al., 2005; Levy-Booth et al., 2007; Wögerbauer, 2007; Pietramellara et al., 2009; Brigulla and Wackernagel, 2010). In laboratory-settings, experimental studies of single bacterial species have demonstrated that bacteria can take up DNA fragments from plants and integrate them into bacterial genomes under highly optimized conditions (e.g., Gebhard and Smalla, 1998; De Vries et al., 2001; Kay et al., 2002; Ceccherini et al., 2003). In contrast, in natural settings, sampling-based studies of agricultural soils, run-off water, and GIT contents have found spread of transgenes from GM-plants, but negative or inconclusive evidence for HGT (Gebhard and Smalla, 1999; Netherwood et al., 2004; Mohr and Tebbe, 2007; Demanèche et al., 2008; Douville et al., 2009).

Most research on HGT from GM-plants to bacteria has been performed via an assay after a limited time period following transgene exposure, perhaps in part because only limited explicit considerations of the population dynamics of HGT events have been presented to guide sampling design and data analysis (Heinemann and Traavik, 2004; Nielsen and Townsend, 2004; Nielsen et al., 2005). Given the low mechanistic probability of occurrence, horizontally transferred non-mobile DNA will initially be present at an exceedingly low frequency in the overall population. It may therefore take months, years, or even longer for the few initial transformants to divide and numerically out-compete non-transformed cells of the population to reach frequencies that can be efficiently detected by sampling efforts. The generation time of bacterial populations is therefore of high importance for detection efforts. Cell division time varies with species and environments and can be as short as <1 h in nutrient rich environments such as the GIT and up to several weeks in nutrient limited environments such as soil.

The time lag between initial occurrence and potential detection will be present even though the relevant HGT events lead to positive selection of transformants (Nielsen and Townsend, 2001, 2004; Heinemann and Traavik, 2004; Pettersen et al., 2005). Quantifying this time lag and determining the relationship between HGT frequencies and probability of detection requires mathematical models with dependency on several key parameters: HGT frequencies, changes in relative fitness of the transformants, bacterial population sizes, and generation times in nature. A few studies have accordingly begun to characterize the effects of natural selection and the probability of fixation of HGT events in bacterial populations (Nielsen and Townsend, 2001, 2004; Pettersen et al., 2005).

Here we integrate previous theory into a cohesive probabilistic framework that addresses current methodological shortcomings in the detection of HGT events and guides experimental design of future sampling of bacterial populations. Our analysis yields a simple formulation for the probability of detection given that a HGT actually occurred, and facilitates computation of the statistical power of an experimental sampling design.

We apply the model to four different scenarios that are relevant for experimental monitoring of complex bacterial communities, accounting for both the adaptive dynamics of natural selection and the unknown timing of HGT events. In scenarios 1 and 2, the effects of variable DNA exposure are considered (i.e., exposed sub-population versus the total population of bacteria). Sampling occurs at the end of the DNA exposure period. In scenarios 3 and 4, the sampling is delayed until sometime after the DNA exposure of the bacterial recipients has ended. The total population size (N) and the strength of selection (m) varies in the scenarios (between $N = 10^6$–10^{12}, and $m = 10^{-10}$–1). The m parameter represents the relative cost or advantage conferred by the HGT event to the transformant bacterium compared to untransformed members of the same population. In nature, m values would range from the reciprocal of the population size (weak positive selection) to near infinity (strong positive selection). The latter would for instance be caused by antibiotic treatment leading to death of all susceptible non-transformed cells. However, for most traits much lower values of m are expected. The m value of a given trait is not a constant and will depend on the environmental conditions. For instance, an antibiotic resistance trait can be highly advantageous in the presence of antibiotics (high positive m) but confer a fitness cost in the absence of antibiotics (negative m value; c.f. Johnsen et al., 2011).

MODELING

Immediately following an HGT event into a large bacterial population, the lineage of bacterial cells carrying the novel transferred gene is highly vulnerable to extinction due to natural stochasticity in cell survival over the first generations (Fisher, 1922, 1930; Haldane, 1927; Johnson and Gerrish, 2002; Pettersen et al., 2005). Subsequently, after the transformant population has established at higher numbers, it can be assumed to follow a fairly deterministic path, given continued directional selection. For a selected variant in transit to fixation with Malthusian relative fitness m per generation over t_g generations, the current frequency p of a mutant starting at frequency p_0 can be modeled deterministically as

$$\frac{p}{1-p} = \frac{p_0}{1-p_0} e^{mt_g} \tag{1}$$

(Hartl and Clark, 1997; Nielsen and Townsend, 2004). With a single HGT event, the frequency of the transformant in a haploid population becomes $1/N$ where N is the overall number of bacterial cells in the population of interest. Thus, for the frequency of a transformant (p) subsequent to a HGT event,

$$\frac{p}{1-p} = \frac{1}{N-1} e^{mt_g} \tag{2}$$

(Nielsen and Townsend, 2004). Solved for p, Eq. 2 yields

$$p = \frac{e^{mt_g}}{N - 1 + e^{mt_g}}. \tag{3}$$

Because HGT events are relatively rare and presumably independent, we assume that the time delay until a HGT occurs is exponentially distributed, parameterized by a rate that incorporates the number of bacteria exposed, x, the rate of HGT per exposed bacterium, r, and the time, t_x, during which exposure may occur (See Nielsen and Townsend, 2001 for a detailed description of these factors). Accordingly, the time to the next HGT, T_x, would be distributed as

$$f_{T_x}(t_x) = rxe^{-rxt_x}. \tag{4}$$

The probability of fixation of a new variant gene in a haploid population has been characterized as

$$\frac{1 - e^{-2m}}{1 - e^{-2Nm}}, \tag{5}$$

(Kimura, 1957, 1962; Moran, 1961; Gillespie, 1974; see Patwa and Wahl, 2008, for a review of alternate cases).

Following the exponentially distributed occurrence rate (Eq. 4), filtered by the fixation process (Eq. 5), the timing until the occurrence, T, of the first HGT that is to be eventually fixed in the population, would be distributed as

$$f_T(t) = \left(\frac{1-\mathrm{e}^{-2m}}{1-\mathrm{e}^{-2Nm}}\right) rx\mathrm{e}^{-\left(\frac{1-\mathrm{e}^{-2m}}{1-\mathrm{e}^{-2Nm}}\right)rxt}. \tag{6}$$

Given that an HGT occurs that is on its way to fixation, what is the probability that such a transfer will be detected? This probability depends in part on the sample size of the monitoring effort, n. Here, n is treated as the number of bacteria in the environment sampled in a perfect assay for possession of the HGT event. If the frequency of the primary transformant and its offspring in the population at a given time is p, then the probability of detection is

$$1 - (1 - p)^n. \tag{7}$$

Because the frequency p of a HGT event/transformant that is under strong positive selection deterministically increases with time until it is fixed in the population (**Figure 1**), the probability of detection depends on the amount of time t since the first HGT event occurred, which depends on the time of first exposure to DNA of concern, T. The later the samples are taken, the greater the probability that a selected HGT event on its way to fixation will be detected.

The probability of detection for a HGT event on its way to fixation with selection coefficient m at time t_g after the original transfer event is derived by substituting Eq. 3 for p into Eq. 7 (c.f. Nielsen and Townsend, 2004). Assuming the value of Eq. 3 is very small (i.e., population size is large and selection coefficient is sufficiently small), a useful approximation for the probability of detection of a HGT event on its way to fixation is

$$1 - \left(1 - \frac{\mathrm{e}^{mt_\mathrm{g}}}{N - 1 + \mathrm{e}^{mt_\mathrm{g}}}\right)^n \approx 1 - \left(1 - n\frac{\mathrm{e}^{mt_\mathrm{g}}}{N - 1 + \mathrm{e}^{mt_\mathrm{g}}}\right) = \frac{n\mathrm{e}^{mt_\mathrm{g}}}{N - 1 + \mathrm{e}^{mt_\mathrm{g}}}. \tag{8}$$

However, for practical implementation, the probability term from Eq. 3 may not be known to be small. Furthermore, the unknown timing of the successful HGT is a key factor in the probability of detection. Therefore it would be best to integrate over all possible timings in order to calculate a representative probability of detection of HGT events. Noting that in this case $t_\mathrm{g} = t_\mathrm{s} - t$, this integration, from Eqs 4, 5, and 8, is

$$\int_0^{t_x} \left(\frac{1-\mathrm{e}^{-2m}}{1-\mathrm{e}^{-2Nm}}\right) rx\mathrm{e}^{-\left(\frac{1-\mathrm{e}^{-2m}}{1-\mathrm{e}^{-2Nm}}\right)rxt} \times \left(1 - \left(1 - \frac{\mathrm{e}^{m(t_\mathrm{s}-t)}}{N - 1 + \mathrm{e}^{m(t_\mathrm{s}-t)}}\right)^n\right) \mathrm{d}\,t, \tag{9}$$

or, moving factors that do not depend upon time t out of the integral,

$$\left(\frac{1-e^{-2m}}{1-e^{-2Nm}}\right) rx \int_0^{t_x} \left(1 - \left(1 - \frac{\mathrm{e}^{m(t_\mathrm{s}-t)}}{N - 1 + \mathrm{e}^{m(t_\mathrm{s}-t)}}\right)^n\right) \times \mathrm{e}^{-\left(\frac{1-\mathrm{e}^{-2m}}{1-\mathrm{e}^{-2Nm}}\right)rxt} \mathrm{d}\,t. \tag{10}$$

Equation 10 yields a prediction of the probability of occurrence and detection of a HGT event, and may be parameterized across a range of rates of HGT.

For experimental design purposes (or for prediction for policy purposes), it may be important to calculate not just the full probability of detection, but also the restricted, higher probability of detection given that a successful HGT has occurred. This calculation can be achieved by dividing the result of Eq. 10 by the probability of any successful HGT event over the time t_x,

$$\left(\frac{1-\mathrm{e}^{-2m}}{1-\mathrm{e}^{-2Nm}}\right) rx \int_0^{t_x} \mathrm{e}^{-\left(\frac{1-\mathrm{e}^{-2m}}{1-\mathrm{e}^{-2Nm}}\right)rxt} \mathrm{d}\,t \approx 1 - \mathrm{e}^{-\left(1-\mathrm{e}^{-2m}\right)rxt_x}. \tag{11}$$

The approximation is valid provided N is large compared to m. Setting this approximation aside for generality, the larger probability of detection given that a successful HGT has occurred is then

$$\frac{\int_0^{t_x} \left(1 - \left(1 - \frac{\mathrm{e}^{m(t_\mathrm{s}-t)}}{N-1+\mathrm{e}^{m(t_\mathrm{s}-t)}}\right)^n\right) \mathrm{e}^{-\left(\frac{1-\mathrm{e}^{-2m}}{1-\mathrm{e}^{-2Nm}}\right)rxt} \mathrm{d}\,t}{\int_0^{t_x} \mathrm{e}^{-\left(\frac{1-\mathrm{e}^{-2m}}{1-\mathrm{e}^{-2Nm}}\right)rxt} \mathrm{d}\,t}. \tag{12}$$

MATERIALS AND METHODS

We applied this model to estimate the probability of successfully finding HGT events (e.g., antibiotic resistance genes or transgenes) in bacterial populations under different scenarios representative of various environmental conditions. The total population sizes

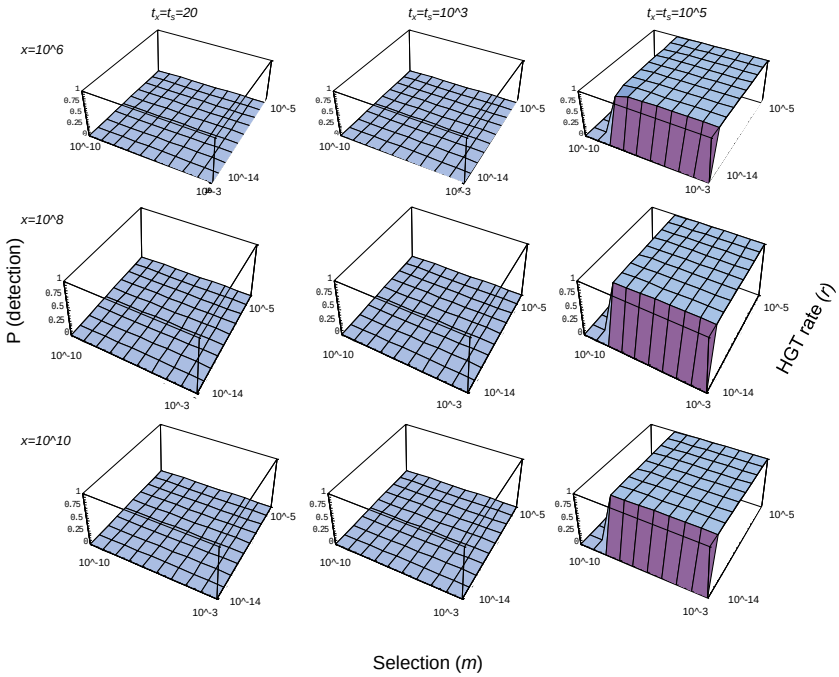


FIGURE 1 | Scenario 1: HGT in large populations, no sampling delay; weak positive selection. Probabilities of detection of transformants in a large bacterial population $N = 10^{12}$ with HGT rates ranging from $r = 10^{-14}$ to 10^{-5}, and weak positive selection of transformants ranging from $m = 10^{-10}$ to 10^{-3}. The proportion of DNA exposed bacteria is low, medium and high ($x = 10^6$, 10^8, and 10^{10}, respectively, out of the 10^{12} total bacterial population, from top to bottom), and the time period of DNA exposure is the same as the time to sampling: $t_x = t_s = 20$, 10^3, and 10^5, from left to right. Sample size $n = 10{,}000$ bacteria.

ranged from 10^6 (small) to 10^{12} (large). We assume only a fraction of the bacterial population that was sampled was exposed to novel genetic material (0.0001–1% in large populations, and 0.1–10% in small populations); resulting in HGT rates (and hence, transformant rates) ranging from 10^{-14} to 10^{-5}. Moreover, as explained above, we considered only transformants that have relative fitness gains, as expressed by a positive selection coefficient, including weak positive selection ($m = 10^{-10}$–10^{-3}) or strong positive selection ($m = 10^{-3}$–1). Our analysis excludes secondary transmissions, a process that may need explicit consideration in cases of plasmid transfer (Landis et al., 2000).

Four different environmental scenarios were examined that broadly represent the population dynamics of HGT events in bacterial populations. The scenarios encompassed: (i) large and small bacterial populations, (ii) strong and weak selection of the HGT events (transformants), and (iii) immediate or delayed sampling, i.e., if the sampling of the larger bacterial population was performed at the end of the DNA exposure, or delayed in time until long after the DNA exposure had ended. Within these scenarios, we varied the HGT rate, the selection coefficient, the ratio of exposed to total population of bacteria, the time period of exposure, and the time until the bacteria was sampled in the field (**Table 1**).

Since approx. 10,000 bacteria represents the upper limit of the number of individual isolates that can be practically assayed in a research laboratory (Nielsen and Townsend, 2004), we assume this sample size ($n = 10{,}000$) for all our scenario calculations, even though effective sample sizes in actual studies to date have been smaller. In scenarios where several samples were taken from a field at several time points, sample size will be proportionally reduced at each sampling point. This ensures comparability among experimental designs.

In scenarios 1 and 2, the focus is on the effects of variable DNA exposure level (exposed sub-population versus the total population of bacteria) and on the strength of selection. We keep the time span of exposure equal to the time span before sampling, i.e., sampling occurs at the end of the DNA exposure (i.e., $t_x = t_s$). In scenarios 3 and 4, the sampling is delayed for considerable amounts of time after the DNA exposure of the bacterial recipients has ended (i.e., $t_x < t_s$).

All calculations were performed and graphics were drawn in the Mathematica 4.1 software (Wolfram Research, IL, USA). The Mathematica notebook containing these calculations is available in the Appendix.

RESULTS

SCENARIO 1. DETECTION OF BACTERIAL TRANSFORMANTS IN LARGE POPULATIONS

Scenario 1 represents a large bacterial population (e.g., abundant members of the soil bacterial community or the GIT of an animal population). Of the total population, only a sub-fraction of 0.0001, 0.01, and 1% is actually exposed to DNA (e.g., due to limited release/exposure of DNA from the defined source and or DNA degradation in soil or the GIT; Nielsen et al., 2007; Nordgård et al., 2007; Rizzi et al., 2012). Those bacteria exposed can acquire DNA

Table 1 | Parameters and their ranges used in this study.

Parameter	Symbol	Range	Comments/reference
Total population size	N	10^6–10^{12}	Overall size of population that is susceptible to HGT in the exposed environment; note this bacterial population may therefore not be limited to a particular species.
Number of exposed bacteria, in large (and small) populations	x	10^6–10^{10} (10–10^5)	The number of the overall susceptible population that will be exposed to the donor DNA source; A smaller fraction of those exposed is transformed. See HGT rate below
Selection coefficient	m	10^{-10}–1	Relative measure of Malthusian fitness in populations with overlapping generations
Time to sampling	t_s	20–10^5	The time (in bacterial generations) since the beginning of DNA exposure to the time of sampling
Time of exposure	t_x	20–10^5	Time of exposure to DNA source (in bacterial generations)
HGT rate	r	10^{-14}–10^{-5}	Frequency of gene transfer into the bacterial population
Sample size	n	10,000	Nielsen and Townsend (2004)

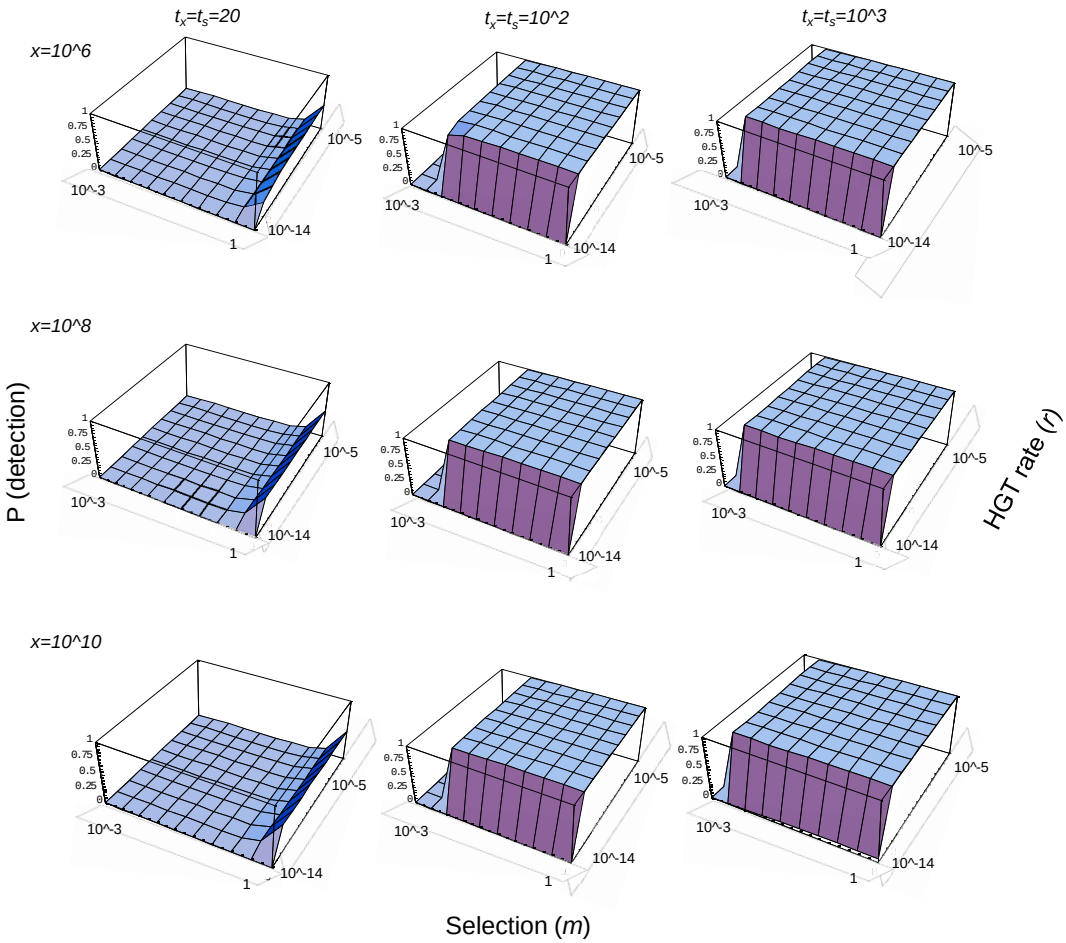


FIGURE 2 | Scenario 1: HGT in large populations, no sampling delay; strong positive selection. Probability of detection of transformants in a large bacterial population, $N = 10^{12}$, with a HGT rate ranging from $r = 10^{-14}$ to 10^{-5}, and strong positive selection on transformants ranging from $m = 10^{-3}$ to 1. The number of bacteria exposed to DNA is low, medium, and high ($x = 10^6$, 10^8, and 10^{10}, respectively, out of the 10^{12} total bacterial population, from top to bottom), and the time period of DNA exposure is the same as time to sampling: $t_x = t_s = 20$, 10^2, and 10^3, from left to right. Sample size $n = 10{,}000$ bacteria.

at rates ranging from extremely low (below what is usually experimentally measurable in the laboratory) to very high. Detection is likely only when sampling was performed after a long period of exposure (**Figures 1** and **2**). The strength of directional selection is of considerable importance. However the determining factor is time of sampling after onset of exposure. To achieve a 90% or greater probability of detection, given that transfer has occurred, requires a selection coefficient greater than $m = 10^{-4}$ (**Figure 1**).

This observation suggests a time interval from the onset of a DNA exposure, until detection is possible, of 11 years in the GIT to up to 3,000 years in soil and (given 10^5 bacterial generations, with generation times of 1 h to 2 weeks, respectively). **Figures 1** and **2** illustrate that an increase in the proportion of exposed bacteria is of little importance when compared to prolonging the time period of DNA exposure and sampling. Given enough time, even weakly but positively selected HGT events (e.g., antibiotic resistance gene or transgene) resulting from DNA exposure to only a small fraction of the total population and with a low HGT rate, is likely to establish in the bacterial population.

SCENARIO 2. DETECTION OF BACTERIAL TRANSFORMANTS IN SMALL POPULATIONS

Scenario 2 considers a small bacterial population. Such a scenario can be representative of fluctuating colonization or infection patterns such as microcolonies on plant, skin, or soil surfaces (e.g., Kinkel et al., 1995; Morris et al., 1997; Monier and Lindow, 2004), or alternatively situations where only a subset of the species/strains in the overall DNA exposed microbial community are capable of acquiring DNA. In this scenario, a sub-fraction of 0.001, 0.1, and 10% of the overall capable population is exposed to DNA and can acquire DNA at rates ranging from extremely low to high frequencies ($r = 10^{-14}$–10^{-5}).

When selection is weak, the HGT events and, hence, resulting transformants, are likely to be detected only after relatively long exposure times, i.e., more than 1,000 generations, and only when the fraction of exposed bacteria is about 0.1% or higher (**Figure 3**). In situations where positive selection is stronger, the HGT event is detectable in the short-term, i.e., after 20 generations, given that the fraction of bacteria that are exposed is high (**Figure 4**, bottom panel). A combination of an intermediate fraction of bacteria exposed (0.1%) and an intermediate time for DNA exposure (1,000 generations) gives a relatively high probability of detecting HGT events (**Figure 4**, middle panel). Even in cases where the fraction of bacteria exposed to the DNA is very low, long-term exposure ($t_x > 10^5$ generations) and strong positive selection will lead to establishment of transformants, i.e., at detectable levels (**Figure 4**, upper panel). Our longest generation time examined, 10^5, represents a continual exposure period of 10 years (with a bacterial division time of 1 h or less) to more than 3,000 years (with a bacterial division time of 11 days or more).

THE EFFECT OF DELAYED SAMPLING AFTER SHORT-TERM TRANSIENT EXPOSURE TO DNA

In the following scenarios (three and four, weak and strong selection, respectively), the bacteria are exposed to DNA for only a short period of time (20–100 generations, e.g., representing a time period of a less than a day to a few years, depending on bacterial growth rate). In all cases, the bacterial population is sampled after exposure. That is, a time delay before sampling is introduced after the end of the exposure period that provides additional time for directional selection (of a range of intensities) to act on the transformed cells. These scenarios illustrate situations where the sampled microbial community (e.g., agricultural soil, GIT) is only temporarily exposed to the DNA source in question (e.g., soil or GIT bacteria by seasonal crop cultivation or consumption patterns). We examined large populations ($N = 10^{12}$) only, and applied different parameter values for the weak and strong selection scenarios. For the weak positive selection scenario, the exposure time was 100 generations, the exposed population was of size $n = 10^8$ and the time lag before sampling ranged from 10^4 to 10^5 generations). For the strong selection scenario, the DNA exposure time was extremely short (20 bacterial generations), the exposed population was 10^6, and the time lag before sampling ranged from none (i.e., sampling at the end of exposure), 30 bacterial generations, or 80 bacterial generations later.

SCENARIO 3. DELAYED SAMPLING – LARGE POPULATIONS AND WEAK POSITIVE SELECTION

In situations where potential transformants experience only weak positive selection (10^{-10}–10^{-3}), with 0.01% of the bacterial population exposed to DNA, and an exposure time of 100 generations, no HGT events could be detected either at the end of the exposure or after a delayed sampling (10^4 generations after DNA exposure; **Figure 5**, left). However, a further 5- to 10-fold increase in the time delay before sampling (to 5×10^4 and 10^5 generations) yielded increasing probabilities of detecting the HGT events (**Figure 5**, middle and right). Thus, theoretically, in environmental situations where the bacterial generation time is very short (e.g., in a mammalian gut system), HGT events arising from limited, transient DNA exposure can be detected, providing they are positively selected and have had the necessary time to increase in relative numbers within the overall population. However, even the most rapidly dividing bacterial populations would need more than 10 years to comprise 5×10^4 and 10^5 generations (the 10-year figure would assume a bacterial division time of 30 min). Supposing this scenario represented an environment with intermittent antibiotic treatments, the effect of the length of the time period before transformants become detectable would be sensitive to any inconstancy of selection. The time period before the transformant population either increases in proportion to detectability or is lost from the population would therefore be different, and typically longer, in a situation with more variable selection dynamics.

SCENARIO 4. DELAYED SAMPLING – LARGE POPULATIONS AND STRONG SELECTION

Under strong positive selection, even HGT events occurring as a consequence of exposure of a very low overall proportion of the population (here 0.0001%) over a short period of time ($t = 20$ generations) can be detected (**Figure 6**). The time of sampling nevertheless remains a significant factor in the probability of detection. Sampling at the end of the exposure ($T = 20$) yields a low probability of detection (**Figure 6**, left). In contrast, introducing a time lag before sampling, here 30 and 80 generations after the end of exposure ($T = 50$ and 100, respectively), results in a sharp increase in the likelihood of detection (**Figure 6**, middle and right). The starkness of this result may at first seem surprising; however positive selection that increases the frequency of the transformed bacteria is the main characteristic that makes detection possible; other factors in this scenario are of negligible importance. Strong directional selection makes such HGT events less affected

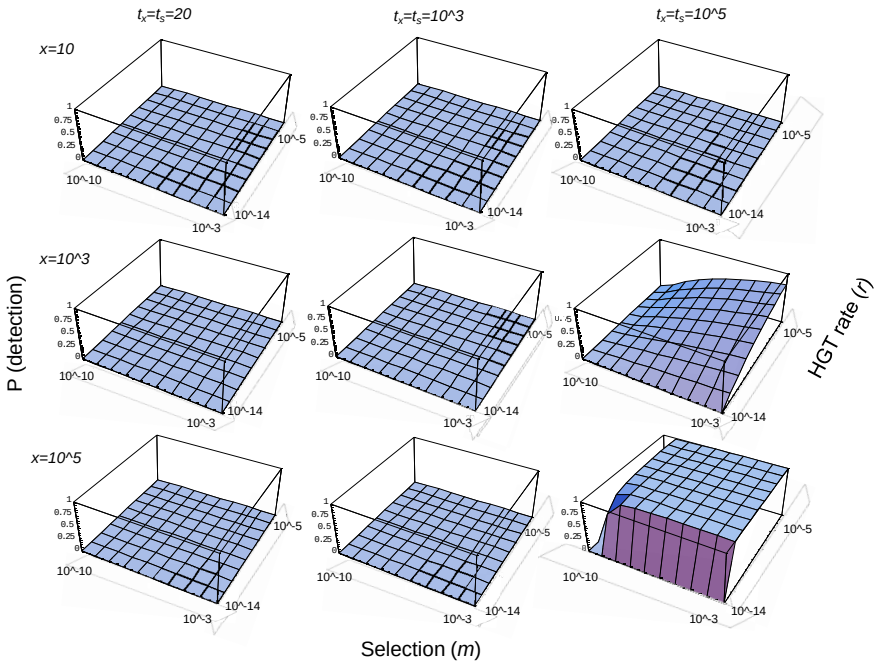


FIGURE 3 | Scenario 2: HGT in small populations, no sampling delay; weak positive selection. Probability of detection of transformants in a small bacterial population, $N = 10^6$, with HGT rates ranging from $r = 10^{-14}$ to 10^{-5}, and weak positive selection on transformants ranging from $m = 10^{-10}$ to 10^{-3}. The number of bacteria exposed to the DNA is low, medium, and high ($x = 10$, 10^3, and 10^5, respectively, out of the 10^6 total bacterial population, from top to bottom), and the time period of exposure is the same as the time to sampling; $t_x = t_s = 20$, 10^3, and 10^5, from left to right. Sample size $n = 10{,}000$ bacteria.

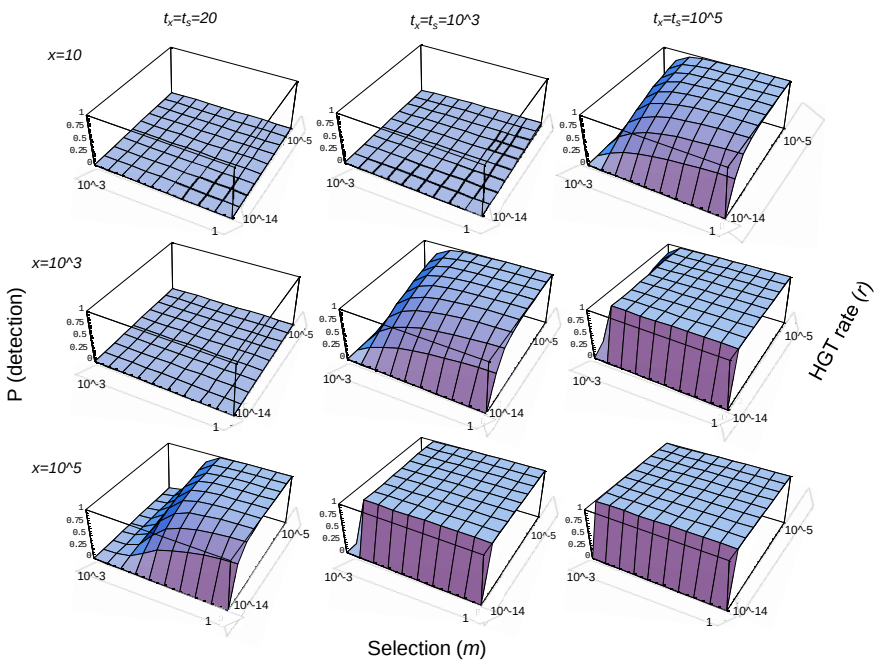


FIGURE 4 | Scenario 2: HGT in small populations, no sampling delay; strong positive selection. Probability of detection of transformants in a small bacterial population, $N = 10^6$, with HGT rates ranging from $r = 10^{-14}$ to 10^{-5}, and strong positive selection on transformants ranging from $m = 10^{-3}$ to 1. The number of bacteria exposed to DNA is low, medium, and high ($x = 10$, 10^3, and 10^5, respectively, out of the 10^6 total bacterial population, from top to bottom), and the time period of exposure is the same as the time to sampling: $t_x = t_s = 20$, 10^3, and 10^5, from left to right. Sample size $n = 10{,}000$ bacteria.

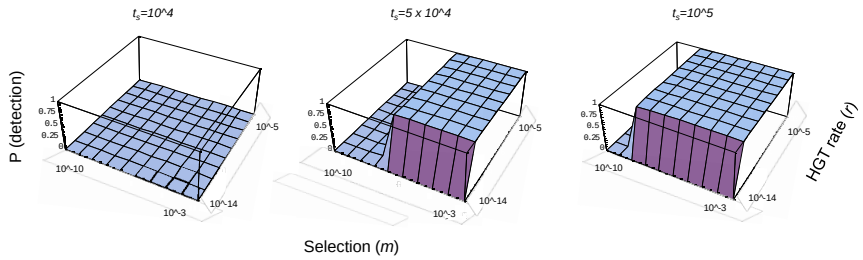


FIGURE 5 | Scenario 3: HGT in large populations with delayed sampling; weak positive selection. Probability of detection of transformants in a large bacterial population, $N = 10^{12}$, with a HGT rate ranging from $r = 10^{-14}$ to 10^{-5} experiencing a short exposure of $t_x = 100$ generations, with delayed sampling. The proportion of the bacteria exposed to DNA is medium (0.01%) and the selection on transformants is weak, ranging from $m = 10^{-10}$ to 10^{-3}. The time of sampling, encompassing the time of DNA exposure t_x, is, from left to right, $t_s = 10^4$, 5×10^4, and 10^5 generations. Sample size $n = 10{,}000$ bacteria.

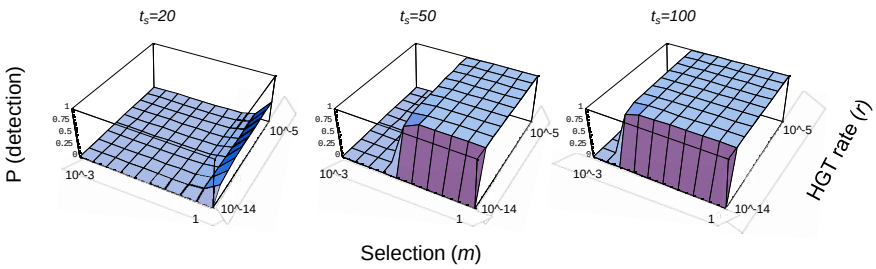


FIGURE 6 | Scenario 4: HGT in large populations with delayed sampling; strong positive selection. Probability of detection of bacterial transformants in a large population $N = 10^{12}$ with a HGT ranging from $r = 10^{-14}$ to 10^{-5}, experiencing a short DNA exposure time $t_x = 20$, with sampling at the end of exposure or delayed. The proportion of the bacteria exposed is low (0.0001%). The selection on transformant bacteria is strong, ranging from $m = 10^{-3}$ to 1. The time of sampling after the onset of DNA exposure is $t_s = 20$, 50, and 100 generations. Sample size $n = 10{,}000$ bacteria.

by genetic drift and the impact of the events therefore closer reflect the distribution of their initial occurrence, similarly to the fate of strongly beneficial mutations (Barret et al., 2006).

APPLICATION TO GMP–HGT MONITORING STUDIES

Application of the model presented here to sampling parameters derived from published field monitoring studies of bacterial populations exposed to GMPs shows limited potential to recover HGT events, had they occurred (**Figure 7**). Only transgenes conferring strong positive selection coefficients (approx. $m = 0.09$ or higher) are likely to be detected with high probability by sampling of microbial communities with limited exposure to the GM-plant material (over 365 generations, representing 1 year of exposure). In contrast, HGT of transgenes conferring 10-fold weaker positive selection coefficients ($m = 0.009$) can be detected with reasonable probability after limited sampling of microbial communities given a 10-fold longer exposure.

The 10-year long exposure period in a field-based monitoring study by Demanèche et al. (2008) represented the longest exposure period before HGT assay to date. Demanèche and colleagues in France examined samples from agricultural sites that had been cultivated with a GM maize variety over a 10 years time period. In addition, soil samples taken from a conventional field and pasture were included (three sample types) in the analyses. The GM maize variety (event *Bt*-176) harbored the $bla_{\text{TEM-116}}$ allele, conferring

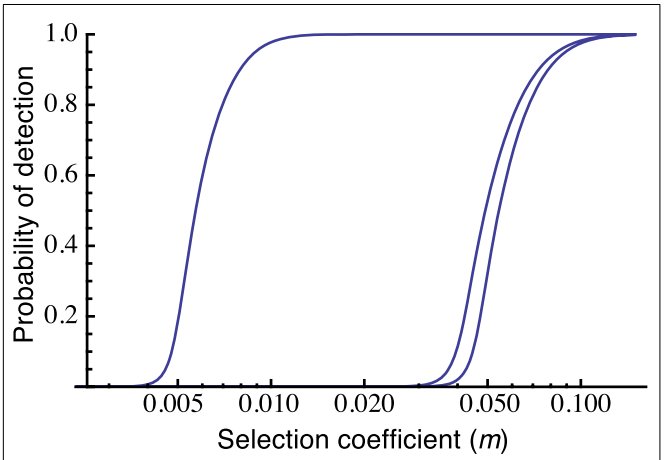


FIGURE 7 | Calculation of the probability of detecting HGT in the field, parameterized with sample sizes from published studies, for selection coefficients ranging from $m = 0$ to 0.1. Graphs from right to left: a sample size of 600 (Paget et al., 1998), and a sample size of 4000 (Gebhard and Smalla, 1999). The two right graphs assume sampling after $t_s = 365$ generations. The graph on the left side represents a sample size $n = 192$ after $t_s = 3{,}650$ generations (Demanèche et al., 2008). In all cases, A HGT rate of $r = 10^{-8}$ per generation and a population size of $N = 10^{10}$ bacteria was assumed.

bacterial resistance to certain beta-lactam antibiotics. The outcome of the study was that a range of TEM-type alleles could be found in soil samples taken both from GM and non-GM, conventional sites. The study recovered up to 2.5×10^5 culturable bacteria per gram soil sample and reported up to 6.5% of the ampicillin resistance among the culturable fraction.

A total of 576 resistant colonies were further analyzed with PCR targeted toward bla_{TEM}. Of the 576 amplifications performed on samples from the three different field types, 505 were TEM-positive, representing 87.7% of the resistant fraction. DNA of 80 of the 505 TEM-positive samples was sequenced. Of these, 10 were found to represent the $bla_{\text{TEM-116}}$ allele that is also present in the GM maize event *Bt*-176. However, only 4 out of the 10 positive samples originated from the soils cultivated with the transgene. Thus, the authors concluded that the resistance gene was already prevalent in soil and that there was no evidence for horizontal dissemination from the GM-plant variety over the 10-year time period. In **Figure 7**, we assume a sample size of 192 (1/3rd of the 576 resistant samples analyzed by PCR) and a bacterial generation time of one per day (3,650 generations). It is noted that bacterial generation time will vary according to species and strain, as well to seasonal and tempo-spatial effects in soil. Application of our model to the study of Demanèche et al. (2008) suggests that HGT events with m values as low as 0.009 can be detected with at least 90% probability. At these weak levels of selection, the key factor determining the detection probability is the number of bacterial generations, in this case assumed to be 1 per day. Increasing the sample size above $n = 192$ would have little effect on the probability of detection.

DISCUSSION

We have presented a probabilistic framework for detection of initially rare HGT events/transformants by sampling of larger bacterial populations. This result expands on earlier studies (Nielsen and Townsend, 2001, 2004; Pettersen et al., 2005) to derive a quantitative approach for analysis of the time scale over which HGT events take place and can be detected. Our population genetic framework facilitates practical implementation as well as a more detailed examination of the relative role of the key factors determining the fate of horizontally acquired genes in bacterial populations. The utility of the quantitative approach presented here is, although dependent on some knowledge of the rates of the relevant processes, independent of the specific mechanism of HGT (e.g., transduction, conjugation, transformation). The model is therefore equally applicable to understanding HGT processes between bacterial species/strains/cells as it is applicable to HGT events occurring between unrelated species. Quantitative adjustments to the DNA exposure and HGT rate can accommodate diverse mechanisms. Furthermore, the model identifies parameter values that should guide further hypothesis formation and experimental design.

SELECTION

The design of sampling approaches aimed at detecting rare HGT processes is deeply challenging, because the fundamental task is to detect a very low probability event with a very small sample size in a very large population. Field studies over limited time periods are correspondingly not likely to identify rare HGT events in large and complex bacterial communities (**Figure 7**). The detection of HGT events is therefore most often feasible only if the few initial transformants have a growth advantage so they increase their relative proportion in the overall population. However, there are methodological challenges to the implementation of defined selective conditions at the DNA exposure stage when rare transformants arise.

In laboratory systems, the use of antibiotics at concentrations below the minimal inhibitory concentrations (MIC) can possibly apply such directional selection, and hence enrichment of rare transformants present in large, complex microbial communities. However, it is a non-trivial problem to experimentally achieve sub-lethal concentrations of antibiotics that confer directional selection of rare transformants without simultaneously limiting the viability of the overall bacterial population.

In field systems, directional selection and enrichment of initially rare transformants will depend on the prevailing environmental conditions. There are usually few opportunities to introduce directional selection with controlled selection coefficients.

As exemplified in this study, directional selection typically dominates determination of the probability of detection. Strong sampling designs would therefore avoid focus on the detection of the initial HGT events (and associated HGT frequencies), but rather attempt to detect positively selected descendants of the primary transformants. A shift in focus to the detection of descendants precludes precise determination of HGT frequencies. However, frequencies are poor predictors of the short and long-term (evolutionary) impact of HGT events. As long as such events occur repeatedly, other factors will determine the biological impact of these events (Pettersen et al., 2005).

Our calculations are based on a fixed selection coefficient m. However, the strength of selection will frequently fluctuate over space and time due to environmental variables, as well as variability among bacterial genotypes attributable to gene-by-environment interactions (Kimura, 1954; Barker and Butcher, 1966). Thus, selection coefficients will be inexact and will rarely be amenable to robust quantification over variable environments. Furthermore, the genome of a given bacterial transformant will be exposed to other HGT events and mutational processes that may change the initial beneficial fitness effects of a given HGT event (Lenski et al., 1991; Gerrish, 2001; Heffernan and Wahl, 2002; Rozen et al., 2002; Barret et al., 2006; Johnsen et al., 2011).

In practice, host and environmental variation prevents precise and meaningful quantification of m values. Theoretical modeling approaches, however, offer the opportunity to examine the effects of broad ranges of m, therefore providing opportunities to identify threshold values and to predict the dynamics of rare HGT events in larger bacterial populations.

FIXATION

Our approach quantifies detection of HGTs that are on their way to fixation (Kimura, 1962), whereas the biological importance of HGT events arises at population proportions much less than one. For instance, the prevalence of a pathogenic strain carrying an HGT event encoding antibiotic resistance is of highest

interest when its relative proportion among sensitive strains is <0.1–0.3; as higher proportions will lead to changes in clinical prescription guidance for first line antibiotic therapy (Daneman et al., 2008). Random or seasonal variations in local population sizes may also cause particular genotypes (e.g., transformants) to fluctuate at low frequencies above or below detection for long periods of time (Gerrish and Lenski, 1998). Genetic drift and uneven survival rates in structured bacterial populations are important in determining the fate of transformants (Heffernan and Wahl, 2002; Pettersen et al., 2005). The event of key importance is therefore when the transformant proportion rises to the point where subsequent evolution is largely deterministic based on the current level of directional selection (Rouzine et al., 2001).

The probability of fixation of a transformant by genetic drift alone is governed by the inverse of population size. Given geographically dispersed and large population sizes, the fixation of a horizontally acquired gene/transgene in a bacterial population has been viewed as unlikely (Berg and Kurland, 2002). However, see also views by Majewski and Cohan (1999), Cohan (2002, 2005), and Novozhilov et al. (2005). The likelihood of fixation of a neutral HGT event may differ from the likelihood of fixation of a neutral mutation; this is because mutations occur routinely and repeatedly in large bacterial populations, whereas HGT events may be much more tempo-spatially variable.

SPATIAL CONSIDERATIONS

Although our model accounts for the effects of natural selection over time, it contains no inherent spatial component. Samples should be collected with consideration that rare horizontal transfers are not expected to occur and be distributed evenly in large, structured bacterial populations. Similarly, antibiotic resistance genes or transgenes are likely to be initially present only in a limited number of patches (e.g., patients/hospitals, or soil sites/fields); representing metapopulations of the larger global population (Maynard Smith et al., 2000). Initial frequencies will match their occurrence, but subsequent frequencies will correspond to the outcome of spatially variable directional selection and genetic drift. Migration between patches may also be of variable intensity and directionality. Uneven distribution patterns need to be considered in the sampling design.

FIELD MONITORING

The analyses of published GMP field monitoring studies (**Figure 7**) indicated that detection of HGT events could only be achieved under circumstances of strong positive selection of the hypothesized transformants. Selection coefficients as high as $m = 0.05$, by evolutionary genetic standards, represent an extraordinary adaptive event. In the laboratory, selection coefficients as small as $m = 0.01$ can be measured, and over evolutionary time, selection coefficients as small as the inverse of the effective population size (here, this would be as small as 10^{-10} bacteria per gram sample) are of importance in determining the genome composition of organisms. The retrospective analyses of these studies also suggest that increasing the sample size massively does little to increase the probability of detecting a HGT event that has occurred. However, increasing the delay between exposure and testing permits detection of HGT events characterized by much lower selection coefficients.

Most of the field sampling-based HGT studies published so far have been based on a number of implicit assumptions on the characteristics of the biological system investigated. A more formal theoretical analysis of the population genetic aspects of the system investigated will contribute to make these assumptions explicit; and therefore provide improved clarity and robustness to future experimental design. The model presented here aims to provide guidance on future field-based sampling incorporating key population genetic factors. The multiple levels of, and importance of population genetic considerations in understanding horizontal gene flow have recently been reviewed by Baquero and Coque, 2011, and references within) and Zur Wiesch et al. (2011).

CLINICAL SETTINGS

The Research topic for this particular issue of the journal is on resistance genes in the open environment, not in clinical settings. The practical scenarios examined in this study are therefore taken from non-clinical environments. However, the general insight of the presented study is also conceptually relevant to the general aspects of the population genetics of horizontal gene flow in clinical environments. This generality arises because, as we indicate in the Section "Discussion," the model design does not rely on a given DNA transfer mechanism or particular environmental conditions. Our model examines the relationship between the four essential components determining the fate of initially rare HGT events in larger populations: (exposed) population size, HGT rates, bacterial generation time and selective advantage. These four population parameters are essential to the fate of HGT events occurring both in clinical settings among pathogens, as well as in non-clinical settings among non-pathogens. Despite the general insight to clinical scenarios that our model might provide, certain characteristics of the lifestyle of clinical pathogenic populations render specific calculations based on our model to be inappropriate. These characteristics include:

1. Exceptionally strong selective environments are caused by the use of high doses of antibiotics for treatment of bacterial infections. An acquisition of a resistance gene under antibiotic treatment is exceptionally advantageous, as, 100% of the susceptible population is likely to die. Thus, the relative growth advantage is immense, leading to very rapid population expansion of the transformant population and the absence of competitors. Such strong positive selection is not comparable to the much weaker levels of positive selection for most other traits in non-clinical environments. Moreover, clinical antibiotic usage is also highly time-limited, producing strong fluctuations in the selection for a given resistance trait over time, that would require dynamic epidemiological modeling. We assume constant selection over time in our model.
2. The infectious lifestyle of some pathogens leads to exceptionally rapid changes in their population sizes (during infections) followed by strong bottlenecks (during transmission). Thus, depending on the pathogen in question, the transformed cells may or may not be competing with non-transformed members of their populations. Thus, the fitness

effects may have a different context in clinical environments depending on the characteristics of the infectious pathogen in question.

The infection pattern of the pathogen in question will also determine its initial population size *N*, a value that would be very low for a strict pathogen (e.g., tuberculosis) but perhaps initially somewhat larger for opportunistic pathogens (e.g., *Clostridium difficile*). However, our model assumes a more stable environment with a constant large population size, and is based on a competitive growth advantage of the transformant (relative to non-transformed members of the same populations; present in the same environment). This growth advantage will materialize as higher cell division rates for the transformant; the rate difference expressed through the *m* (Malthusian fitness parameter) value. Thus, a materialized growth advantage requires the presence of a much larger non-transformed population.

In summary, our model is not designed to capture the intense short-term positive selection, population expansions (infections), coupled with bottlenecks (insufficient antibiotic treatment, and or transmission of a few bacteria to the next patient) that lead to different ranges of population genetic parameters and other model assumptions. From our point of view, such characteristics cannot be included in our model without addressing the etiology of infections and resistance patterns of individual pathogenic strains. Such developments are of high interest for further work.

From the application of our model in the examination of various environmental scenarios, cases and literature examples, it can be concluded that some interspecies HGT is likely to occur over time and spatial scales not amenable to direct experimental observation. The model suggests sampling-based detection of the descendants (offspring) of the initial transformants is achievable; emphasizing that the probability of detection can only correspond to a calculable level of selection, and that a powerful experimental design requires a delayed sampling strategy.

The recent publications by Gallet et al. (2012) and Toprak et al. (2012) present innovative laboratory approaches for quantification of weak positive selection, or for selection of initially rare but positively selected bacterial phenotypes.

ACKNOWLEDGMENTS

Thomas Bøhn and Kaare Magne Nielsen acknowledge financial support from Genøk, the Research Council of Norway and the University of Tromsø. Kaare Magne Nielsen acknowledges EU COST Action network FP0905, and the AGES, FEAR project, Austria.

REFERENCES

Aminov, R. I. (2010). A brief history of the antibiotic era: lessons learned and challenges for the future. *Front. Microbiol.* 1:134. doi: 10.3389/fmicb.2010.00134

Aminov, R. I. (2011). Horizontal gene exchange in environmental microbiota. *Front. Microbiol.* 2:158. doi: 10.3389/fmicb.2011.00158

Aminov, R. I., and Mackie, R. I. (2007). Evolution and ecology of antibiotic resistance genes. *FEMS Microbiol. Lett.* 271, 147–161.

Baquero, F., and Coque, T. M. (2011). Multilevel population genetics in antibiotic resistance. *FEMS Microbiol. Rev.* 35, 705–706.

Barker, J. S. F., and Butcher, J. C. (1966). A simulation study of quasi-fixation of genes due to random fluctuation of selection intensities. *Genetics* 53, 261–268.

Barret, R. D. H., M'Gonigle, L. K., and Otto, S. P. (2006). The distribution of beneficial mutant effects under strong selection. *Genetics* 174, 2071–2079.

Bensasson, D., Boore, J. L., and Nielsen, K. M. (2004). Genes without frontiers. *Heredity* 92, 483–489.

Berg, O. C., and Kurland, G. C. (2002). Evolution of microbial genomes: sequence acquisition, and loss. *Mol. Biol. Evol.* 19, 2265–2276.

Bergstrom, C. T., Lipsitch, M., and Levin, B. R. (2000). Natural selection, infectious transfer and the existence conditions of bacterial plasmids. *Genetics* 155, 505–519.

Bertolla, F., and Simonet, P. (1999). Horizontal gene transfers in the environment: natural transformation as a putative process for gene transfers between transgenic plants and micro-organisms. *Res. Microbiol.* 150, 5409–5420.

Brigulla, M., and Wackernagel, W. (2010). Molecular aspects of gene transfer and foreign DNA acquisitions in prokaryotes with regard to safety issues. *Appl. Microbiol. Biotechnol.* 86, 1027–1041.

Ceccherini, M. T., Poté, J., Kay, E., Tran Van, V., Maréchal, J., Pietramellara, G., Nannipieri, P., Vogel, T. M., and Simonet, P. (2003). Degradation and transformability of DNA from transgenic plants. *Appl. Environ. Microbiol.* 69, 673–678.

Cohan, F. M. (2002). "Clonal structure," in *Encyclopedia of Evolution*, ed. M. Pagel (New York: Oxford University Press), 159–163.

Cohan, F. M. (2005). "Periodic selection and ecological diversity in bacteria," in *Selective Sweeps*, ed. D. Nurminsky (Georgetown: Landes Bioscience), 78–93.

Daneman, N., Low, D. E., McGeer, A., Green, K. A., and Fisman, D. N. (2008). At the threshold, defining clinically meaningful resistance thresholds for antibiotic choice in community-acquired pneumonia. *Clin. Infect. Dis.* 46, 1131–1138.

De Vries, J., Meier, P., and Wackernagel, W. (2001). The natural transformation of *Pseudomonas stutzeri* and *Acinetobacter sp.* by kanamycin resistance genes (nptII) present in plasmids and the genome of transgenic plants depends on homologous sequences in the recipient cells. *FEMS Microbiol. Lett.* 195, 211–215.

Demanèche, S., Sanguin, H., Poté, J., Navarro, E., Bernillon, D, Mavingui, P., Wildi, W., Vogel, T. M., and Simonet, P. (2008). Antibiotic resistant soil bacteria in transgenic plant fields. *Proc. Natl. Acad. Sci. U.S.A.* 105, 3957–3962.

Didelot, X., and Maiden, M. C. (2010). Impact of recombination on bacterial evolution. *Trends Microbiol.* 18, 315–322.

Douville, M., Gagné, F., Andre, C., and Blaise, C. (2009). Occurrence of the transgenic corn cry1Ab gene in freshwater mussels (*Elliptio complanata*) near corn fields: evidence of exposure by bacterial ingestion. *Ecotoxicol. Environ. Saf.* 72, 17–25.

EFSA. (2004). Opinion of the scientific panel on genetically modified organisms on the use of antibiotic resistance genes as marker genes in genetically modified plants. *EFSA J.* 48, 1–18.

EFSA. (2009). Use of antibiotic resistance genes as marker genes in genetically modified plants. Scientific opinion of the panel on genetically modified organisms (GMO) and the panel on biological hazards (BIOHAZ). *EFSA J.* 1034, 1–82.

Eisen, J. (2000). Horizontal gene transfer among microbial genomes: new insights from complete genome analysis. *Curr. Opin. Genet. Dev.* 10, 606–611.

Elena, S. F., Ekunwe, L., Hajela, N., Oden, S. A., and Lenski, R. E. (1998). Distribution of fitness effects caused by random insertion mutations in *Escherichia coli*. *Genetica* 102–103, 349–358.

Feil, E. J., and Spratt, B. G. (2001). Recombination and the population structure of bacterial pathogens. *Annu. Rev. Microbiol.* 55, 561–590.

Fisher, R. A. (1922). On the dominance ratio. *Proc. R. Soc. Edinb.* 52, 399–433.

Fisher, R. A. (1930). *The Genetical Theory of Natural Selection.* Oxford: Clarendon Press, 38–41.

Gallet, R., Cooper, T. F., Elena, S. F., and Lenormand, T. (2012). Measuring selection coefficients below 10^{-3}: method, questions, and prospects. *Genetics* 190, 175–186.

Gebhard, F., and Smalla, K. (1998). Transformation of *Acinetobacter* sp. strain BD413 by transgenic sugar beet DNA. *Appl. Environ. Microbiol.* 64, 1550–1554.

Gebhard, F., and Smalla, K. (1999). Monitoring field releases of genetically modified sugar beets for persistence of transgenic plant DNA and horizontal gene transfer. *FEMS Microbiol. Ecol.* 28, 261–272.

Gerrish, P. (2001). The rhythm of microbial adaptation. *Nature* 413, 299–302.

Gerrish, P., and Lenski, R. E. (1998). The fate of competing beneficial mutations in an asexual population. *Genetica* 102/103, 127–144.

Gillespie, J. H. (1974). Natural selection for within-generation variance in offspring number. *Genetics* 76, 601–606.

Haldane, J. B. S. (1927). A mathematical theory of natural and artificial selection. Part V. Selection and mutation. *Proc. Camb. Philos. Soc.* 28, 838–844.

Hartl, D. L., and Clark, A. G. (1997). *Principles of Population Genetics*, 3rd Edn. Sunderland: Sinauer Associates Inc.

Heffernan, J. M., and Wahl, L. M. (2002). The effects of genetic drift in experimental evolution. *Theor. Popul. Biol.* 62, 349–356.

Heinemann, J. A., Kurenbach, B., and Bleyendaal, N. (2011). Evaluation of horizontal gene transfer monitoring experiments conducted in New Zealand between 2004 and 2009. *J. Org. Syst.* 6, 3–19.

Heinemann, J. A., and Traavik, T. (2004). Problems in monitoring horizontal gene transfer in field trials of transgenic plants. *Nat. Biotechnol.* 22, 1105–1109.

Imhof, M., and Schlötterer, C. (2001). Fitness effects of advantageous mutations in evolving *Escherichia coli* populations. *Proc. Natl. Acad. Sci. U.S.A.* 98, 1113–1117.

Johnsen, P. J., Townsend, J., Bøhn, T., Simonsen, G. S., Sundsfjord, A., and Nielsen, K. M. (2009). Factors affecting the reversal of antimicrobial resistance. *Lancet Infect. Dis.* 9, 357–364.

Johnsen, P. J., Townsend, J. P., Bøhn, T., Simonsen, G. S., Sundsfjord, A., and Nielsen, K. M. (2011). Retrospective evidence for a biological cost of vancomycin resistance in the absence of glycopeptide selective pressures. *J. Antimicrob. Chemother.* 66, 608–610.

Johnson, T., and Gerrish, P. J. (2002). The fixation probability of a beneficial allele in a population dividing by binary fission. *Genetica* 115, 283–287.

Jorgensen, F., and Kurland, C. G. (1987). Death rates of bacterial mutants. *FEMS Microbiol. Lett.* 40, 43–46.

Kay, E., Vogel, T. M., Bertolla, F., Nalin, R., and Simonet, P. (2002). In situ transfer of antibiotic resistance genes from transgenic (transplastomic) tobacco plants to bacteria. *Appl. Environ. Microbiol.* 68, 3345–3351.

Keese, P. (2008). Risks from GMOs due to horizontal gene transfer. *Environ. Biosafety Res.* 7, 123–149.

Kimura, M. (1954). Process leading to quasi-fixation of genes in natural populations due to random fluctuation of selection intensities. *Genetics* 39, 280–295.

Kimura, M. (1957). Some problems of stochastic processes in genetics. *Ann. Math. Stat.* 28, 882–901.

Kimura, M. (1962). On the probability of fixation of mutant genes in a population. *Genetics* 47, 713–719.

Kimura, M., and Ohta, T. (1969). Average number of generations until extinction of an individual mutant gene in a finite population. *Genetics* 61, 763–771.

Kinkel, L. L., Wilson, M., and Lindow, S. E. (1995). Effects of scales on estimates of epiphytic bacterial populations. *Microb. Ecol.* 29, 283–297.

Kuo, C. H., and Ochman, H. (2010). The extinction dynamics of bacterial pseudogenes. *PLoS Genet.* 6, e1001050. doi: 10.1371/journal.pgen.1001050

Landis, W. G., Lenart, L. A., and Spromberg, J. A. (2000). Dynamics of horizontal gene transfer and the ecological risk assessment of genetically engineered organisms. *Hum. Ecol. Risk Assess.* 6, 875–899.

Lawrence, J. G. (2002). Gene transfer in bacteria: speciation without species? *Theor. Popul. Biol.* 61, 449–460.

Lawrence, J. G., Hendrix, R. W., and Casjens, S. (2001). Where are the pseudogenes in bacterial genomes? *Trends Microbiol.* 9, 535–540.

Lenski, R. E., Rose, M. R., Simpson, S. C., and Tadler, S. C. (1991). Long-term experimental evolution in *Escherichia coli*. 1. Adaptation and divergence during 2 000 generations. *Am. Nat.* 138, 1315–1341.

Levy-Booth, D. J., Campbell, R. G., Gulden, R. H., Hart, M. M., Powell, J. R., Klironomos, J. N., Pauls, K. P., Swantonb, C. J., Trevors, J. T., and Dunfield, K. E. (2007). Cycling of extracellular DNA in the soil environment. *Soil Biol. Biochem.* 39, 2977–2991.

Majewski, J., and Cohan, F. M. (1999). Adapt globally, act locally: the effect of selective sweeps on bacterial sequence diversity. *Genetics* 152, 1459–1474.

Maynard Smith, J., Feil, E. J., and Smith, N. H. (2000). Population structure and evolutionary dynamics of pathogenic bacteria. *Bioessays* 22, 1115–1122.

Mira, A., Ochman, H., and Moran, N. A. (2001). Deletion bias and the evolution of bacterial genomes. *Trends Genet.* 17, 589–596.

Mohr, K., and Tebbe, C. (2007). Field study results on the probability and risk of a horizontal gene transfer from transgenic herbicide-resistant oilseed rape pollen to gut bacteria of bees. *Appl. Microbiol. Biotechnol.* 75, 573–582.

Monier, J. M., and Lindow, S. E. (2004). Frequency, size, localization of bacterial aggregates on bean leaf surfaces. *Appl. Environ. Microbiol.* 70, 346–355.

Moran, P. A. P. (1961). The survival of a mutant under general conditions. *Proc. Camb. Philos. Soc.* 57, 304–314.

Morris, C. E., Monier, J. M., and Jacques, M. A. (1997). Methods for observing microbial biofilms directly on leaf surfaces and recovering them for isolation of culturable microorganisms. *Appl. Environ. Microbiol.* 63, 1570–1576.

Nakamura, Y., Itoh, T., Matsuda, H., and Gojobori, T. (2004). Biased biological functions of horizontally transferred genes in prokaryotic genomes. *Nat. Genet.* 36, 760–766.

Netherwood, T., Martín-Orúe, S. M., O'Donnell, A. G., Gockling, S., Graham, J., Mathers, J. C., and Gilbert, H. J. (2004). Assessing the survival of transgenic plant DNA in the human gastrointestinal tract. *Nat Biotechnol.* 22, 204–209.

Nielsen, K. M., Berdal, K. G, Kruse, H., Sundsfjord, A., Mikalsen, A., Yazdankhah, S., and Nes, I. (2005). *An Assessment of Potential Long-Term Health Effects Caused by Antibiotic Resistance Marker Genes in Genetically Modified Organisms Based on Antibiotic Usage and Resistance Patterns in Norway*. VKM-Report, Norwegian Scientific Committee for Food Safety, Oslo.

Nielsen, K. M., Johnsen, P. J., Bensasson, D., and Daffonchio, D. (2007). Release and persistence of extracellular DNA in the open environment. *Environ. Biosafety Res.* 6, 37–53.

Nielsen, K. M., and Townsend, J. P. (2001). "Environmental exposure, horizontal transfer and selection of transgenes in bacterial populations," in *Enhancing Biocontrol Agents and Handling Risks*, Vol. 339, eds M. Vurro, J. Gressel, T. Butt, G. E. Harman, A. Pilgeram, R. J. St. Leger, and D. L. Nuss, (Amsterdam: NATO Science Series, IOS Press), 145–158.

Nielsen, K. M., and Townsend, J. P. (2004). Monitoring and modeling horizontal gene transfer. *Nat. Biotechnol.* 22, 1110–1114.

Nordgård, L., Nguyen, T., Midtvedt, T., Benno, Y., Traavik, T., and Nielsen, K. M. (2007). Lack of detectable uptake of DNA by bacterial gut isolates grown in vitro and by *Acinetobacter baylyi* colonizing rodents in situ. *Environ. Biosafety Res.* 6, 149–160.

Novozhilov, A. S., Karev, G. P., and Koonin, E. V. (2005). Mathematical modelling of evolution of horizontally transferred genes. *Mol. Biol. Evol.* 22, 1721–1732.

Ochman, H., Lawrence, J. G., and Groisman, E. A. (2000). Lateral gene transfer and the nature of bacterial innovation. *Nature* 405, 299–304.

Orr, H. A. (2005). The genetic theory of adaptation. *Nat. Genet.* 6, 119–127.

Paget, E., Lebrun, M., Freyssinet, G., and Simonet, P. (1998). The fate of recombinant plant DNA in soil. *Eur. J. Soil Biol.* 34, 81–88.

Patwa, Z., and Wahl, L. M. (2008). The fixation probability of beneficial mutations. *J. R. Soc. Interface* 5, 1279–1289.

Pettersen, A. K., Primicero, R., Bøhn, T., and Nielsen, K. M. (2005). Modeling suggests frequency estimates are not informative for predicting the long-term effect of horizontal gene transfer in bacteria. *Environ. Biosafety Res.* 4, 222–233.

Pietramellara, G., Ascher, J., Borgogni, F., Ceccherini, M.-T., Guerri, G., and Nannipieri, P. (2009). Extracellular DNA in soil and sediment: fate and ecological relevance. *Biol. Fertil. Soils* 45, 219–235.

Pontiroli, A., Rizzi, A., Simonet, P., Daffonchio, D., Vogel, T. M., and Monier, J. M. (2009). Visual evidence of horizontal gene transfer between plant and bacteria in the phytosphere of transplastomic tobacco. *Appl. Environ. Microbiol.* 75, 3314–3322.

Pontiroli, A., Simonet, P., Frostegard, A., Vogel, T. M., and Monier, J. M. (2007). Fate of transgenic plant DNA in the environment. *Environ. Biosafety Res.* 6, 15–35.

Remold, S. K., and Lenski, R. E. (2004). Pervasive joint influence of epistasis and plasticity on mutational effects in *Escherichia coli*. *Nat. Genet.* 36, 423–426.

Rizzi, A, Raddadi, N., Sorlini, C., Nordgård, L., Nielsen, K. M., and Daffonchio, D. (2012). The stability and degradation of dietary DNA in the gastrointestinal tract of mammals – implications for horizontal gene transfer and the biosafety of GMOs. *Crit. Rev. Food Sci. Nutr.* 52, 142–161.

Rouzine, I. M., Rodrigo, A., and Coffin, J. M. (2001). Transition between stochastic evolution and deterministic evolution in the presence of selection: general theory and application to virology. *Microbiol. Mol. Biol. Rev.* 65, 151–185.

Rozen, D. E., de Visser, J. A., and Gerrish, P. J. (2002). Fitness effects of fixed beneficial mutations in microbial populations. *Curr. Biol.* 12, 1040–1045.

Spratt, B. G., Hanage, W. P., and Feil, E. J. (2001). The relative contribution of recombination and point mutation to the diversification of bacterial clones. *Curr. Opin. Microbiol.* 4, 602–606.

Thomas, C. M., and Nielsen, K. M. (2005). Mechanisms and barriers to horizontal gene transfer between bacteria. *Nat. Rev. Microbiol.* 3, 711–721.

Toprak, E., Veres, A., Michel, J. B., Chait, R., Hartl, D. L., and Kishony R. (2012). Evolutionary paths to antibiotic resistance under dynamically sustained drug selection. *Nat. Genet.* 44, 101–105.

Townsend, J. P., Nielsen, K. M., Fisher, D., and Hartl, D. L. (2003). Horizontal acquisition of divergent chromosomal DNA in bacteria: effects of mutator phenotypes. *Genetics* 164, 13–21.

Wögerbauer, M. (2007). "Risk assessment of antibiotic resistance marker genes in genetically modified organisms," in BMGFJ – Forschungsberichte der Sektion IV, Band 5/2007, Vienna, Austria.

Zur Wiesch, P. A., Kouyos, R., Engelstadter, J., Regoes, R. R., and Bonhoeffer, S. (2011). Population biological principles of drug-resistance evolution in infectious diseases. *Lancet Infect. Dis.* 11, 236–247.

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 12 November 2011; accepted: 16 January 2012; published online: 20 February 2012.

Citation: Townsend JP, Bøhn T and Nielsen KM (2012) Assessing the probability of detection of horizontal gene transfer events in bacterial populations. Front. Microbio. ***3****:27. doi: 10.3389/fmicb.2012.00027*

This article was submitted to Frontiers in Antimicrobials, Resistance and Chemotherapy, a specialty of Frontiers in Microbiology.

APPENDIX

$$\text{In[1]} := \mathbf{p/(1-p) = (p_0) \,/\, (1-p_0)^{*}\, E\text{\^{}}(mt)}$$

$$\text{Out[1]} = \frac{p}{1-p} = \frac{\mathbb{E}^{m\,t} p_0}{1-p_0}$$

$$\text{In[2]} := \mathbf{ExpandDenominator}\left[\frac{p}{1-p} = \frac{\mathbb{E}^{mt} p_0}{1-p_0} /. p_0 \to 1/bign\right]$$

$$\text{Out[2]} = \frac{p}{1-p} = \frac{\mathbb{E}^{mt}}{-1+bign}$$

$$\text{In[3]} := \mathbf{Extract}\left[\mathbf{Flatten}\left[\mathbf{Solve}\left[\frac{p}{1-p} = \frac{\mathbb{E}^{mt}}{-1+bign}, p\right]\right], 1\right]$$

$$\text{Out[3]} = p \to \frac{\mathbb{E}^{mt}}{-1+bign+\mathbb{E}^{mt}}$$

$$\text{In[4]} := \mathbf{r\,x\,E\text{\^{}}(-r\,x\,t)}$$

$$\text{Out[4]} = \mathbb{E}^{-r\,t\,x} r\,x$$

$$\text{In[5]} := \frac{1-\mathbb{E}^{-2\,m}}{\mathbb{E}^{-2\,bign\,m}}$$

$$\text{Out[5]} = \frac{1-\mathbb{E}^{-2\,m}}{1-\mathbb{E}^{-2\,bign\,m}}$$

$$\text{In[6]} := \mathbb{E}^{-r\,t\,x} r\,x /. r \to \frac{1-\mathbb{E}^{-2\,m}}{1-\mathbb{E}^{-2\,bign\,m}} * r$$

$$\text{Out[6]} = \frac{\mathbb{E}^{-\frac{(1-\mathbb{E}^{-2m})\,r\,t\,x}{1-\mathbb{E}^{-2\,bign\,m}}} (1-\mathbb{E}^{-2\,m}) r\,x}{1-\mathbb{E}^{-2\,bign\,m}}$$

$$\text{In[7]} := \mathbf{1-(1-p)\text{\^{}}n}$$

$$\text{Out[7]} = 1-(1-p)^{n}$$

$$\text{In[8]} := \textbf{Print}\left[\mathbf{1-(1-p)^n/.p\to\frac{\mathbb{E}^{mt}}{-1+bign+\mathbb{E}^{mt}}}, \text{″}\approx\text{″}, \mathbf{1-\left(1-n*\left(p/.p\to\frac{\mathbb{E}^{mt}}{-1+bign+\mathbb{E}^{mt}}\right)\right)}\right]$$

$$1-\left(1-\frac{\mathbb{E}^{m\,t}}{-1+bign+\mathbb{E}^{m\,t}}\right)^{n}\approx\frac{\mathbb{E}^{m\,t}n}{-1+bign+\mathbb{E}^{m\,t}}$$

$$\textbf{equation9[r\_, m\_, bign\_, x\_, ts\_, n\_, tx\_] :=}$$

$$\textbf{NIntegrate}\left[-\mathbb{E}^{-r*\frac{1-\mathbb{E}^{-2\,m}}{1-\mathbb{E}^{-2\,bign\,m}}*tx}\left(-1+\left(\frac{-1+bign}{-1+bign+\mathbb{E}^{m(ts-t)}}\right)^{n}\right)r*\frac{1-\mathbb{E}^{-2\,m}}{1-\mathbb{E}^{-2\,bign\,m}}x,\{t,0,tx\}\right];$$

$$\textbf{off[NIntegrate :: nlim];}$$

$$\textbf{equation9[r, m, bign, x, ts, n, tx]}$$

$$\text{Out[11]} = \textbf{NIntegrate}\left[-\frac{1}{1-\mathbb{E}^{-2\,bign\,m}}\mathbb{E}^{-\frac{r(1-\mathbb{E}^{-2\,m})tx}{1-\mathbb{E}^{-2\,bign\,m}}}\left(-1+\left(\frac{-1+bign}{-1+bign+\mathbb{E}^{m(ts-t)}}\right)^{n}\right)r(1-\mathbb{E}^{-2\,m})\,x,\,\{t,\,0,\,tx\}\right]$$

$$\text{In[12]} := \textbf{On[NIntegrate :: nlim];}$$

$$\textbf{equation9[10\^{} - 5, 10\^{} - 2, 10\^{}9, 10\^{}3, 10\^{}3, 10\^{}5, 10\^{}3]}$$

$$\text{Ou[13]} = 0.0271281$$

$$\text{In[14]} := \textbf{equation10[r\_, m\_, bign\_, x\_, ts\_, n\_, tx\_] :=}$$

$$\mathbf{r*x*\frac{1-\mathbb{E}^{-2\,m}}{1-\mathbb{E}^{-2\,bign\,m}}*NIntegrate\left[-\left(-1+\left(\frac{-1+bign}{-1+bign+\mathbb{E}^{m(ts-t)}}\right)^{n}\right)\mathbb{E}^{-r*\frac{1-\mathbb{E}^{-2\,m}}{1-\mathbb{E}^{-2\,bign\,m}}*tx},\,\{t,\,0,\,tx\}\right];}$$

$$\textbf{off[NIntegrate :: nlim];}$$

$$\textbf{equation10[r, m, bign, x, ts, n, tx]}$$

$$\text{Out[16]} = \frac{1}{1-\mathbb{E}^{-2\,bign\,m}}(1-\mathbb{E}^{-2\,m})\,r\,x\,\text{NIntegrate}\left[-\left(-1+\left(\frac{-1+bign}{-1+bign+\mathbb{E}^{m\,(ts-t)}}\right)^{n}\right)\mathbb{E}^{-\frac{r(1-\mathbb{E}^{-2\,m})tx}{1-\mathbb{E}^{-2\,bign\,m}}},\,\{t,\,0,\,tx\}\right]$$

$$\textbf{equation11[r\_, m\_, bign\_, x\_, ts\_, tx\_] :=}$$

$$\textbf{NIntegrate}\left[\mathbf{r\,x\frac{1-\mathbb{E}^{-2\,m}}{1-\mathbb{E}^{-2\,bign\,m}}\mathbb{E}^{-\frac{1-\mathbb{E}^{-2\,m}}{1-\mathbb{E}^{-2\,bign\,m}}*rxt},\,\{t,\,0,\,tx\}}\right];$$

$$\textbf{equation11[r, m, bign, x, ts, tx]}$$

$$\text{Out[18]} = \text{NIntegrate}\left[\frac{r\,x\,(1-\mathbb{E}^{-2\,m})\mathbb{E}^{-\frac{\{1-\mathbb{E}^{-2\,m}\}r\,x\,t}{1-\mathbb{E}^{-2\,bign\,m}}}}{1-\mathbb{E}^{-2\,bign\,m}},\,\{t,\,0,\,tx\}\right]$$

$$\textbf{equation12[r\_, m\_, bign\_, x\_, ts\_, n\_, tx\_,] :=}$$

$$\textbf{NIntegrate}\left[-\left(-1+\left(\frac{-1+bign}{-1+bign+\mathbb{E}^{m\,(ts-t)}}\right)^{n}\right)\mathbb{E}^{-r*\frac{(1-\mathbb{E}^{-2\,m})tx}{1-\mathbb{E}^{-2\,bign\,m}}*tx},\,\{t,\,0,\,tx\}\right]/$$

$$\textbf{NIntegrate}\left[\mathbb{E}^{-\frac{(1-\mathbb{E}^{-2\,m})}{1-\mathbb{E}^{-2\,bign\,m}}*r\,x\,t},\,\{t,\,0,\,tx\}\right];$$

$$\textbf{equation12[r, m, bign, x, ts, n, tx]}$$

$$\text{Out}[20] = \text{NIntegrate}\left[-\left(-1+\left(\frac{-1+\text{bign}}{-1+\text{bign}+\mathbb{E}^{\text{m (ts-t)}}}\right)^{\text{n}}\right)\mathbb{E}^{-\frac{(1-\mathbb{E}^{-2\,\text{m}})\text{tx}}{1-\mathbb{E}^{-2\,\text{bign m}}}},\ \{\text{t},\ 0,\ \text{tx}\}\right]/$$
$$\text{NIntegrate}\left[\mathbb{E}^{-\frac{(1-\mathbb{E}^{-2\,\text{m}})\,\text{r x t}}{1-\mathbb{E}^{-2\,\text{bign m}}}},\ \{\text{t},\ 0,\ \text{tx}\}\right]$$

In[21] : = **equation12[10^ − 5, 10^ − 2, 10^9, 10^3, 10^3, 10^5, 10^3]**

Out[21] = 0.151013