frontiers | Research Topics

Livestock and its role in the emergence, spread, and evolution of antimicrobial resistance: Animal-to-human or animal-to-environment transmission

Edited by
João Pedro Rueda Furlan, Kohei Makita, William Calero-Cáceres and Eliana Guedes Stehling

Published in
Frontiers in Veterinary Science

ISSN 1664-8714
ISBN 978-2-8325-3463-2
DOI 10.3389/978-2-8325-3463-2

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: frontiersin.org/about/contact

Livestock and its role in the emergence, spread, and evolution of antimicrobial resistance: Animal-to-human or animal-to-environment transmission

Topic editors

João Pedro Rueda Furlan — University of São Paulo, Brazil
Kohei Makita — Rakuno Gakuen University, Japan
William Calero-Cáceres — Technical University of Ambato, Ecuador
Eliana Guedes Stehling — University of São Paulo, Ribeirão Preto, Brazil

Citation

Furlan, J. P. R., Makita, K., Calero-Cáceres, W., Stehling, E. G., eds. (2023). *Livestock and its role in the emergence, spread, and evolution of antimicrobial resistance: Animal-to-human or animal-to-environment transmission*. Lausanne: Frontiers Media SA. doi: 10.3389/978-2-8325-3463-2

Table of contents

frontiers | Frontiers in Veterinary Science

TYPE Editorial
PUBLISHED 29 August 2023
DOI 10.3389/fvets.2023.1270955

OPEN ACCESS

EDITED AND REVIEWED BY
Michael Ward,
The University of Sydney, Australia

*CORRESPONDENCE
Eliana Guedes Stehling
✉ elianags@usp.br
William Calero-Cáceres
✉ wr.calero@uta.edu.ec
Kohei Makita
✉ kmakita@rakuno.ac.jp
João Pedro Rueda Furlan
✉ jpedro.rueda@usp.br

RECEIVED 01 August 2023
ACCEPTED 21 August 2023
PUBLISHED 29 August 2023

CITATION
Stehling EG, Calero-Cáceres W, Makita K and Furlan JPR (2023) Editorial: Livestock and its role in the emergence, spread, and evolution of antimicrobial resistance: animal-to-human or animal-to-environment transmission. *Front. Vet. Sci.* 10:1270955. doi: 10.3389/fvets.2023.1270955

Editorial: Livestock and its role in the emergence, spread, and evolution of antimicrobial resistance: animal-to-human or animal-to-environment transmission

Eliana Guedes Stehling[1]*, William Calero-Cáceres[2]*, Kohei Makita[3]* and João Pedro Rueda Furlan[1]*

[1]Department of Clinical Analyses, Toxicology and Food Science, School of Pharmaceutical Sciences of Ribeirão Preto, University of São Paulo, Ribeirão Preto, Brazil, [2]UTA-RAM-One Health, Department of Food and Biotechnology Science and Engineering, Universidad Técnica de Ambato, Ambato, Ecuador, [3]Veterinary Epidemiology Unit, Graduate School of Veterinary Medicine, Rakuno Gakuen University, Ebetsu, Japan

KEYWORDS

bacterial pathogens, antimicrobial resistance, molecular epidemiology, genomic evolution, food-producing animals, One Health

Editorial on the Research Topic
Livestock and its role in the emergence, spread, and evolution of antimicrobial resistance: animal-to-human or animal-to-environment transmission

The emergence of multidrug-resistant bacterial pathogens is a global multifactorial and multisectoral problem. The intensive use of antimicrobials in livestock has been associated with an increase in antimicrobial resistance (AMR). In this regard, antimicrobial-resistant strains are constantly emerging from this sector, raising concerns about animal-to-human and animal-to-environment transmission. This Research Topic includes original, brief report, and review research focused on AMR in the veterinary sector.

Cross-species and inter-host transmission of multidrug-resistant gram-negative bacteria

Ramírez-Castillo et al. discussed the spread of carbapenem resistance in terrestrial food-producing animals, seafood, aquaculture, wildlife, companion animals, and their environments. Overall, poultry, swine, and cattle have carried acquired carbapenemases, including KPC, NDM, IMP, VIM, and OXA. These carbapenemases were occurred mainly in *Enterobacterales*, *Acinetobacter* species, and *Pseudomonas aeruginosa*. In seafood and aquaculture, intrinsic or acquired carbapenemases have been detected in *Enterobacterales*, *Shewanella algae*, *Vibrio* species, and clinically important non-fermenting gram-negative bacilli. A high prevalence of clinically significant carbapenemases has been observed in wildlife and companion animals. Furthermore, carbapenemases were also identified

in their surrounding environments. These findings show the spread of carbapenem-resistant strains in the veterinary sector and highlight the cross-species transmission of carbapenem resistance.

In Andaman and Nicobar Islands of India, the occurrence of β-lactamase-producing *Enterobacteriaceae* strains from broilers and native fowl was investigated by Bhowmick et al.. Among the identified species, *Klebsiella pneumoniae* was the most prevalent, followed by *Salmonella enterica*, and *Escherichia coli*. These species showed resistance to several antimicrobials, highlighting the β-lactams agents. In this regard, β-lactamase-encoding genes, including bla_{CTX-M}, were identified. The rate of β-lactamase-producing strains was significantly higher in Andaman than in Nicobar birds. These results revealed that β-lactamases are circulating in the fowl population, including those living in remote locations with low anthropogenic activity. Furthermore, a partial clonal relationship of sequences of β-lactamases with human strains from the Indian subcontinent was observed, providing evidence of the inter-host transmission.

Antimicrobial-resistant *Staphylococcus aureus*, enterococci, and *Campylobacter jejuni* of bovine origin

The European study conducted by Nemati et al. demonstrated the key clinical properties of bovine *Staphylococcus aureus*, including contagiousness and AMR. The bovine adhesion-like protein, encoded by *adlb* gene, was distributed among the different genotypes and clonal complexes (CCs) from ten European countries. Overall, *S. aureus* strains were inhibited by all antimicrobials tested, but some strains were resistant to important antimicrobials, including oxacillin. In this regard, MDR strains were detected in Belgium, Austria, Italy, and Germany. All penicillin-resistant strains showed the simultaneous presence of all *bla* operon genes searched. These results demonstrate that contagiousness and AMR seem to be correlated with different genotypes and CCs and that the prevalence of penicillin resistance is country dependent.

The review of Strong et al. addressed the factors associated with increase or decrease in the prevalence of antimicrobial-resistant enterococci applicable to the Canadian farm-to-fork beef continuum. For this proposal, articles discussing various factors associated with AMR were selected. The AMR was related to certain heavy metals and antimicrobial supplementation. In many instances, unique genes and phenotypic resistance patterns were nuanced. Furthermore, the interpretation of minimum inhibitory concentration and intrinsic resistance varied among enterococci. Several issues were identified, limiting the interpretability and comparison of factors at the broader One-Health scope. Therefore, studies focused on identifying research gap, as well as the standardization of laboratory methodologies are encouraged, which will contribute to future transdisciplinary projects and applications.

In *Campylobacter jejuni* strains, Goulart et al. investigated the growth kinetics and competition, as well as fluoroquinolone (FQ) resistance development. FQ-resistant strains had statistically significant increases over FQ-susceptible strains in growth in competition experiments carried out in mixed cultures without antimicrobials. In addition, FQ-susceptible strains developed resistance to ciprofloxacin more readily when exposed to low levels of the antimicrobial and at high initial bacterial cell density. Accordingly, these findings indicate that FQ-resistant strains may have a slightly higher fitness advantage over the FQ-susceptible strains and provides an explanation for the high prevalence of FQ-resistant *C. jejuni* strains in cattle production.

The use of antimicrobials in livestock: trends and challenges

The Danish pig sector is one of the most important in the world and antimicrobial use (AMU) should be monitored. In this context, Moura et al. investigated which antimicrobials were used, how, and for which reasons. In 2020, there was practically no use of polymyxins, extended-spectrum cephalosporins, and fluoroquinolones. The use of orally administered antimicrobials for gastrointestinal indications in weaned piglets was highlighted. The substitution of group treatments for individual treatments, as well as the promotion of animal health and disease prevention, can enable further reductions in the AMU in the pig sector.

To understand the risk of AMR in livestock house aerosol and its association with AMU, Kobayashi et al. performed a study on the aerosol of the piggeries of Japanese farms. The results revealed that the AMR rate for critically important antimicrobials was positively associated with the AMU of them. The observed positive associations show that the AMR rate may be decreased by reducing the AMU. Therefore, these findings are expected to help establish countermeasures for AMR from aerosol bacteria in swine farms.

In Colombia, Roldan-Henao et al. evaluated the productivity and seroprevalence of *Salmonella* in pigs administered with organic acids (OA) compared to pigs given antimicrobial growth promoters (AGP). This pilot study indicated that administrating OA and cleaning the water pipes improve productivity in pigs and delay exposure to *Salmonella* species when compared with AGP. Although this study must be repeated before definite conclusions can be drawn, OA shows promise and may replace AGP, reducing AMU and AMR.

Indonesia is an important producer of broilers and empirical evidence has shown that the broiler industry uses excessive amounts of antimicrobials; however, quantitative data on AMU at the farm level is not available. Sani et al. compared on-farm AMU monitoring methods and assessed which monitoring method is most suitable for obtaining information on quantitative AMU at the farm level. Using four different indicators, this study demonstrated considerable differences in the ranking of AMU. Besides, collecting farm-level AMU data and adding it to a database can help with monitoring AMU trends.

In summary, these studies provided important findings on the transmission of AMR in Gram-negative and Gram-positive bacteria, as well as new insights into AMU in the veterinary sector. The Guest Editors of this Research Topic hope that these results will further motivate scientists to study and discuss the impact of livestock in the emergence, spread, and evolution of AMR.

Author contributions

ES: Formal analysis, Writing—original draft, Writing—review and editing. WC-C: Formal analysis, Writing—original draft. KM: Formal analysis, Writing—original draft. JF: Formal analysis, Writing—original draft, Writing—review and editing.

Acknowledgments

The Guest Editors express their gratitude to all contributors of this Research Topic for their excellent contributions and the reviewers for their work in evaluating the submitted articles. ES and JF thank the National Council for Scientific and Technological Development (CNPq; 304905/2022-4 and 150712/2022-7) for fellowships.

Conflict of interest

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.

Publisher's note

frontiers | Frontiers in Veterinary Science

TYPE Original Research
PUBLISHED 05 January 2023
DOI 10.3389/fvets.2022.1075133

OPEN ACCESS

EDITED BY
William Calero-Cáceres,
Technical University of Ambato, Ecuador

REVIEWED BY
Raoudha Dziri,
Tunis El Manar University, Tunisia
Yasmine Hasanine Tartor,
Zagazig University, Egypt

*CORRESPONDENCE
Indranil Samanta
✉ isamanta76@gmail.com;
✉ drisamanta@wbuafscl.ac.in
Ananda Tiwari
✉ ananda.tiwari@helsinki.fi

SPECIALTY SECTION
This article was submitted to Veterinary Epidemiology and Economics, a section of the journal Frontiers in Veterinary Science

RECEIVED 20 October 2022
ACCEPTED 28 November 2022
PUBLISHED 05 January 2023

CITATION
Bhowmick S, Pal S, Sunder J, Sujatha T, De AK, Mondal T, Singh AD, Joardar SN, Batabyal K, Dutta TK, Bandyopadhyay S, Tiwari A and Samanta I (2023) Exploring broilers and native fowls of Andaman and Nicobar Islands as a source of β-lactamase-producing *Enterobacteriaceae* even with limited anthropogenic activities and docking-based identification of catalytic domains in novel β-lactamase variants. *Front. Vet. Sci.* 9:1075133. doi: 10.3389/fvets.2022.1075133

Exploring broilers and native fowls of Andaman and Nicobar Islands as a source of β-lactamase-producing *Enterobacteriaceae* even with limited anthropogenic activities and docking-based identification of catalytic domains in novel β-lactamase variants

Sneha Bhowmick[1,2], Surajit Pal[1], Jai Sunder[2], T. Sujatha[2], Arun Kumar De[2], Tousif Mondal[1], Abhishek D. Singh[3], Siddhartha Narayan Joardar[1], Kunal Batabyal[1], Tapan Kumar Dutta[4], Samiran Bandyopadhyay[5], Ananda Tiwari[6]* and Indranil Samanta[1]*

[1]Department of Veterinary Microbiology, West Bengal University of Animal and Fishery Sciences, Kolkata, West Bengal, India, [2]Animal Science Division, ICAR-Central Island Agricultural Research Institute, Port Blair, Andaman and Nicobar Islands, India, [3]Department of Veterinary Public Health, West Bengal University of Animal and Fishery Sciences, Kolkata, West Bengal, India, [4]Department of Veterinary Microbiology, Central Agricultural University, Aizawl, Mizoram, India, [5]ICAR-Indian Veterinary Research Institute, Eastern Regional Station, Kolkata, West Bengal, India, [6]Department of Food Hygiene and Environmental Health, University of Helsinki, Helsinki, Finland

Objectives: The present study was conducted to detect the occurrence of β-lactamase and biofilm-producing *Escherichia coli*, *Salmonella*, and *Klebsiella* in broilers and native fowl reared in the Andaman and Nicobar Islands, India. The study also included molecular docking experiments to confirm the nature of the catalytic domains found in the β-lactamase variants obtained and to reveal the clonal relationship of the isolates with human clinical strains from the database.

Materials and methods: A total of 199 cloacal swabs were collected from five poultry breeds/varieties (broiler, *Vanraja*, *Desi*, *Nicobari*, and layer) in three districts of the Andaman and Nicobar Islands. *E. coli*, *Salmonella enterica*, and *Klebsiella pneumoniae* were isolated by standard techniques and confirmed by PCR. Phenotypical β-lactamase producers were identified by a double-disc test. The genes (bla_{CTX}, bla_{SHV}, bla_{TEM}, and bla_{AmpC}) were screened, and selected sequences of β-lactamase variants were submitted to DDBJ.

Homology modeling, model validation, and active site identification of different β-lactamase variants were done by the SWISS-MODEL. Molecular docking was performed to identify the catalytic domains of the β-lactamase variants. The selected β-lactamase sequences were compared with the Indian ESBL sequences from human clinical strains in NCBI-GenBank.

Results: In total, 425 *Enterobacteriaceae* strains were isolated from the collected samples. *Klebsiella pneumoniae* (42.58%) was found to be the most prevalent, followed by *Salmonella enterica* (30.82%) and *E. coli* (26.58%). The phenotypical antibiogram of all 425 isolates showed the highest resistance against oxytetracycline (61–76%) and the lowest against gentamicin (15–20%). Phenotypical production of β-lactamase enzymes was observed in 141 (33.38%) isolates. The isolation rate of β-lactamase producing *E. coli*, *Salmonella enterica*, and *Klebsiella pneumoniae* was significantly higher ($p < 0.05$) in the birds reared in the South Andaman district (25.6, 17.5, and 18.7%, respectively) than in Nicobar (11.5, 7.6, 7.1%, respectively). Genotyping of the β-lactamase-producing isolates revealed the maximum possession of bla_{TEM}, followed by bla_{SHV} and bla_{CTX-M}. The nucleotide sequences were found to be similar with $bla_{CTX-M-15}$, bla_{SHV-11}, bla_{SHV-27}, $bla_{SHV-228}$, bla_{TEM-1}, and bla_{AmpC} in BLAST search. Distribution of studied biofilm-associated genes in *Enterobacteriaceae* strains from different varieties of the birds revealed that the layer birds had the maximum possession, followed by *Vanraja*, *Desi*, broilers, and *Nicobari* fowls. The phylogenetic analysis of selected sequences revealed a partial clonal relationship with human clinical strains of the Indian subcontinent. Molecular docking depicted the Gibbs free energy release for 10 different macromolecules (proteins) and ligand (antibiotic) complexes, ranging from −8.1 (SHV-27 + cefotaxime) to −7 (TEM-1 + cefotaxime) kcal/mol.

Conclusion and relevance: The study revealed β-lactamase variants circulating in the fowl population of the Andaman and Nicobar Islands (India), even in remote places with low anthropogenic activity. Most of the strains possessed bla_{TEM-1}, followed by $bla_{CTX-M-15}$. Possession of bla_{SHV-11}, bla_{SHV-27}, and $bla_{SHV-228}$ in poultry *Enterobacteriaceae* strains was not reported earlier from any part of the world. The phylogenetic analysis revealed a partial clonal relationship of β-lactamase sequences with the human clinical strains isolated from the Indian subcontinent.

KEYWORDS

Andaman and Nicobar, docking, clonal, ESBL, poultry

Introduction

Antimicrobial resistance in livestock is a global challenge as the bacteria possessing the resistance genes can be disseminated into the human food chain through cross-contamination by means of occupational exposure, contaminated environment, and consumption of animal-origin foods. Extended spectrum-β-lactamase (ESBL) and AmpC-β-lactamase (ACBL) producing *Enterobacteriaceae* are the most reported antimicrobial-resistant bacteria in humans and livestock in the last two decades (1). Poultry was identified as the major reservoir of ESBL-producing *Enterobacteriaceae* in comparison to pigs, cattle, and other members of the livestock family (2). The poultry as a reservoir of ESBL-producing bacteria acts as a challenge for the farmers and slaughterhouse workers or meat vendors, as increased gut colonization of the resistant bacteria was detected in people who had more contact with the birds than the community (3). A recent whole-genome sequencing-based study also evidenced the transmission of ESBL-producing bacteria from poultry to the human population (4).

The ESBL enzyme generates resistance to β-lactam antibiotics, including higher-generation cephalosporins and

monobactams. AmpC β-lactamase-producing bacteria (ACBL) can develop resistance against β-lactam antibiotics in addition to β-lactamase inhibitors like clavulanic acid. There are three classical ESBLs, i.e., TEM (except TEM-1, TEM-2, and TEM-13), SHV (except SHV-1, SHV-2, and SHV-11), and CTX-M (5). CTX-M-15 is the most common ESBL genotype prevalent currently among the human clinical isolates with a rising trend of CTX-M-1, frequently originating from livestock and poultry (6). Poultry acts as the major reservoir of CTX-M-1, SHV-12, TEM-52, and AmpC β-lactamases (7).

Anthropogenic activities were found to be associated with the development of an ESBL-"resistome" in the environment including aquatic settings either due to the dissemination of ESBL-determinants or the bacteria carrying the genes associated with direct human activities and/or the release of the antimicrobials in the sub-therapeutic level in the environment because of indirect human activities (8–10). Persistence of ESBL-producers on the abiotic or biotic surface, associated with the development of "resistome", is dependent on the capacity to form biofilms, as they help in the survival of the bacterial colony against physical and chemical stresses, including disinfectants, host phagocytosis, and antibiotics (11). However, a recent study identified antimicrobial resistance genes in the commensals present in soil exposed to low anthropogenic activities (12).

Several studies found variants of ESBL in *Enterobacteriaceae* in healthy or diseased poultry birds (6, 13), but limited literature is available about the affinity of the β-lactamases for the precise class of cephalosporins. The present study was conducted to detect the presence of β-lactamase and biofilm-producing *Escherichia coli*, *Salmonella*, and *Klebsiella* in broilers and backyard or native fowl reared in the Andaman and Nicobar Islands (India), even in remote places with low anthropogenic activities. The study also included molecular docking experiments to confirm the nature of the catalytic domains in β-lactamase variants ($bla_{\mathrm{CTX-M}}$, bla_{SHV}, and bla_{TEM}) and phylogenetic analysis to reveal the clonal relationship of the poultry-origin *Enterobacteriaceae* isolates with human clinical strains from the GenBank database. *Enterobacteriaceae* was selected as the study bacteria as the family is included in the World Health Organization (WHO) global priority list under "critical" category as an indicator of antibiotic resistance.

Materials and methods

Sampling

During the period from November 2019 to January 2021, a total of 199 cloacal swabs (Table 1) were collected from five poultry breeds or varieties (broiler, *Vanraja*, *Desi*, *Nicobari*, and layer) irrespective of age and sex in three different districts of the Andaman and Nicobar Islands (India), i.e., South Andaman (S/A) (11.74°N/92.65°E), North and Middle Andaman (N & M/A) (12.65°N/92.89°E), and Nicobar (C/N) (7.12°N/93.78°E). The sample size varied between the districts depending on the accessibility and willingness of the farmers to join the study. The collected swabs taken from live birds were properly labeled and were aseptically transported, maintaining the cold chain, into the bacteriology laboratory of the Animal Science Division, ICAR-CIARI, Andaman and Nicobar Islands (India).

No clinical symptoms were reported by the farmers during the collection of cloacal swabs from the birds. The contract farmers reared the broilers in medium-sized flocks (100–200 birds) with the guidelines, feed, vaccines, and medicines (including antibiotics like doxycycline, neomycin, and cephalexin) provided by the enterprise. The backyard farmers reared *Vanraja*, layer birds, and native fowls such as *Desi* and *Nicobari* in small flocks consisting of 15–20 birds per household with occasional exposure to tetracyclines, neomycin, and fluoroquinolones for therapy under the guidance of local veterinarians, para-veterinarians, and drug shop owners. The backyard farmers reared the birds under a semi-intensive system with daytime roaming around the houses and overnight shelter at the farmer's house. No commercial feed mixture was detected to have been used for feeding. The contract farmers prepared a separate bamboo or brick poultry shed and used feeders and waterers, with occasional cleaning and disinfection of the shed with formalin.

Isolation, identification, and PCR-based confirmation of *Escherichia coli*, *Salmonella*, and *Klebsiella*

The swab samples were transported in a sterile transport medium (transport liquid medium, HiMedia, India) and inoculated into the nutrient broth (HiMedia, India) and incubated at 37°C for 24 h. The loopful of overnight growth was streaked onto MacConkey agar (HiMedia, India) and incubated at 37°C for 24 h. The selected single pink colonies were transferred into eosin methylene blue (EMB) agar (HiMedia, India) and incubated at 37°C for 24 h. The single colonies with a greenish metallic sheen were selected for further morphological and biochemical identification (14). For the isolation of *Salmonella*, the swab samples collected were enriched with overnight growth in selenite broth (HiMedia, India) at 37°C. The loopful of growth was streaked onto brilliant green agar (BGA) (HiMedia, India) and incubated at 37°C. The single reddish colonies were selected for further morphological and biochemical identification (14). Similarly, for the isolation of *Klebsiella*, the growth in nutrient broth was streaked into *Klebsiella* selective agar (HiMedia, India) and incubated at 37°C. The single purple magenta colonies were considered for further morphological and

TABLE 1 Distribution of ESBL-producing *E. coli*, *Salmonella*, and *Klebsiella* in three districts of Andaman and Nicobar Islands (India).

District	Breed	Number of collected samples	Number of *E.coli* isolates	Number of ESBL-*E.coli* isolates (%)	Number of *Salmonella* isolates (%)	Number of ESBL-*Salmonella* isolates (%)	Number of *Klebsiella* isolates (%)	Number of ESBL-*Klebsiella* isolates (%)
South Andaman	*Vanraja*	18	14	8	14	3	17	8
	Desi	20	14	10	14	2	20	4
	Nicobari	20	14	6	11	5	19	2
	Layer	12	9	3	6	2	12	3
	Broiler	30	6	2	27	11	29	17
	Sub-Total	100	57 (57/113, 50.44%)	29* (29/113, 25.6%)	72 (72/131, 54.96%)	23[a] (23/131, 17.55%)	97 (97/181, 53.59%)	34[b] (34/181, 18.78%)
N&M Andaman	*Vanraja*	14	7	3	7	2	12	5
	Desi	35	7	4	13	1	23	4
	Sub-Total	49	14 (14/113, 12.38%)	7 (7/113, 6.19%)	20 (20/131, 15.26%)	3 (3/131, 2.29%)	35 (35/181, 19.33%)	9 (9/181, 4.97%)
Nicobar	*Nicobari*	50	42 (42/113, 37.16%)	13* (13/113, 11.5%)	39 (39/131, 29.77%)	10[a] (10/131, 7.63%)	49 (49/181, 27.07%)	13[b] (13/181, 7.18%)
	Total	199	113 (113/425, 26.58%)	49 (49/113, 43.36%)	131 (131/425, 30.82%)	36 (36/131, 27.48%)	181 (181/425, 42.58%)	56 (56/181, 30.93%)

*Differs significantly at a p-value of <0.05; [a]Differs significantly at a p-value of <0.05; [b]Differs significantly at a p-value of <0.05.

10.3389/fvets.2022.1075133

biochemical identification (14). The tentatively identified *E. coli*, *Salmonella*, and *Klebsiella* isolates were confirmed by 16SrRNA gene-specific PCR (15, 16). *Klebsiella pneumoniae* was also identified by specific PCR with the *Klebsiella* species isolates (17). The PCR products were agarose gel electrophoresed containing ethidium bromide, and the gel was visualized and documented in a gel documentation system (Labmate Asia, India).

TABLE 2 Phenotypical antibiotic resistance in *Enterobacteriaceae* strains isolated from poultry in Andaman and Nicobar Islands (India).

Antibiotics	*E. coli* (%) ($n = 113$)	*S. enterica* (%) ($n = 131$)	*K. pneumoniae* (%) ($n = 181$)
Erythromycin (E)	74 (65.49%)	104 (79.395%)	108 (59.67%)
Tetracycline (TE)	86 (76.11%)	102 (77.86%)	112 (61.88%)
Chloramphenicol (C)	35 (30.97%)	48 (36.64%)	55 (30.39%)
Amoxicillin/clavulanic acid (AMC)	87 (76.99%)	65 (49.62%)	111 (61.33%)
Gentamicin (GEN)	18 (15.93%)	24 (18.32%)	38 (20.99%)
Sulphafurazole (SF)	50 (44.25%)	54 (41.22%)	71 (39.23%)
Ampicillin/cloxacillin (AX)	72 (63.72%)	76 (58.02%)	111 (61.33%)
Co-trimoxazole (COT)	82 (72.57%)	68 (51.91%)	109 (60.22%)
Amikacin (AK)	43 (56.64%)	37 (28.24%)	92 (50.83%)
Ampicillin (AMP)	64 (56.64%)	70 (53.44%)	83 (45.86%)
Ciprofloxacin (CIP)	82 (72.57%)	77 (58.78%)	128 (70.72%)
Oxytetracycline (O)	92 (81.42%)	106 (80.92%)	125 (69.06%)

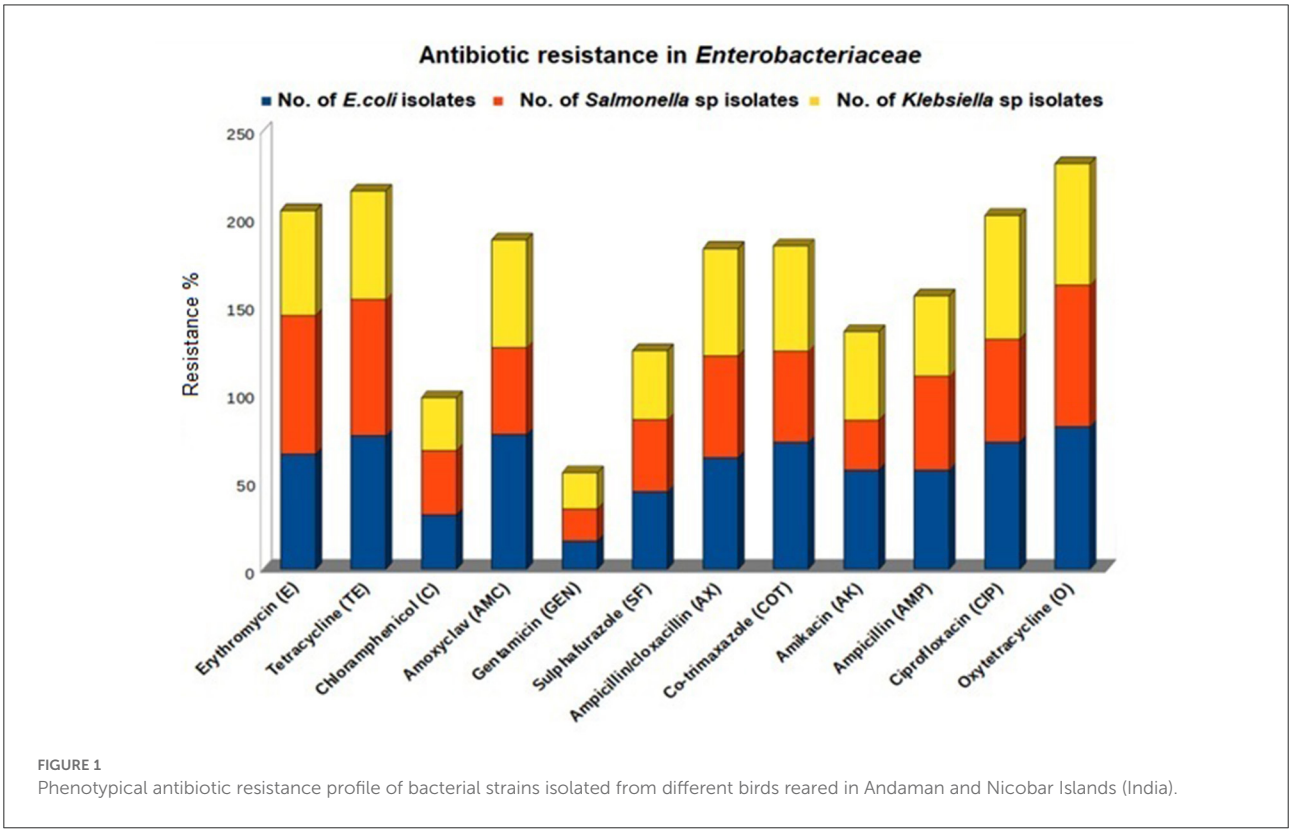


FIGURE 1
Phenotypical antibiotic resistance profile of bacterial strains isolated from different birds reared in Andaman and Nicobar Islands (India).

Antibiogram

All the *E. coli*, *Salmonella enterica*, and *Klebsiella pneumoniae* isolates were screened for antibiotic sensitivity with ceftazidime (CAZ) (30 μg), cefotaxime (CTX) (30 μg), ceftriaxone (CTR) (30 μg), cefpodoxime (CPD) (10 μg), cefoxitin (CX) (30 μg), aztreonam (AT) (30 μg), erythromycin (E) (15 μg), tetracycline (TE) (30 μg), chloramphenicol (C) (30 μg), amoxicillin/clavulanic acid (AMC) (20/10 μg), gentamicin (GEN) (10 μg), sulphafurazole (SF) (300 μg), ampicillin/cloxacillin (AX) (10 μg), ampicillin (AMP) (10 μg), co-trimoxazole (COT) (23.75/1.25 μg), amikacin (AK) (30 μg), ciprofloxacin (CIP) (5 μg), and oxytetracycline (O) (30 μg). The interpretation of the susceptibility or resistance was calculated as per the CLSI recommendation (18).

Double disc diffusion test

The bacterial isolates with a zone of inhibition (ZOI) diameter of ≤22 mm for ceftazidime, ≤27 mm for cefotaxime, ≤25 mm for ceftriaxone, ≤17 mm for cefpodoxime, and ≤27 mm for aztreonam were considered for disc diffusion testing to detect phenotypical ESBL or AmpC production. For confirmation of ESBL production, the isolates that showed an increase of ≥5 mm in ZOI diameter when tested with CTZ and CAZ alone and in combination with ceftazidime/clavulanic acid (CAC/CAZ) (30/10 μg) and cefotaxime/clavulanic acid (CEC/CTX) (30 /10 μg) (18).

Cefoxitin (CX) (30 μg) disc screening was used for the initial detection of AmpC producers, and the isolates with ZOI diameter ≥18 mm were considered for the cefoxitin-cloxacillin double disc test. For confirmation of AmpC production, the isolates showed an increase of ≥4 mm in ZOI diameter when tested with cefoxitin alone and in combination with cefoxitin/cloxacillin (19).

PCR-based detection of ESBL and chromosomal AmpC genes

All the isolates showing phenotypical β-lactamase production were screened for the presence of $bla_{\text{CTX-M}}$, bla_{SHV}, bla_{TEM}, and bla_{AmpC} genes by PCR (20, 21). The PCR products were electrophoresed with ethidium bromide (0.5 μg/ml) and the gel was visualized and documented in a gel documentation system (Labmate Asia, India). The commercial source (Xcelris Genomics, India) was used for the sequencing of selected PCR products as representative of each breed or variety of the birds or the districts studied. The sequence homology was detected by the standard nucleotide BLAST algorithm (https://blast.ncbi.nlm.nih.gov/Blast.cgi?CMD=Web&PAGE_TYPE=BlastHome). The sequences were submitted to the DNA Data Bank of Japan (DDBJ; www.ddbj.nig.ac.jp).

Detection of biofilm-associated genes

All the 425 isolates were subjected to PCR-based detection of biofilm-associated genes, namely, *csgA*,

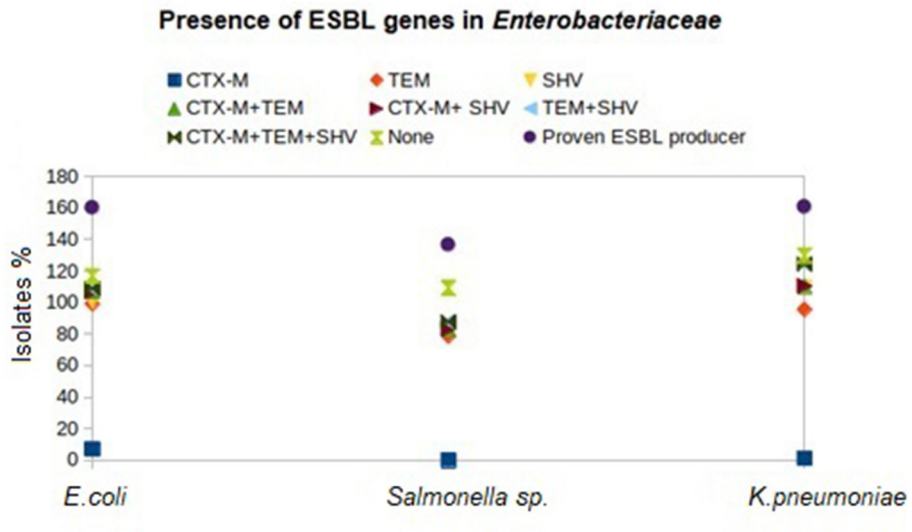


FIGURE 2
Distribution of β-lactamase genes in different birds reared in Andaman and Nicobar Islands (India).

TABLE 3 Accession numbers of nucleotide sequences of ESBL/AmpC genes possessed by *E. coli*, *Salmonella enterica*, and *Klebsiella pneumoniae* strains isolated from different birds in Andaman and Nicobar Islands (India).

Bacteria	ESBL type	Source	Strain no	Place	Accession number
E. coli	SHV-11	Desi bird	DPDB15	Diglipur, N&M/Andaman	LC655953
E. coli	TEM-1	Desi bird	BEBDB8	Beodnabad, S/Andaman	LC659951
E. coli	TEM-1	Layer	TBLB4	Terylabad, S/Andaman	LC659952
E. coli	TEM-1	Vanraja	BEBVR8	Beodnabad, S/Andaman	LC659953
E. coli	TEM-1	Vanraja	RGVR8	Nimbudera, N&M/Andaman	LC659954
E. coli	TEM-1	Nicobari	BLNB39	Big Lapathy, Nicobar	LC659955
E. coli	TEM-1	Vanraja	RGVR6	Nimbudera, N&M/Andaman	LC659960
E. coli	CTX-M-15	Nicobari	MPNB15	Manpur, S/Andaman	LC660645
E. coli	CTX-M-15	Desi bird	BEBDB8	Beodnabad, S/Andaman	LC660646
E. coli	CTX-M-15	Nicobari	SLNB30	Small Lapathy, Nicobar	LC660647
E. coli	AmpC	Desi bird	BEBDB5	Beodnabad, S/Andaman	LC661855
E. coli	AmpC	Desi bird	RGDB7	Rangat, N&M/Andaman	LC661856
E. coli	AmpC	Desi bird	DPDB21	Diglipur, N&M/Andaman	LC661857
E. coli	AmpC	Vanraja	RGVR5	Nimbudera, N&M/Andaman	LC661858
E. coli	AmpC	Vanraja	RGVR7	Nimbudera, N&M/Andaman	LC661859
E. coli	AmpC	Nicobari	KYKNB10	Kinyuka, Nicobar	LC661860
E. coli	AmpC	Nicobari	BLNB39	Big Lapathy, Nicobar	LC661861
S. enterica	SHV-228	Broiler	CABR1	Calicut, S/Andaman	LC656726
S. enterica	SHV-228	Nicobari	AHNB8	Dollygunj, S/Andaman	LC656727
S. enterica	TEM-1	Layer	AHLB9	Dollygunj, S/Andaman	LC656923
S. enterica	AmpC	Broiler	INBR28	Indiranagar, S/Andaman	LC661874
S. enterica	AmpC	Desi bird	KGDB21	Kodiyaghat, S/Andaman	LC661875
S. enterica	AmpC	Vanraja	BEBVR7	Beodnabad, S/Andaman	LC661876
S. enterica	AmpC	Layer	AHLB10	Dollygunj, S/Andaman	LC661877
S. enterica	AmpC	Desi bird	DPDB11	Diglipur, N&M/Andaman	LC661878
S. enterica	AmpC	Vanraja	RGVR8	Nimbudera, N&M/Andaman	LC661879
K. pneumoniae	SHV-27	Desi bird	BTDB29	Baratang, N&M/Andaman	LC653140
K. pneumoniae	SHV-11	Nicobari	KYKNB10	Kinyuka, Nicobar	LC655875
K. pneumoniae	TEM-1	Layer	TBLB2	Terylabad, S/Andaman	LC659956
K. pneumoniae	TEM-1	Nicobari	KGNB4	Kodiyaghat, S/Andaman	LC659957
K. pneumoniae	TEM-1	Desi bird	RCDB16	Rangachang, S/Andaman	LC659958
K. pneumoniae	TEM-1	Desi bird	DPDB20	Diglipur, N&M/Andaman	LC659959
K. pneumoniae	TEM-1	Nicobari	BLNB33	Big Lapathy, Nicobar	LC659961
K. pneumoniae	TEM-1	Nicobari	TLNB45	Tamaloo, Nicobar	LC659962
K. pneumoniae	TEM-1	Vanraja	LPVR16	LalPahad, S/Andaman	LC659963
K. pneumoniae	TEM-1	Vanraja	DPVR14	Diglipur, N&M/Andaman	LC659964
K. pneumoniae	CTX-M-15	Desi bird	DPDB19	Diglipur, N&M/Andaman	LC660643
K. pneumoniae	CTX-M-15	Desi bird	DPDB22	Diglipur, N&M/Andaman	LC660644
K. pneumoniae	AmpC	Nicobari	PKNB15	Perka,C/N	LC661862

(Continued)

TABLE 3 (Continued)

Bacteria	ESBL type	Source	Strain no	Place	Accession number
K. pneumoniae	AmpC	Nicobari	BLNB33	Big Lapathy, Nicobar	LC661863
K. pneumoniae	AmpC	Nicobari	TLNB45	Tamaloo, Nicobar	LC661864
K. pneumoniae	AmpC	Broiler	MPBR9	MaccaPahad, S/Andaman	LC661865
K. pneumoniae	AmpC	Layer	AHLB8	Dollygunj, S/Andaman	LC661866
K. pneumoniae	AmpC	Nicobari	KGNB4	Kodiyaghat, S/Andaman	LC661867
K. pneumoniae	AmpC	Desi bird	BEBDB6	Beodnabad, S/Andaman	LC661868
K. pneumoniae	AmpC	Vanraja	LPVR17	LalPahad, S/Andaman	LC661869
K. pneumoniae	AmpC	Desi bird	RGDB6	Rangat, N&M/Andaman	LC661870
K. pneumoniae	AmpC	Desi bird	DPDB20	Diglipur, N&M/Andaman	LC661871
K. pneumoniae	AmpC	Vanraja	RGVR6	Nimbudera, N&M/Andaman	LC661872
K. pneumoniae	AmpC	Vanraja	DPVR14	Diglipur, N&M/Andaman	LC661873

sdiA, and *rpoS* (22, 23). The commercial source (Xcelris Genomics, India) was used for the sequencing of selected PCR products. The sequence homology was detected by the standard nucleotide BLAST algorithm (https://blast.ncbi.nlm.nih.gov/Blast.cgi?CMD=Web&PAGE_TYPE=BlastHome).

Homology modeling, model validation, and active site identification of different ESBL variants

Available PDB structures of CTX-M-15 (PDB id: 4HBU), SHV-11 (PDB id: 6NFD), and TEM-1 (PDB id: 1BTL) were pulled out from the Research Collaboratory for Structural Bioinformatics Protein Data Bank (RCSB-PDB) database (https://www.rcsb.org/). A position-specific iterated basic local alignment search tool (PSI-BLAST) was performed to find out suitable templates for SHV-28 (template PDB id: 3D4F) and SHV-228 (template PDB id: 3OPL) (https://www.ebi.ac.uk/Tools/sss/psiblast/). Protein homology modeling was performed by using the SWISS-MODEL server (https://swissmodel.expasy.org/). Other structural assessments and stereochemical qualities (Supplementary Figure 1) were verified by the PROCHECK server (https://www.ebi.ac.uk/thornton-srv/software/PROCHECK/). Catalytic active sites for the crystal structures and the modeled proteins were further deposited to DoGSiteScorer, a web server for automatic binding site detection, under the proteins plus (http://dogsite.zbh; uni-hamburg.de/) to get the potential pockets for molecular docking analyses (Supplementary Figure 2).

Docking of third-generation cephalosporins with the ESBL variants

Molecular docking was performed on the Autodock Vina Windows Desktop Suite (https://autodock.scripps.edu/download-autodock4/) as described earlier (24). Three-dimensional SDF file structures of cefotaxime ($C_{16}H_{17}N_5O_7S_2$; PubChem id: 5742673) and cefpodoxime ($C_{15}H_{17}N_5O_6S_2$; PubChem id: 6335986) were retrieved from the PubChem database (https://pubchem.ncbi.nlm.nih.gov/). Receptor energy minimization was done in Swiss-PdbViewer (https://spdbv.unil.ch/energy_tut.html), and the ligand structures were optimized by the Avogadro desktop suite (https://avogadro.cc/). Two-dimensional macromolecule + ligand complexes were visualized by LIGPLOT (https://www.ebi.ac.uk/thornton-srv/software/LigPlus/install.html) analysis, and 3D complexes were made in the PyMOL (https://www.schrodinger.com/products/pymol) desktop suite.

Partial clonal relationship of poultry origin β-lactamases producing *Enterobacteriaceae* strains with human clinical isolates

The selected β-lactamase sequences from the present study were compared with the ESBL sequences of clinical *Enterobacteriaceae* strains isolated from human patients in India and Indian subcontinents (Bangladesh, Myanmar, China, Thailand), available in the NCBI-Genbank database (National Centre for Biotechnology Information; https://www.ncbi.nlm.nih.gov/genbank/). The phylogenetic tree was constructed by the maximum likelihood (ML) method using

molecular evolutionary genetics analysis (MEGA-X; https://www.megasoftware.net/) and analyzed in iTOL v6 (https://itol.embl.de/).

Statistical analysis

The chi-square test (SPSS Inc.) was applied to reveal the statistical differences in the occurrence of β-lactamase-producing *E. coli*, *Salmonella*, and *Klebsiella* strains among the studied fowl population reared in the South Andaman and Nicobar districts.

Results

In total, 425 *Enterobacteriaceae* strains were isolated from the collected samples ($n = 199$). *K. pneumoniae* (42.58%) was found to be the most prevalent, followed by *Salmonella enterica* (30.82%) and *E. coli* (26.58%) (Table 1). *E. coli*, *Salmonella*, and *Klebsiella* were tentatively identified by biochemical tests and confirmed with 16S-rRNA gene-specific PCR.

Phenotypical antibiotic resistance profiling of all 425 isolates showed the highest resistance against oxytetracycline (61–76%), amoxicillin/clavulanic acid (61–76%), and co-trimoxazole (60–72%), and the lowest resistance was observed against gentamicin (15–20%). *E. coli* (81.42%) and *Salmonella* (80.92%) showed the highest phenotypical resistance against oxytetracycline, whereas *Klebsiella* showed the highest resistance against ciprofloxacin (70.72%) (Table 2; Figure 1).

Out of 425 isolates, phenotypical production of β-lactamase enzymes was observed by double disc test in 141 (33.38%) isolates. Production of β-lactamase enzymes was detected maximum in *E. coli* (43.36%) isolates, followed by the *Salmonella* (27.48%) and *Klebsiella* (30.93%) strains (Table 1). The isolation rate of β-lactamase-producing *Enterobacteriaceae* was significantly higher ($p < 0.05$) in the birds reared in the South Andaman district than in the Nicobar district (Table 1). Using the cefoxitin-cloxacillin double disc synergy (CC-DDS) test, phenotypical AmpC production was found in 28.24% (120/425) bacterial isolates. *Klebsiella* (51.33%) was the highest AmpC producer, followed by *Salmonella* (36.28%) and *E. coli* (18.58%).

Genotyping of the β-lactamase-producing isolates revealed maximum possession of bla_{TEM} by *E. coli* (92.04%), *Salmonella* (78.63%), and *Klebsiella* (94.48%) isolates followed by bla_{SHV} and $bla_{\text{CTX-M}}$ (Figure 2). None of the *Salmonella* isolates possessed $bla_{\text{CTX-M}}$. Moreover, none of the *E. coli*, *Salmonella*, and *Klebsiella* isolates possessed all the studied ESBL genes ($bla_{\text{CTX-M}}$, bla_{TEM}, and bla_{SHV}) together. Furthermore, $bla_{\text{TEM}} + bla_{\text{SHV}}$ genotype was possessed by the maximum number of isolates, followed by the genotype $bla_{\text{TEM}} + bla_{\text{CTX-M}}$. All the phenotypical AmpC-producing isolates possessed bla_{AmpC} in PCR. The nucleotide sequences of the PCR products were compared and found similar with $bla_{\text{CTX-M-15}}$

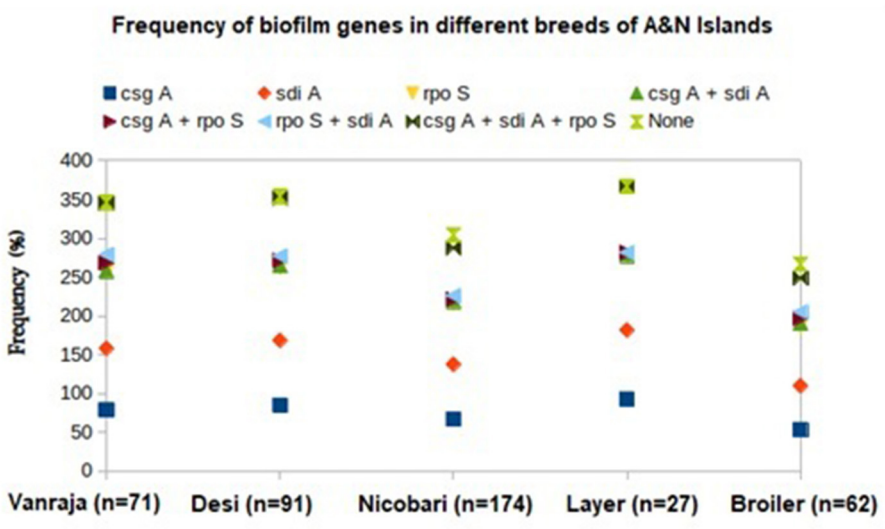


FIGURE 3
Distribution of biofilm-associated genes in different birds reared in Andaman and Nicobar Islands (India).

(98.1% cognate), bla_{SHV-11} (99.45% cognate), bla_{SHV-27} (98.01–99.38% cognate), $bla_{SHV-228}$ (99.38% cognate), bla_{TEM-1} (99.33% cognate), and bla_{AmpC} (99.88% cognate) in the BLAST search. The sequences were published by DDBJ with accession numbers (https://getentry.ddbj.nig.ac.jp/) (Table 3).

The distribution of biofilm-associated genes (*csgA*, *rpoS*, and *sdiA*) in the studied *Enterobacteriaceae* strains from different breeds or varieties of the birds revealed the maximum possession mostly by layer birds, followed by the other varieties of the studied birds (Figure 3). The *csgA* was detected with the highest frequency in the isolates from layer birds (92.5%), followed by *Desi* (84.6%), *Vanraja* (79%), *Nicobari* (66.6%), and broiler (53.2%). The *sdiA* was detected with the highest frequency in the isolates from layer birds (88.8%), followed by *Desi* (83.5%), *Vanraja* (79%), *Nicobari* (70.6%), and broiler (56.4%). The *rpoS* was detected with the highest frequency in the isolates from *Vanraja* (99%), followed by layer birds (96.3%), *Desi* (94.5%), *Nicobari* (78.7%), and broiler (77.4%).

The phylogenetic analysis revealed a partial clonal relationship of β-lactamase sequences of the present study (Table 3), i.e., 15 bla_{TEM-1} (LC659951-64 and LC656923), 2 $bla_{SHV-228}$ (LC656726-27), 5 $bla_{CTX-M-15}$ (LC660643-47), 1 bla_{SHV-27} (LC653140), and 2 bla_{SHV-11} (LC655875 and LC655953) sequences with $bla_{CTX-M-15}$, bla_{SHV-11}, and bla_{SHV-27} and bla_{TEM-1} sequences possessed by clinical strains isolated from human patients

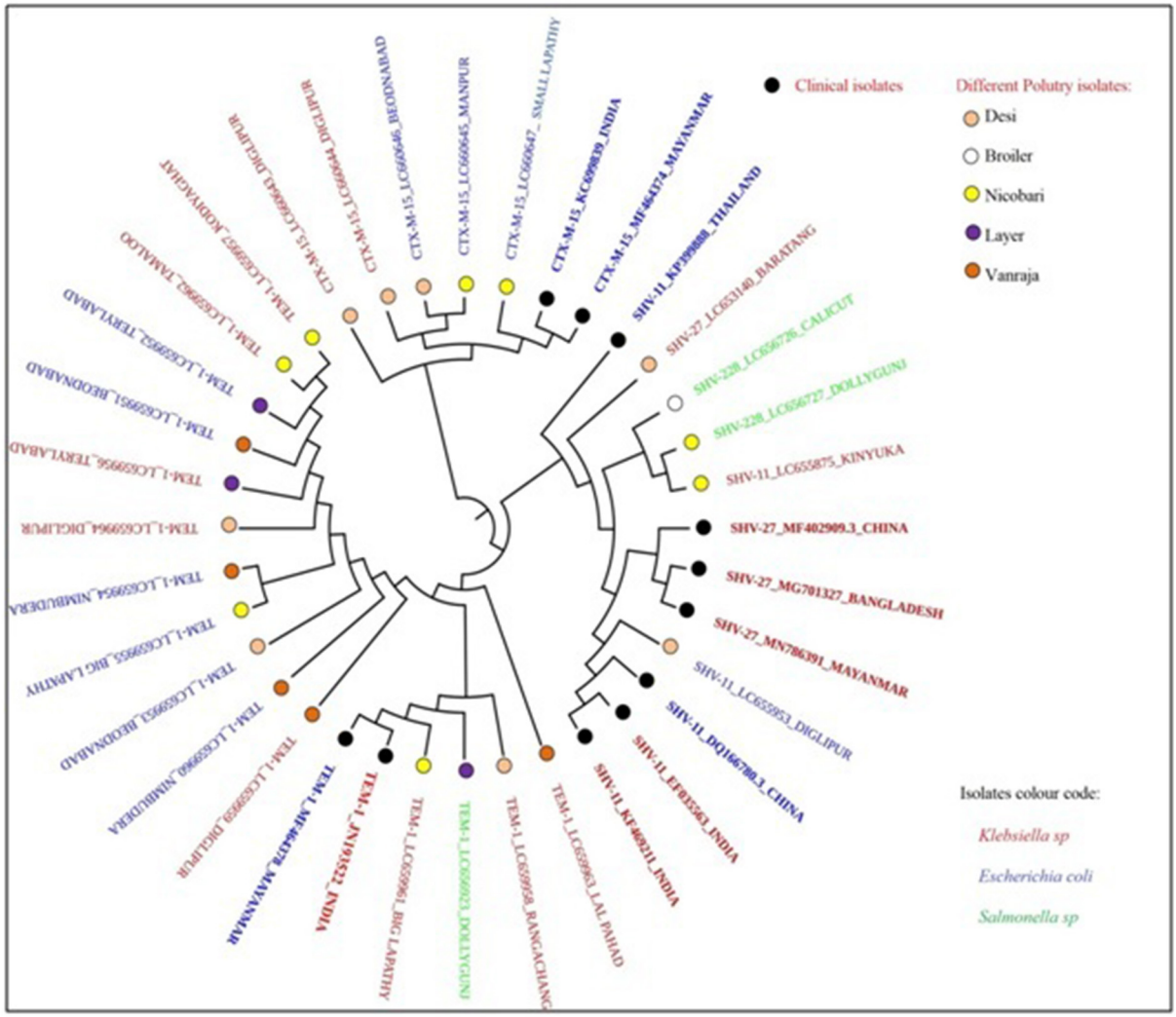


FIGURE 4
Clonal relationship of β-lactamase-producing *Enterobacteriaceae* isolated from locally reared fowls in Andaman and Nicobar Islands (India) with human clinical isolates.

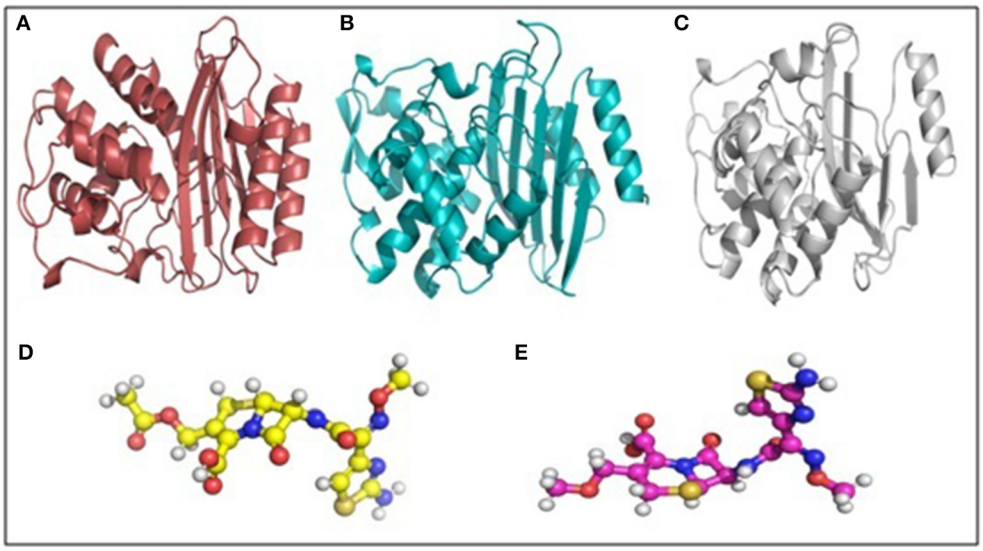


FIGURE 5
Macromolecule and ligand representation (3D) in PyMOL. These color codes have been mentioned throughout the study. **(A)** Cartoon representation of TEM-1 in ruby color. **(B)** Cartoon representation of CTX-M-15 in cyan color. **(C)** Cartoon representation of SHV in gray color. **(D)** Ball and stick representation of cefotaxime (ligand bonds are in golden color). **(E)** Ball and stick representation of cefpodoxime (ligand bonds are in lilac color).

in India, Bangladesh, China, Myanmar, and Thailand (Figure 4).

Molecular docking depicted the Gibbs free energy release for 10 different macromolecules (proteins) and ligand (antibiotic) complexes, ranging from −8.1 (SHV-27+cefotaxime) to −7 (TEM-1+cefotaxime) kcal/mol. The color code of the drug and receptor molecules was maintained throughout the study (Figure 5). A summary of all the 10 complexes and participating amino acid residues in molecular interaction is described in Table 4. Different ligand + receptor complexes (2D Ligplot Plus and 3D PyMOL) are described in Figures 6–10.

Discussions

In the present study, *K. pneumoniae* was found to be the most prevalent in the cloacal swabs of the birds collected from different districts of A&N Islands (India), followed by *Salmonella* (30.82%) and *E. coli* (26.58%). Similar isolation rates of *Klebsiella* were reported earlier from poultry (43.8–72.3%) and bovine milk samples (45.2%) in other parts of India (25, 26). The isolation rate of *Salmonella* and *E. coli* in the present study was found to be corroborative with earlier reports (27, 28). However, the recovery of *Salmonella*, *E. coli*, and *Klebsiella* from poultry varied in different geographical regions depending on isolation protocol, sample size, and animal husbandry practices (29).

Antibiogram profiling of all 425 isolates showing maximum resistance against tetracycline is corroborative with the previous findings in Bangladesh (30), Iran (31), Malaysia (32), and Egypt (33). The resistance of poultry origin-*Enterobacteriaceae* to quinolone antibiotics (ciprofloxacin) from South China (34), Spain (35), and Egypt (36) was also reported, where co-resistance to ciprofloxacin and tigecycline was reported. Resistance to quinolones is often linked to tetracycline as the tetracycline molecule activates mutations in the *mar* operon, which results in more expression of the MarA protein increasing multidrug resistance (37). Most of the isolates in the present study showed resistance to three or more antibiotics and were considered multidrug resistant (38). The most common MDR pattern was found as E-TE-C-AMC-SF-COT-AMP-AX-CIP-O (9.7% in *E. coli*, 6.1% in *Salmonella*, and 5% in *Klebsiella*). All three studied bacterial strains (86.19%) were found susceptible to gentamicin, indicating the possible future usage of gentamicin for the treatment of bacterial infections in poultry in the A&N Islands.

Phenotypical β-lactamase production was detected maximum in *E. coli* (43.36%) isolates, followed by *Salmonella* (27.48%) and *Klebsiella* (30.93%). The majority of the ESBL producers in poultry belonged to the *E. coli* and *Salmonella* group of bacteria throughout the world (7). The occurrence of ESBL-producing *Enterobacteriaceae* was

TABLE 4 Summary of *in silico* analyses.

Results of Autodock Vina				Amino acid residues¥	
ESBLs (Protein) name		Ligand structure	Gibbs free energy (Kcal/mol)	Hydrogen bonding	Hydrophobic interactions
CTX-M-15		**Cefotaxime**	**−7.5**	**Ser 70**, **Asn 132**, Lys 234, **Ser 237**, **Gly 238**, **Pro 268**	**Ser 130**, **Asn 170**, **Thr 235**, **Gly 236**, **Gly 239**, Ser 272, **Lys 269**, **Ala 270**
		Cefpodoxime	**−7.1**	**Ser 70**, **Asn 132**, **Asn 170**, **Ser 237**, **Ala 270**, **Pro 268**	Asn 104, **Ser 130**, **Thr 235**, **Gly 236**, **Gly 238**, **Gly 239**, **Lys 269**
SHV	**SHV-11**	**Cefotaxime**	**−7.8**	Asp 100, **Ala 233**, **Arg 239**	**Ser 66**, **Tyr 101**, **Ser 126**, **Thr 163**, **Asn 166**, **Val 212**, **Thr 231**, **Gly 232**, **Glu 235**
		Cefpodoxime	**−8.0**	**Ala 233**, Gly 234, **Glu 235**, **Arg 239**	**Ser 66**, **Tyr 101**, **Ser 126**, **Thr 163**, **Asn 166**, **Val 212**, **Thr 231**, **Gly 232**, Mse 266
	SHV-27	**Cefotaxime**	**−8.1**	**Ile 32**, Met 34, **Ile 230**, **Ala 232**	**Gly 30**, Glu 33, **Phe 51**, **Thr 56**, Pro 168, **Met 171**, Ala 172, **Arg 228**, Gly 229, **Val 231**
		Cefpodoxime	**−7.5**	**Ile 32**, **Thr 56**	**Gly 30**, Met 31, **Phe 51**, Pro 52, Met 53, Met 54, Thr 166, **Met 171**, **Arg 228**, **Ile 230**, **Val 231**, **Ala 232**
	SHV-228	**Cefotaxime**	**−7.3**	**Ser 126**, **Thr 231**, **Ala 233**, **Glu 235**, Arg 239	**Ser 66**, **Asn 166**, **Val 212**, **Gly 232**, Gly 234
		Cefpodoxime	**−7.1**	**Ser 126**, Asn 128, Thr 163, **Thr 231**, **Ala 233**	**Ser 66**, Tyr 101, **Asn 166**, **Val 212**, **Gly 232**, **Glu 235**
TEM-1		**Cefotaxime**	**−7**	**Ser 130**, **Pro 167**, **Ala 237**, **Arg 244**	**Glu 104**, **Tyr 105**, **Asn 170**, **Val 216**, **Ser 235**, **Gly 236**, **Glu 240**
		Cefpodoxime	**−7.3**	**Ser 130**, **Ser 235**, **Ala 237**, **Arg 244**	Ser 70, **Glu 104**, **Tyr 105**, **Pro 167**, **Asn 170**, **Val 216**, **Gly 236**, **Glu 240**

¥ amino acids, being reported necessary for these catalytic activities, are mentioned in bold.

in accordance with those reported in Thailand (24.9%) (39), Lebanon (28%) (29), Ghana (29%) (4), and Denmark (27%) (40), lower than the prevalence rate reported in Germany (81–85%) (41) and Spain (79%) (42), and higher than Nicaragua (13%) (43) and Finland (14%) (44). The occurrence of ESBL-producing *Enterobacteriaceae* in poultry varies widely according to geographical location and antibiotic exposure, and the plasmids play a significant role in the clonal spread of ESBL genes in the poultry production system as the vertical route has less importance (44).

The isolation rate of β-lactamase producing *Enterobacteriaceae* was significantly higher ($p < 0.05$) in the birds reared in the South Andaman district than in Nicobar, which is correlated with more anthropogenic activities as the total human population and population density of South Andaman is significantly higher than the Nicobar (45). Anthropogenic activities were found to be directly correlated with the generation of ESBL-resistome in the environment either due to the dissemination of ESBL-producing bacteria or the release of the antimicrobials at the sub-therapeutic level in the environment (8–10). However, the occurrence of β-lactamase-producing *Enterobacteriaceae* in the birds reared in the Nicobar Islands with the minimum anthropogenic activities is an important finding as it may be correlated with increased soil salinity and high incidence of migratory

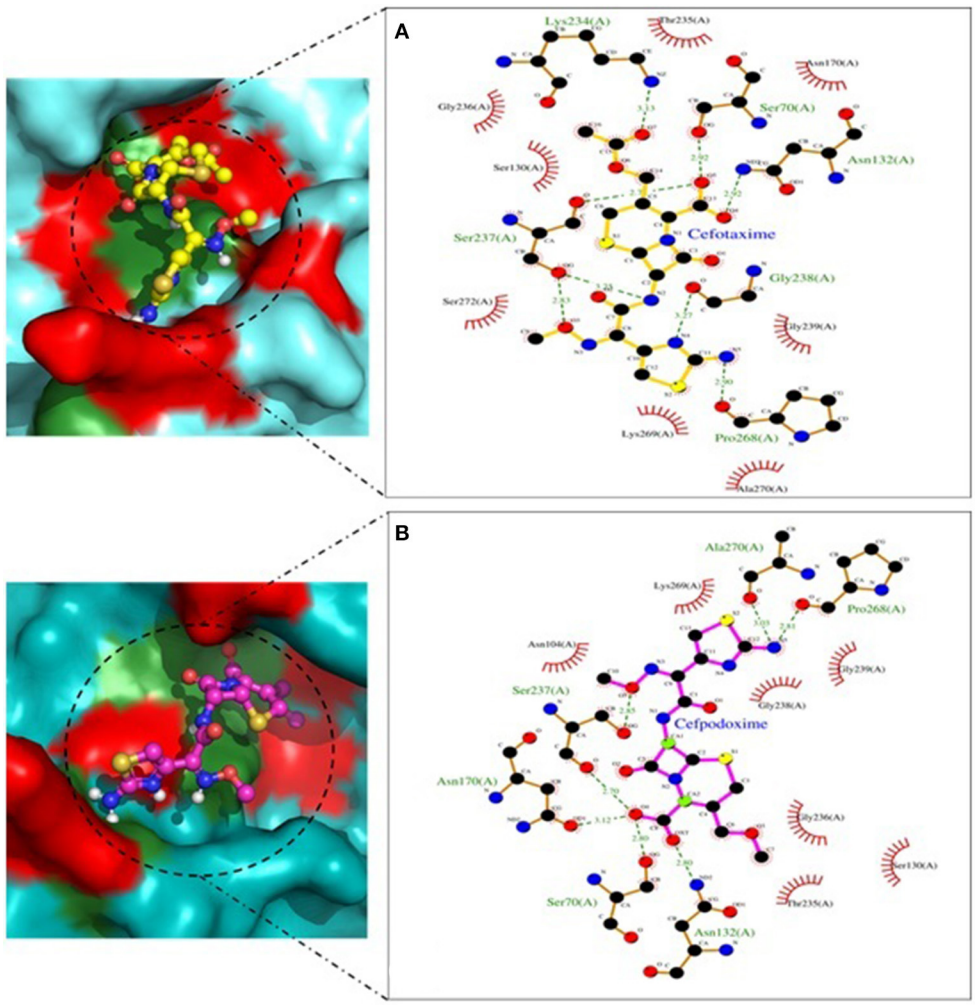


FIGURE 6
CTX-M-15 involved in interactions. **(A)** A binding map for cefotaxime+CTX-M-15. **(B)** A binding map for cefpodoxime+CTX-M-15.

birds in the islands after tsunami (46). Increased translation of multiple antibiotic resistance operons and transfer of ESBL gene containing plasmid was detected in soil bacteria to cope with the salinity stress as the stressors and the antimicrobials use the same bacterial cellular components or processes (47, 48). An increased presence of migratory birds after tsunami was associated with the generation of feeding habitats by the submergence of agricultural fields (49).

The nucleotide sequencing of the PCR products revealed that the variants of the β-lactamase circulating in the fowl population of A&N Islands were TEM-1 with the highest frequency, followed by CTX-M-15, SHV-11, SHV-27, and SHV-228. Similarly, TEM-1 was reported with a maximum frequency in *E. coli* strains isolated from diseased poultry in China (50) and in *Salmonella* strains isolated from poultry or poultry products in the Netherlands (51). Although TEM-1 is not considered as a classical ESBL, it is reported with high frequency in human clinical isolates throughout the world, and TEM-1-encoded enzyme was sometimes detected to demonstrate ESBL properties (52, 53). The high prevalence of TEM-1 in the fowl population of the present study also indicated the probable presence of subclinical bacterial infections, which was overlooked by the farmers who were not trained in poultry farming (13). The possession of $bla_{\mathrm{CTX-M-15}}$ is mostly associated with clinical *Enterobacteriaceae* isolates originated from both human and animal populations worldwide (54). CTX-M-15-producing *Enterobacteriaceae* were earlier

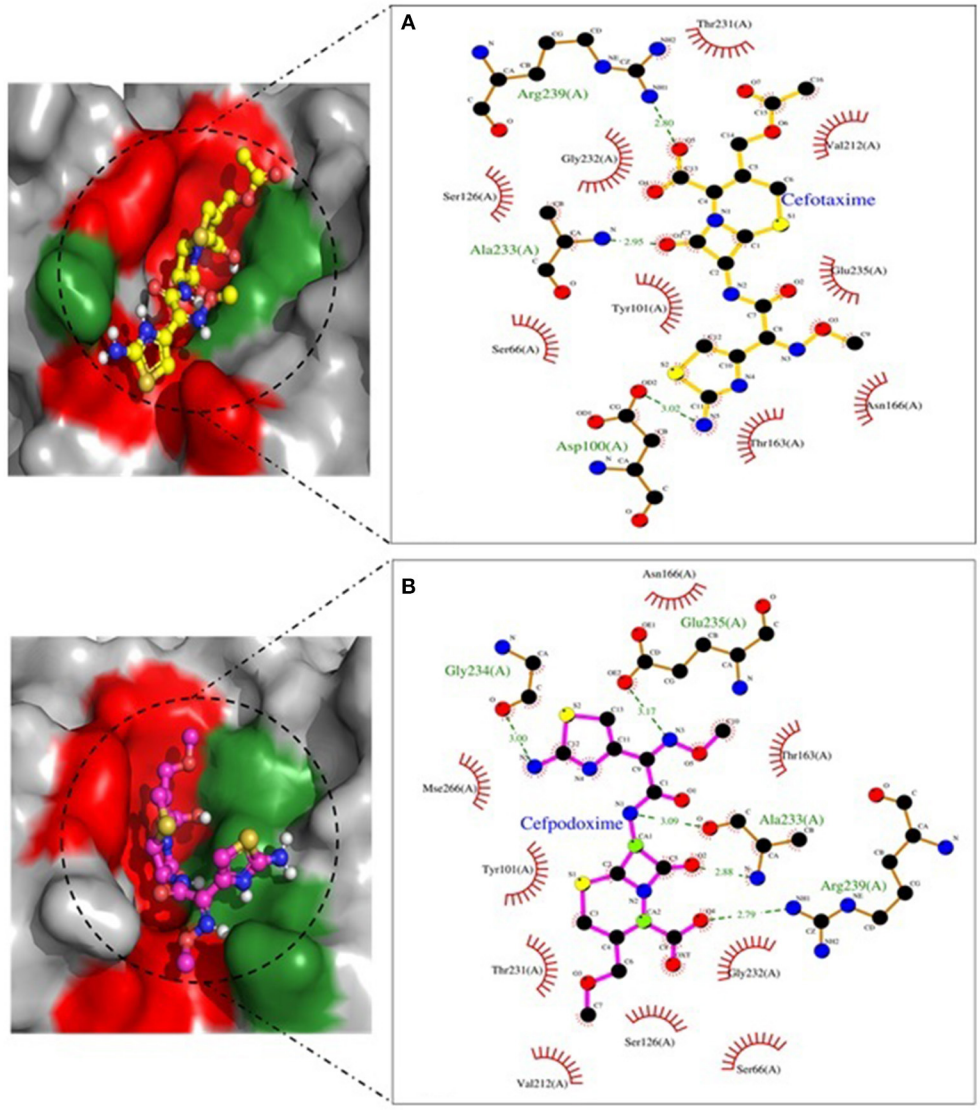


FIGURE 7
SHV-11 involved in interactions. **(A)** A binding map for cefotaxime+SHV-11. **(B)** A binding map for cefpodoxime+SHV-11.

reported in poultry from different parts of the globe (41, 42, 55). The SHV-27 was earlier reported in *Klebsiella* strains isolated from neonatal blood in Brazil, and the enzyme was found to show resistance against cefotaxime, ceftazidime and aztreonam (56). However, SHV-27, SHV-11, and SHV-228 were not reported from poultry in any part of the world.

Using the cefoxitin-cloxacillin double disc synergy (CC-DDS) test, phenotypical AmpC enzyme production was found to be 28.24% (120/425). In India, earlier studies revealed the occurrence of chromosomal AmpC (bla_{AmpC}) in *Enterobacteriaceae* strains isolated from poultry, cattle with mastitis, pig and farm environments, and ducks (57). Other than therapeutic exposure to cefotaxime and ceftazidime, which was not detected in the present study, the occurrence of AmpC-producing bacteria might be associated with clonal transmission from the environment, as observed in a transmission dynamics study of ESBL-producing *Enterobacteriaceae* (39). The co-existence of ESBL and AmpC enzymes was detected in 10.82% (46/425) of the isolates, which is consistent with the earlier findings in the poultry production system (29, 58).

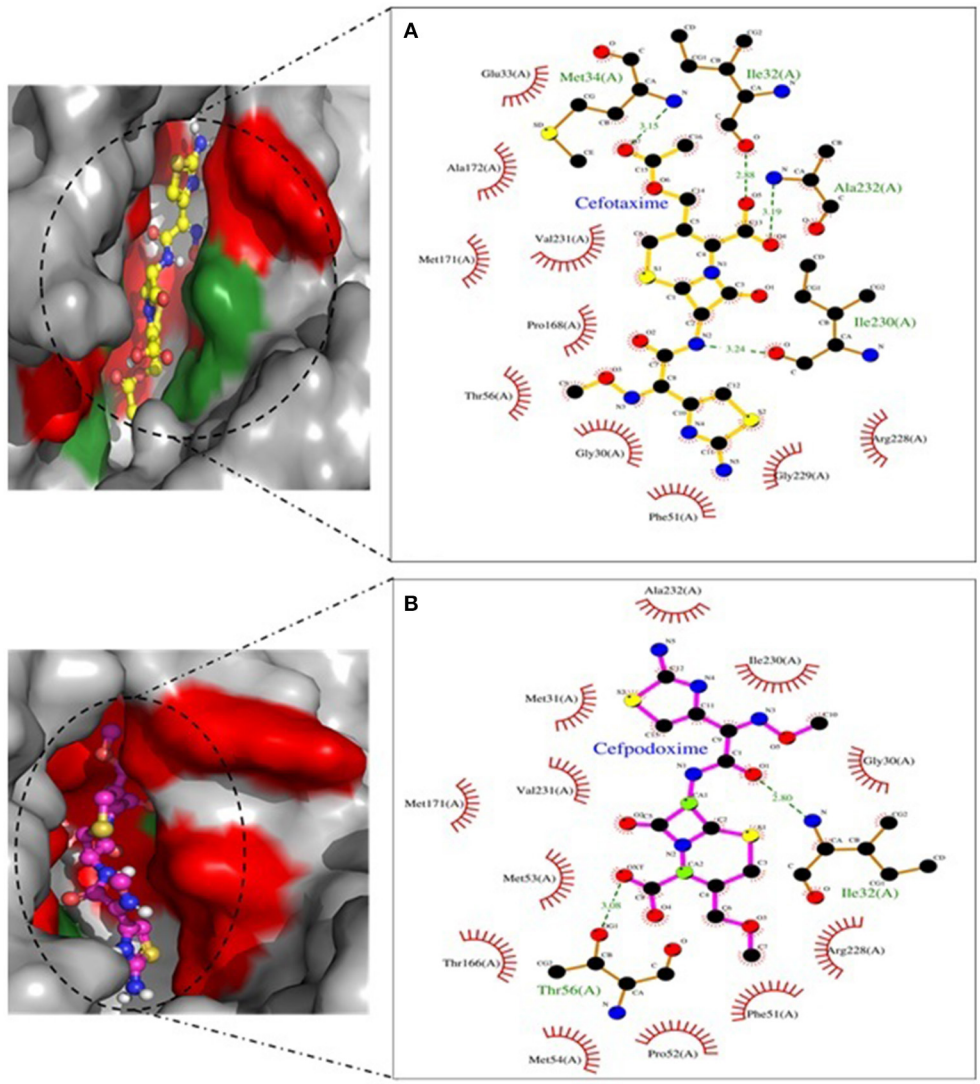


FIGURE 8
SHV-27 involved in interactions. **(A)** A binding map for cefotaxime+SHV-27. **(B)** A binding map for cefpodoxime+SHV-27.

The generation of environmental resistomes is dependent on the persistence of ESBL/AmpC-producers on the abiotic or biotic surface with the capacity to form biofilms, as it helps in the survival of the bacterial colony against physical and chemical stresses (11). The present study detected a high prevalence (76%) of biofilm-associated genes in the *Enterobacteriaceae* strains isolated from the studied fowl population, indicating their possible environmental origin, although the soil microbial profile and the phenotypical biofilm-forming capacity of the strains were not validated.

The phylogenetic analysis revealed a partial clonal relationship between the fowl origin *Enterobacteriaceae* isolates and human clinical strains from the Indian subcontinent. Earlier studies revealed genetic relatedness of strains, similarity in types of β-lactamase genes, and/or associated plasmids in *E. coli* strains originating from animals and humans depicting the transmission probabilities (59).

Molecular docking interaction in the present study demonstrated the probable interactions among the different macromolecule-ligand complexes. The ligands with the minimum binding energy have the highest affinity of β-lactamases for cefotaxime and cefpodoxime. In our study, SHV-27 variants possessed the highest activity against cefotaxime. Improved docking scores were observed for

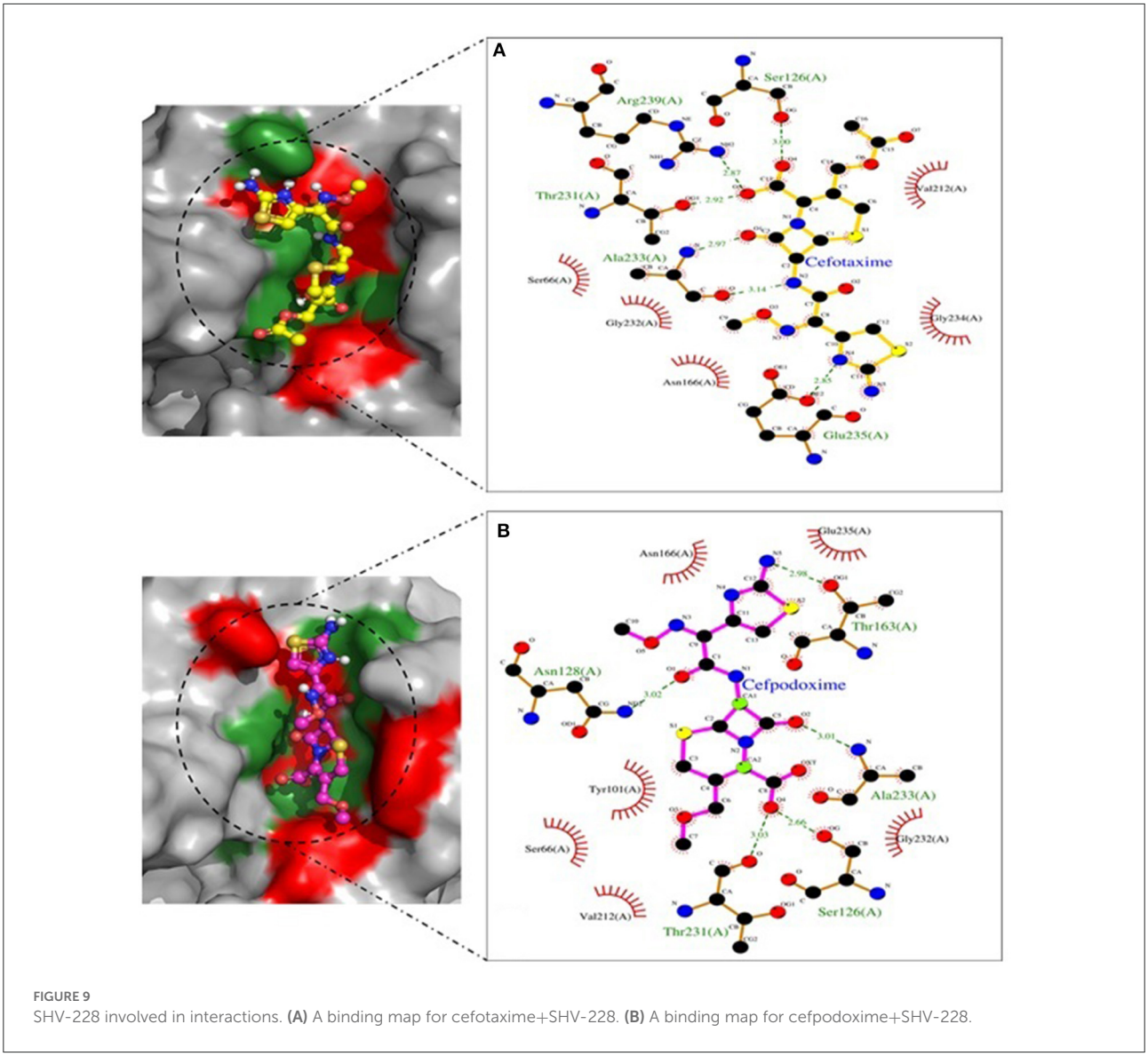


FIGURE 9
SHV-228 involved in interactions. **(A)** A binding map for cefotaxime+SHV-228. **(B)** A binding map for cefpodoxime+SHV-228.

the SHV variants because of the size and volume of its catalytic pocket and its druggability (Supplementary Figure 2). Antibiotic degradation by bla_{SHV} in the present study has also revealed the participation of almost equivalent amino acids in terms of hydrophobic contacts (Ser 66, Tyr 101, Asn 166, and Val 212), which further emphasizes the structural homology of the other related variants. The study suffers from limitations related to sequencing, clonality analysis, and restricted numbers of isolates. The future characterization of this geographical location with the advent of next-generation sequencing can reveal the picture in detail.

The present study thus described the occurrence of β-lactamase/AmpC-producing *Enterobacteriaceae* in the local fowl population, even with the exposure of limited anthropogenic activities. Most of the strains possessed bla_{TEM-1}, followed by $bla_{CTX-M-15}$. The possession of bla_{SHV-11}, bla_{SHV-27}, and $bla_{SHV-228}$ in poultry *Enterobacteriaceae* strains was not reported earlier. ESBL variants were modeled by the SWISS-MODEL and verified. Ligand with the minimum binding energy has the highest affinity of β-lactamases for cefotaxime and cefpodoxime. Phylogenetic analysis of the fowl origin ESBL-producing *Enterobacteriaceae* strains revealed a partial clonal relationship with the clinical isolates from human patients.

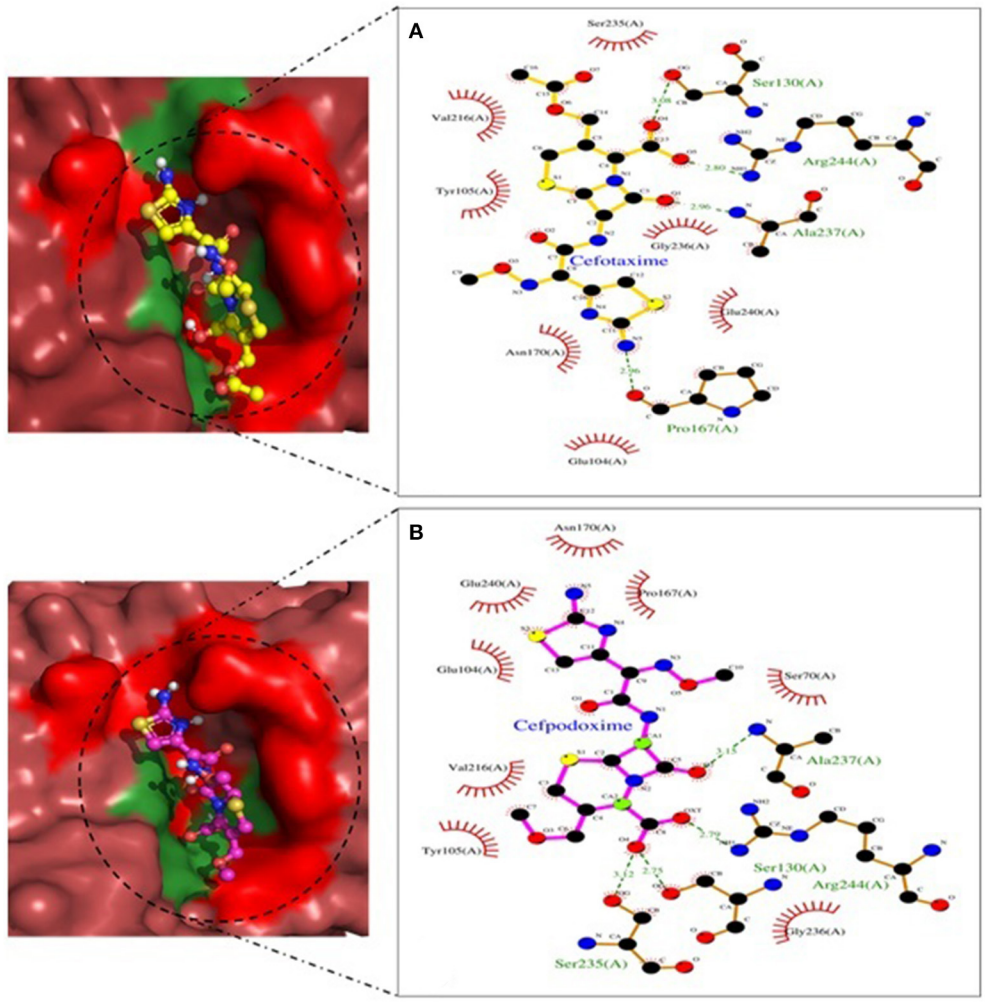


FIGURE 10
TEM-1 involved in interactions. **(A)** A binding map for cefotaxime+TEM-1. **(B)** A binding map for cefpodoxime+TEM-1.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/Supplementary material.

Ethics statement

The animal study was reviewed and approved by Institutional Animal Ethics Committee, WBUAFS. Written informed consent was obtained from the owners for the participation of their animals in this study.

Author contributions

SBh collected the samples and did all the laboratory works. SP conducted bioinformatics analysis. JS and IS supervised the study. TS, AD, SJ, KB, TD, SBa, and AT conceptualized the study. TM and AS helped in the analysis. IS, SBh, and AT wrote the primary and revised manuscripts. All authors contributed to the article and approved the submitted version.

Acknowledgments

The authors provide sincere gratitude to the Director, ICAR-Central Island Agricultural Research Institute, Port Blair-744105, Andaman and Nicobar Islands, India, for providing infrastructure and facilities. It was a collaborative work among

WBUAFS (India), ICAR-CIARI (India), and the University of Helsinki (Finland).

Conflict of interest

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.

Publisher's note

All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations, or those of the publisher, the editors and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fvets.2022.1075133/full#supplementary-material

SUPPLEMENTARY FIGURE 1
RC plot analyses for the modeled SHV-27 protein structure.

SUPPLEMENTARY FIGURE 2
Summary of main chain parameters for the modeled SHV-27 protein structure.

SUPPLEMENTARY FIGURE 3
Summary of side chain parameters for the modeled SHV-27 protein structure.

SUPPLEMENTARY FIGURE 4
RMSD profile for the modeled SHV-27 protein structure.

SUPPLEMENTARY FIGURE 5
RC plot analyses for the modeled SHV-228 protein structure.

SUPPLEMENTARY FIGURE 6
Summary of main chain parameters for the modeled SHV-228 protein structure.

SUPPLEMENTARY FIGURE 7
Summary of side chain parameters for the modeled SHV-228 protein structure.

SUPPLEMENTARY FIGURE 8
RMSD profile for the modeled SHV-228 protein structure.

SUPPLEMENTARY FIGURE 9
Surface view of macromolecules. **(A)** TEM-1 (in ruby color) showing the opening of the catalytic pocket marked in yellow color. **(B)** CTX-M-15 (in cyan color) showing the opening of the catalytic pocket marked in yellow color. **(C)** SHV (in gray color) showing the opening of the catalytic pocket marked in yellow color.

References

1. Bevan ER, Jones AM, Hawkey PM. Global epidemiology of CTX-M β-lactamases: temporal and geographical shifts in genotype. *J Antimicrob Chemother.* (2017) 72:2145–55. doi: 10.1093/jac/dkx146

2. Wadepohl K, Müller A, Seinige D, Rohn K, Blaha T, Meemken D, et al. Association of intestinal colonization of ESBL-producing *Enterobacteriaceae* in poultry slaughterhouse workers with occupational exposure-A German pilot study. *PLoS ONE.* (2020) 15:e0232326. doi: 10.1371/journal.pone.0232326

3. Price LB, Graham JP. Lackey LG, Roess A, Vailes R, Silbergeld E. Elevated risk of carrying gentamicin-resistant *Escherichia coli* among US poultry workers. *Environ hlth Pers.* (2007) 115:1738–42. doi: 10.1289/ehp.10191

4. Falgenhauer L, Imirzalioglu C, Oppong K, Akenten CW, Hogan B, Krumkamp R, et al. Detection and characterization of ESBL-producing *Escherichia coli* from humans and poultry in Ghana. *Front Microbiol.* (2019) 9:3358. doi: 10.3389/fmicb.2018.03358

5. EFSA Panel on Biological Hazards. Scientific opinion on the public health risks of bacterial strains producing extended-spectrum β-lactamases and/or AmpC β-lactamases in food and food-producing animals. *EFSA J.* (2011) 9:2322. doi: 10.2903/j.efsa.2011.2322

6. Valentin L, Sharp H, Hille K, Seibt U, Fischer J, Pfeifer Y, et al. Subgrouping of ESBL-producing *Escherichia coli* from animal and human sources: an approach to quantify the distribution of ESBL types between different reservoirs. *Int J Med Microbiol.* (2014) 304:805–16. doi: 10.1016/j.ijmm.2014.07.015

7. Saliu EM, Vahjen W, Zentek J. Types and prevalence of extended–spectrum beta–lactamase producing *Enterobacteriaceae* in poultry. *Anim Hlth Res Rev.* (2017) 18:46–57. doi: 10.1017/S1466252317000020

8. Guenther S, Ewers C, Wieler LH. Extended-spectrum beta-lactamases producing *E. coli* in wildlife, yet another form of environmental pollution? *Front Microbiol.* (2011) 2:246. doi: 10.3389/fmicb.2011.00246

9. Huang X, Liu C, Li K, Liu F, Liao D, Liu L, et al. Occurrence and distribution of veterinary antibiotics and tetracycline resistance genes in farmland soils around swine feedlots in Fujian Province, China. *Environ Sci Poltn Res.* (2013) 20:9066–74. doi: 10.1007/s11356-013-1905-5

10. Al Salah DMM, Slaveykova VI. Otamonga JP, Poté J. Impact of anthropogenic activities on the occurrence and distribution of toxic metals, extending-spectra β-lactamases and carbapenem resistance in sub-Saharan African urban rivers. *Sci Total Environ.* (2020) 727:138129. doi: 10.1016/j.scitotenv.2020.138129

11. Flemming HC, Wingender J. The biofilm matrix. *Nat Rev Microbiol.* (2010) 8:623–33. doi: 10.1038/nrmicro2415

12. Tang X, Shen M, Zhang Y, Zhu D, Wang H, Zhao Y, et al. The changes in antibiotic resistance genes during 86 years of the soil ripening process without anthropogenic activities. *Chemosphere.* (2021) 266:128985. doi: 10.1016/j.chemosphere.2020.128985

13. Olsen RH, Bisgaard M, Löhren U, Robineau B, Christensen H. Extended-spectrum β-lactamase-producing *Escherichia coli* isolated from poultry: a review of current problems, illustrated with some laboratory findings. *Avian Pathol.* (2014) 43:199–208. doi: 10.1080/03079457.2014.907866

14. Quinn PJ, Carter ME, Markey B, Carter GR. *Veterinary Clinical Microbiology.* London, UK: Wolfe Publication (1994).

15. Wang RF, Cao WW, Cerniglia CE. PCR detection and quantitation of predominant anaerobic bacteria in human and animal fecal samples. *Appl Environ Microbiol.* (1996) 62:1242–7. doi: 10.1128/aem.62.4.1242-1247.1996

16. Brisse S, Verhoef J. Phylogenetic diversity of Klebsiella pneumoniae and *Klebsiella oxytoca* clinical isolates revealed by randomly amplified polymorphic DNA, gyrA and parC genes sequencing and automated ribotyping. *Int J SystEvol Microbiol.* (2001) 51:915–24. doi: 10.1099/00207713-51-3-915

17. Liu Y, Liu C, Zheng W, Zhang X, Yu J, Gao Q, et al. detection of *Klebsiella pneumoniae* in infant formula based on 16S−23S internal transcribed spacer. *Int J Food Microbiol.* (2008) 125:230–5. doi: 10.1016/j.ijfoodmicro.2008.03.005

18. Clinical and Laboratory Standards Institute (CLSI). *Performance Standards for Antimicrobial Susceptibility Testing. Twenty-Fifth Informational Supplement CLSI Document M100*. Wayne, PA: Clinical and Laboratory Standards Institute (2018).

19. Polsfuss S, Bloemberg GV, Giger J, Meyer V, Böttger EC, Hombach M. Practical approach for reliable detection of AmpC beta-lactamase-producing *Enterobacteriaceae*. *J Clin Microbiol.* (2011) 49:2798–803. doi: 10.1128/JCM.00404-11

20. Féria C, Ferreira E, Correia JD, Gonçalves J, Caniça M. Patterns and mechanisms of resistance to β-lactams and β-lactamase inhibitors in uropathogenic *Escherichia coli* isolated from dogs in Portugal. *J Antimicrob Chemother.* (2002) 49:77–85. doi: 10.1093/jac/49.1.77

21. Weill FX, Lailler R, Praud K, Kérouanton A, Fabre L, Brisabois A, et al. Emergence of extended-spectrum-β-lactamase (CTX-M-9)-producing multi resistant strains of *Salmonella enterica* serotype Virchow in poultry and humans in France. *J Clin Microbiol.* (2004) 42:5767–73. doi: 10.1128/JCM.42.12.5767-5773.2004

22. Silva VO, Espeschit IF, Moreira MA. Clonal relationship of *Escherichia coli* biofilm producer isolates obtained from mastitic milk. *Canadian J Microbiol.* (2013) 59:291–3. doi: 10.1139/cjm-2013-0053

23. Adamus-Białek W, Kubiak A, Czerwonka G. Analysis of uropathogenic *Escherichia coli* biofilm formation under different growth conditions. *Acta Biochimica Polonica*. (2015) 62. doi: 10.18388/abp.2015_1127

24. Banerjee A, Pal S, Goswami P, Batabyal K, Joardar SN, Dey S, et al. Docking analysis of circulating CTX-M variants in multi-drug resistant, beta-lactamase and biofilm-producing *E. coli* isolated from pet animals and backyard livestock. *Microbial Patho.* (2022) 170:105700. doi: 10.1016/j.micpath.2022.105700

25. Koovapra S, Bandyopadhyay S, Das G, Bhattacharyya D, Banerjee J, Mahanti A, et al. Molecular signature of extended spectrum β-lactamase producing *Klebsiella pneumoniae* isolated from bovine milk in eastern and north-eastern India. *Infct Gen Evol.* (2016) 44:395–402. doi: 10.1016/j.meegid.2016.07.032

26. Mahanti A, Ghosh P, Samanta I, Joardar SN, Bandyopadhyay S, Bhattacharyya D, et al. Prevalence of CTX-M-Producing *Klebsiella* spp. in broiler, kuroiler, and indigenous poultry in West Bengal State. *India Microb Drug Resist.* (2018) 24:299–306. doi: 10.1089/mdr.2016.0096

27. Karim MR, Giasuddin M, Samad MA, Mahmud MS, Islam MR, Rahman MH, et al. Prevalence of *Salmonella* spp. in poultry and poultry products in Dhaka, Bangladesh. *Int J Anim Biol.* (2017) 3:18–22.

28. Shecho M, Thomas N, Kemal J, Muktar Y. Cloacal Carriage and Multidrug Resistance *Escherichia coli* O157:H7 from Poultry Farms, Eastern Ethiopia. *J Vet Med*. (2017) 2017:8264583. doi: 10.1155/2017/8264583

29. Dandachi I, Sokhn ES, Dahdouh EA, Azar E, El-Bazzal B, Rolain JM, et al. Prevalence and characterization of multi-drug-resistant gram-negative bacilli isolated from Lebanese poultry: a nationwide study. *Front Microbiol.* (2018) 9:550. doi: 10.3389/fmicb.2018.00550

30. Das A, Dhar PK, Dutta A, Jalal MS, Ghosh P, Das T, et al. Circulation of oxytetracycline-and ciprofloxacin-resistant commensal *Escherichia coli* strains in broiler chickens and farm environments, Bangladesh. *Vet World.* (2020) 13:2395. doi: 10.14202/vetworld.2020.2395-2400

31. Jahantigh M, Samadi K, Dizaji RE, Salari S. Antimicrobial resistance and prevalence of tetracycline resistance genes in *Escherichia coli* isolated from lesions of colibacillosis in broiler chickens in Sistan, Iran. *BMC Vet Res.* (2020) 16:1–6. doi: 10.1186/s12917-020-02488-z

32. Ibrahim S, Wei Hoong L, Lai Siong Y, Mustapha Z, CW Zalati CS, Aklilu E, et al. Prevalence of antimicrobial resistance (AMR) *Salmonella* spp. and *Escherichia coli* isolated from broilers in the East Coast of Peninsular. *Malaysia Antibiotics.* (2021) 10:579. doi: 10.3390/antibiotics10050579

33. Elmonir W, Abd El-Aziz NK, Tartor YH, Moustafa SM, Abo Remela EM, Eissa R, et al. Emergence of colistin and carbapenem resistance in extended-spectrum β-lactamase producing *Klebsiella pneumoniae* isolated from chickens and humans in Egypt. *Biol.* (2021) 10:373. doi: 10.3390/biology10050373

34. Wang XM, Liao XP, Zhang WJ, Jiang HX, Sun J, Zhang MJ, et al. Prevalence of serogroups, virulence genotypes, antimicrobial resistance, and phylogenetic background of avian pathogenic *Escherichia coli* in south of China. *Foodborne Patho Dis.* (2010) 7:1099–106. doi: 10.1089/fpd.2010.0542

35. Sáenz Y, Zarazaga M, Briñas L, Lantero M, Ruiz-Larrea F, Torres C. Antibiotic resistance in *Escherichia coli* isolates obtained from animals, foods and humans in Spain. *Int J Antimicrob Agents.* (2001) 18:353–8. doi: 10.1016/S0924-8579(01)00422-8

36. El-Aziz A, Norhan K, Tartor YH, Gharieb R, Erfan AM, Khalifa E, et al. Extensive drug-resistant *Salmonella enterica*. isolated from poultry and humans: prevalence and molecular determinants behind the co-resistance to ciprofloxacin and tigecycline. *Front Microbiol.* (2021) 3 12:738784398. doi: 10.3389/fmicb.2021.738784

37. Ruiz C, Levy SB. Many chromosomal genes modulate MarA-mediated multidrug resistance in *Escherichia coli*. *Antimicrob Agents Chemother.* (2010) 54:2125–34. doi: 10.1128/AAC.01420-09

38. Magiorakos AP, Srinivasan A, Carey RB, Carmeli Y, Falagas ME, Giske CG, et al. Multidrug-resistant, extensively drug-resistant and pan drug-resistant bacteria: an international expert proposal for interim standard definitions for acquired resistance. *Clin Microbiol Infct.* (2012) 18:268–81. doi: 10.1111/j.1469-0691.2011.03570.x

39. Tansawai U, Walsh TR, Niumsup PR. Extended spectrum ß-lactamase-producing *Escherichia coli* among backyard poultry farms, farmers, and environments in Thailand. *Poult Sci.* (2019) 98:2622–31. doi: 10.3382/ps/pez009

40. Agersø Y, Jensen JD, Hasman H, Pedersen K. Spread of extended spectrum cephalosporinase-producing *Escherichia coli* clones and plasmids from parent animals to broilers and to broiler meat in a production without use of cephalosporins. *Foodborne Patho Dis.* (2014) 11:740–6. doi: 10.1089/fpd.2014.1742

41. Blaak H, van Hoek AH, Hamidjaja RA, van der Plaats RQ. Kerkhof-de Heer L, de RodaHusman AM, Schets FM. Distribution, numbers, and diversity of ESBL-producing E coli in the poultry farm environment. *PloS ONE.* (2015) 10:e0135402. doi: 10.1371/journal.pone.0135402

42. Blanc V, Mesa R, Saco M, Lavilla S, Prats G, Miró E, et al. ESBL-and plasmidic class C β-lactamase-producing *E. coli* strains isolated from poultry, pig and rabbit farms. *Vet Microbiol.* (2006) 118:299–304. doi: 10.1016/j.vetmic.2006.08.002

43. Hasan B, Laurell K, Rakib MM, Ahlstedt E, Hernandez J, Caceres M, et al. Fecal carriage of extended-spectrum β-lactamases in healthy humans, poultry, and wild birds in León, Nicaragua—a shared pool of bla CTX-M genes and possible interspecies clonal spread of extended-spectrum β-lactamases-producing *Escherichia coli*. *Microbial Drug Resist.* (2016) 22:682–7. doi: 10.1089/mdr.2015.0323

44. Oikarainen PE, Pohjola LK, Pietola ES, Heikinheimo A. Direct vertical transmission of ESBL/pAmpC-producing *Escherichia coli* limited in poultry production pyramid. *Vet Microbiol.* (2019) 231:100–6. doi: 10.1016/j.vetmic.2019.03.001

45. Census. *Andaman and Nicobar Islands Population 2011–2022.* (2011). Available online at: https://www.census2011.co.in/census/state/andaman$+$and$+$nicobar$+$islands.html (accessed on October 17, 2022).

46. Amaresan N, Kumar K, Madhuri K, Usharani GK. Isolation and characterization of salt tolerant plant growth promoting rhizobacteria from plants grown in tsunami affected regions of Andaman and Nicobar Islands. *Geomicrobiol J.* (2016) 33:942–7. doi: 10.1080/01490451.2015.1128994

47. McMahon MA, Xu J, Moore JE, Blair IS, McDowell DA. Environmental stress and antibiotic resistance in food-related pathogens. *Appl Environ Microbiol.* (2007) 73:211–7. doi: 10.1128/AEM.00578-06

48. Poole K. Stress responses as determinants of antimicrobial resistance in Gram-negative bacteria. *Trends Microbiol.* (2012) 20:227–34. doi: 10.1016/j.tim.2012.02.004

49. Purti N, Shankar VS, Narshimulu G, Halder S, Ramayya C, Singh RP. Study on the diversity of birds in the new abode of wetlands created by the 2004 tsunami in South Andaman. *J Threatened Taxa.* (2022) 14:20811–20. doi: 10.11609/jott.6804.14.4.20811-20820

50. Yuan L, Liu JH, Hu GZ, Pan YS, Liu ZM, Mo J, et al. Molecular characterization of extended-spectrum β-lactamase-producing *Escherichia coli* isolates from chickens in Henan Province, China. *J Med Microbiol.* (2009) 58:1449–53. doi: 10.1099/jmm.0.012229-0

51. Hasman H, Mevius D, Veldman K, Olesen I, Aarestrup FM. β-Lactamases among extended-spectrum β-lactamase (ESBL)-resistant *Salmonella* from poultry, poultry products and human patients in The Netherlands. *J Antimicrob Chemother.* (2005) 56:115–21. doi: 10.1093/jac/dki190

52. Paterson DL, Bonomo RA. Extended-spectrum β-lactamases: a clinical update. *Clin Microbiol Rev.* (2005) 18:657–86. doi: 10.1128/CMR.18.4.657-686.2005

53. Carattoli A, García-Fernández A, Varesi P, Fortini D, Gerardi S, Penni A, et al. Molecular epidemiology of *Escherichia coli* producing extended-spectrum β-lactamases isolated in Rome, Italy. *J Clin Microbiol.* (2008) 46:103–8. doi: 10.1128/JCM.01542-07

54. Haenni M, Ponsin C, Métayer V, Médaille C, Madec JY. Veterinary hospital-acquired infections in pets with a ciprofloxacin-resistant CTX-M-15-producing *Klebsiella pneumoniae* ST15 clone. *J Antimicrob Chemother.* (2012) 67:770–1. doi: 10.1093/jac/dkr527

55. Kawamura K, Goto K, Nakane K, Arakawa Y. Molecular epidemiology of extended-spectrum β-lactamases and *Escherichia coli* isolated from retail foods including chicken meat in Japan. *Foodborne Patho Dis.* (2014) 11:104–10. doi: 10.1089/fpd.2013.1608

56. Corkill JE, Cuevas LE, Gurgel RQ, Greensill J, Hart CA. SHV-27, a novel cefotaxime-hydrolysing β-lactamase, identified in *Klebsiella pneumoniae* isolates from a Brazilian hospital. *J Antimicrob Chemother.* (2001) 47:463–5. doi: 10.1093/jac/47.4.463

57. Banerjee A, Bardhan R, Chowdhury M, Joardar SN, Isore DP, Batabyal K, et al. Characterization of beta-lactamase and biofilm producing *Enterobacteriaceae* isolated from organized and backyard farm ducks. *Lett Appl Microbiol.* (2019) 69:110–5. doi: 10.1111/lam.13170

58. Castellanos LR, Donado-Godoy P, León M, Clavijo V, Arevalo A, Bernal JF, et al. High heterogeneity of *Escherichia coli* sequence types harbouring ESBL/AmpC genes on IncI1 plasmids in the Colombian poultry chain. *PLoS ONE.* (2017) 12:e0170777. doi: 10.1371/journal.pone.0170777

59. Haenni M, Saras E, Métayer V, Médaille C, Madec JY. High prevalence of bla CTX-M-1/IncI1/ST3 and bla CMY-2/IncI1/ST2 plasmids in healthy urban dogs in France. *Antimicrob Agents Chemother.* (2014) 58:5358–62. doi: 10.1128/AAC.02545-14

frontiers | Frontiers in Veterinary Science

TYPE Brief Research Report
PUBLISHED 03 March 2023
DOI 10.3389/fvets.2023.1123137

OPEN ACCESS

EDITED BY
Kohei Makita,
Rakuno Gakuen University, Japan

REVIEWED BY
Ilias Giannenas,
Aristotle University of Thessaloniki, Greece
Ashenafi Feyisa Beyi,
Iowa State University, United States
Akira Fukuda,
Rakuno Gakuen University, Japan

*CORRESPONDENCE
Manuela Roldan-Henao
✉ manuelaroldan@outlook.com

SPECIALTY SECTION
This article was submitted to Veterinary Epidemiology and Economics, a section of the journal Frontiers in Veterinary Science

RECEIVED 14 December 2022
ACCEPTED 13 February 2023
PUBLISHED 03 March 2023

CITATION
Roldan-Henao M, Dalsgaard A, Cardona-Castro N, Restrepo-Rivera L, Veloza-Angulo LC and Alban L (2023) Pilot study of the productivity and *Salmonella* seroprevalence in pigs administered organic acids. *Front. Vet. Sci.* 10:1123137. doi: 10.3389/fvets.2023.1123137

Pilot study of the productivity and *Salmonella* seroprevalence in pigs administered organic acids

Manuela Roldan-Henao[1]*, Anders Dalsgaard[2], Nora Cardona-Castro[3], Lina Restrepo-Rivera[3], Luis Carlos Veloza-Angulo[1] and Lis Alban[2,4]

[1]Department of Veterinary and Animal Sciences, CES University, Medellin, Colombia, [2]Department of Veterinary and Animal Sciences, University of Copenhagen, Copenhagen, Denmark, [3]Instituto Colombiano de Medicina Tropical, CES University, Medellin, Colombia, [4]Department for Food Safety and Veterinary Issues, Danish Agriculture & Food Council, Copenhagen, Denmark

Control of *Salmonella* in pig/pork production is important to protect public health because pork is one of the main sources of human infection. Moreover, antimicrobial use in pig farms should be kept low to minimize development and transmission of antimicrobial resistance. This pilot study evaluated the productivity and *Salmonella* seroprevalence in pigs administered organic acids (OA) compared to pigs given growth promoters in one farm in Antioquia, Colombia. Two groups each consisting of 60 pigs of 6-weeks of age were studied for 4 months. One group was provided feed and water with OA (Selko pH® and Selacid®), whereas the other group (control) received antimicrobial growth promoters according to routine feeding practices (tylosin and zinc bacitracin). Blood samples were taken three times (T1–T3) and pigs were weighted five times to calculate daily weight gain (DWG) and feed conversion ratio (FCR). Initially when the pigs were 6 weeks old (T1), the *Salmonella* seroprevalence was 1.7% in both groups. When the pigs were 11 weeks old (T2), the seroprevalence was significantly lower in pigs provided OA compared to the control group (19 vs. 47%, $P < 0.001$), whereas when the pigs were 23 weeks old (T3), the seroprevalence did not differ between the groups (62 vs. 77%; $P = 0.075$). The cumulative DWG was significantly higher in the intervention group than in the control group (713 vs. 667 g/day; $P < 0.001$). The cumulative FCR did not differ between groups (2.80 vs. 2.77; $P = 0.144$). The pilot study indicates that cleaning the water pipes and administrating OA improve productivity in pigs and delay exposure to *Salmonella* spp. when compared with growth promoters. Thus, OA could replace antimicrobial growth promoters and reduce antimicrobial use and resistance. However, the study should be repeated before firmer conclusions can be drawn.

KEYWORDS

***Salmonella*, organic acids, pigs, seroprevalence, growth promoters, growth performance**

1. Introduction

Salmonellosis is a foodborne, zoonotic disease that is generally self-limiting (1). Worldwide, non-typhoidal *Salmonella* is ascribed to ~93.8 million human cases of acute gastroenteritis and 155,000 deaths annually (2, 3). In the United States, the cost of human salmonellosis is estimated to be around $2.9 billion per year (4). Denmark has carried out intensive programs to control *Salmonella* in the animal production chain since 1990s which has resulted in a low human incidence, i.e.,

11.8 *Salmonella* cases per 100,000 habitants were registered in 2021 (5). In Colombia, human salmonellosis is underreported and considered an endemic disease with sporadic outbreaks. According to the Colombian National Institute of Health, 7,219 *Salmonella* cases were reported between 2000 and 2013 with *S.* Typhimurium (33.7%) being the most common serotype detected followed by *S.* Enteritidis (28.6%), *S.* Dublin (3.3%) and *S.* Derby (2.1%) (6, 7).

The distribution of the different *Salmonella* serotypes varies according to food source and geographical area (6). Infection with *S.* Enteritidis is often associated with consumption of eggs and poultry meat, whereas the other globally important serotype *S.* Typhimurium is related mainly to consumption of pork (6, 8). In 2020, 13.0% of the human cases of salmonellosis reported in the European Union (EU) were due to consumption of contaminated pork. In 2015, the *Salmonella* prevalence was 28.2% on pork carcasses at abattoirs in Colombia with *S.* Typhimurium, *S.* Agama and *S.* Agona being the main serotypes found (9). Comprehensive control of *Salmonella* throughout the food value chain can decrease the incidence of human salmonellosis (6, 10, 11).

Subclinical salmonellosis in pigs constitutes a source of *Salmonella*. After weaning, the pigs excrete *Salmonella* and infect other pigs in the pen. Excretion of *Salmonella* may increase at times of stress such as during transport to the abattoir and in the lairage area, resulting in high risks for contamination of the carcasses during slaughter unless adequate measures are taken (11–14). In Colombia, the between-farm *Salmonella* seroprevalence was 42.9% ($n = 350$) in 2020 in the seven main pig producing provinces (8, 15–17). Half of the *Salmonella* strains tested ($n = 41$) showed concurring resistance to penicillin, cefuroxime, tetracycline and trimethoprim/sulfamethoxazole. These types of antimicrobial resistances (AMR) in *Salmonella* may be ascribed to the routine use of antimicrobials supplemented to pig feed used in Colombia (3, 18) and is of concern for effective treatment of human salmonellosis (16). Moreover, reduced AMR levels are not just of benefit to human health but will also ensure that pigs suffering from diseases caused by bacterial pathogens can be treated.

Although the use of antimicrobial growth promoters is banned in some parts of the world including the EU, they are still allowed and commonly used in Colombia for disease control and to improve growth in livestock. However, use of such growth promoters leads to development of AMR. For this reason, alternatives have been sought to replace the antimicrobial growth promoters including preventive measures focusing on improving the health of pigs while maintaining productivity (19). Without maintenance of productivity, the farmers cannot be expected to change habits and replace antimicrobial growth promoters with alternatives.

Organic acids (OA) can be used to control *Salmonella* and promote growth in pigs. When administrated in water and/or feed, the OA cause a decreased pH to 3.8–4.2 at which the growth of many gastro-intestinal bacteria except lactobacilli is altered or directly inhibited. OA also modulate the intestinal fermentation patterns of feed creating a better gastro-intestinal environment with improved utilization of feed and growth (20–23). These positive effects of OA on feed conversion rate and growth performance are also described in poultry including increased egg production. The ability to decrease *Salmonella* colonization depends on the type of OA used (24). Although the antibacterial effect of OA is well known in theory, published results of efficacy in on-farm studies vary, with some reporting beneficial effects (25–28) while others fail to demonstrate any effect (29–31). Thus, further evidence is needed to establish at which concentrations and combinations OA could be used to control *Salmonella* spp. in pigs and to elucidate their effect on productivity (21, 32).

The purpose of this pilot study was to evaluate the effect of OA on the productivity and the *Salmonella* seroprevalence in pigs from weaning to slaughter. We undertook a clinical trial, comparing the effect of provision of OA with antimicrobial growth promoters in a pig farm in Antioquia, Colombia. The hypothesis was that OA supplements to water and feed were equally effective as the growth promoters. This would open up for a possible replacement of antimicrobial growth promoters with OA in line with the principles of prudent use of antimicrobials.

2. Materials and methods

2.1. Herd description

This study was endorsed by the Institutional Committee Cuidado y Uso de los Animales (CICUA) at the CES University, Medellin, Colombia (Code No. 206/Act No. 38). The study farm produced piglets as well as finisher pigs was selected in Antioquia, Colombia. The farm had a known positive status for *Salmonella*.

The farm had a total of 500 sows. The piglets were weaned at 4 weeks of age, where after they remained for 7 weeks in the weaning facilities. They were then moved to the growing facilities, where they would stay for 6 weeks. Finally, they were moved to the finishing pens where they remained for 6 weeks until slaughter. The feed was produced on the farm. The composition of the feed is shown in Supplementary Table S1. The pens were equipped with portable waterers to measure water consumption.

2.2. Baseline sampling

Prior to the start of the trial, sampling of blood, rectal swabs and fecal material was performed to determine the within-farm *Salmonella* seroprevalence and to confirm presence of *Salmonella* in the herd. In August 2020, blood samples and rectal swabs were taken from 10 lactating sows, 30 weaned piglets, 30 growing pigs and 30 finishing pigs. Subsequently in September and December 2020 as well as in April 2021, a total of 130 samples were collected, processed and analyzed in three different ways. The first 40 samples consisted of rectal swabs, which were transported in Selenite-Cystine medium (Instituto Colombiano de Medicina Tropical (ICMT), Medellin, Colombia) and processed at two different laboratories. The following 30 samples were fecal samples, each with a volume of around 25 g, and collected directly from the rectum of individual pigs to increase the sensitivity of the subsequent laboratory analysis. The fecal samples were placed in sterile plastic bags. The remaining 60 samples consisted of fecal swab samples which were transported in Aimes transport medium (ICMT, Medellin, Colombia).

2.3. Experimental design and sampling

A parallel, randomized, controlled clinical trial was performed at the selected pig farm including 120 individual pigs. The sample size was based on logistical and economic considerations. The piglets were randomly divided into two groups of 60 pigs each. Each individual pig was ear tagged with an identification number to ensure proper follow-up (Figure 1).

The inclusion criteria were piglets close to weaning at ∼28 days of age and healthy at the time of sampling. The exclusion criteria were piglets that presented physical defects or that had received any antimicrobial treatment up to 10 days before the selection of the animals. At the time of sample selection, 250 piglets met the inclusion criteria and the formula $K = N/n$ (K = sample interval, N = total population units and n = sample size) was used to determine the number of animals to be included in the study.

The farm veterinarian oversaw the assignment of pigs to each group in the pens before the sampling was initiated. Each pen was completely separated from other pens preventing the pigs in one group from having physical contact with other pigs. The trial started when the pigs were 6 weeks of age with a follow-up time of 4 months.

In September 2021, water samples were taken and microbiological and physicochemical analyses were performed to determine the dosage of Selko pH® (Trouw Nutrition, Tres Cantos, Madrid) to be added to the water. These water quality analyses were done as the effect of Selko pH® depends on the characteristics of the of water including pH, hardness, concentrations of minerals and organic matter as well as bacterial concentration. The results showed a high degree of fecal contamination of the water with an *E. coli* count of 1,944 CFU/100 ml, fecal coliforms of 3,888 CFU/100 ml, but with no isolation of *Salmonella* spp. It was therefore decided by the owner of the farm to disinfect the water pipes with 0.4 ml/l of citric acid solution (GREEN DAC® ECOLAB, Bogota, Colombia) before beginning the clinical trial to ensure the effect of the OA treatment. Subsequent water samples obtained after cleaning the pipes contained 0 *E. coli* CFU/100 ml, 8 CFU/100 ml of fecal coliforms and absence of *Salmonella* spp. During the clinical trial, the pipes were cleaned every month using citric acid in the same way as described above.

The drinking water for the intervention group was supplemented with Selko pH® that contains E 236 formic acid, E 260 acetic acid, E 295 ammonium formate, E 300 L-Ascorbic acid, E 330 citric acid, E 4 copper and E 6 zinc. Based on the water characteristics it was decided to add 0.8 ml/liter of Selko pH® to the water to ensure the expected effect. This dosage was administered during the first 4 h of the day, every other day throughout the follow-up period. Likewise, Selacid® (Trouw Nutrition®, Tres Cantos, Madrid) that contains E 200 sorbic acid, E 236 formic acid, E 260 acetic acid, E270 lactic acid, E 280 propionic acid, E 295 ammonium formate and E 330 citric acid was added to the feed. Two kg of Selacid® per ton was added to weaner feed, whereas 1.5 kg per ton was used in grower and fattener feed during the entire study. The concentration of the individual compounds in the two commercial products were not declared and such information could not be obtained from the company. In the control group, tylosin phosphate 10% (1 kg per ton) was added to the weaner feed for the first 7 days of the study. Moreover, 15% zinc bacitracin (300 g per ton) was added to the grower feed for about 1 month.

Before starting the intervention with 6 weeks old piglets, initial (T1) blood samples and rectal swabs were obtained from each the 60 piglets. These samples were analyzed in pools of two yielding a total of 30 pooled samples to determine the *Salmonella* seroprevalence and the proportion of pigs excreting *Salmonella.*

Blood samples were taken again when the pigs were 11 weeks old (T2) and at the end of the observation period, when the pigs were 23 weeks of age (T3). At the beginning of the observation period, each pig was weighed (W1). Weighing was repeated when the pigs were 9, 15, 17, and 23 weeks old (W2–W5) and these measurements were used to calculate the daily weight gain (DWG) using the formula: weight in kg gained/#days between weighing. Similar for feed conversion ratio (FCR), the following formula was used: kg consumed/weight in kg gained in the period. Feed consumption was estimated from the data sheet delivered to the farm manager and workers in charge of supplying feed to the pigs, and on which they noted the number of packages of feed supplied to each pen. The amount of feed in kg consumed by pigs in each pen and each group of pigs was then calculated.

2.4. Serological and microbiological analysis

The blood samples were stored and transported in a refrigerator (4–5°C) within 24 h after sample collection. Subsequently, serum was extracted and the ELISA diagnostic kit IDEXX® Swine *Salmonella* Ab (IDEXX, Barcelona, Spain) was used to evaluate the seroprevalence of *Salmonella* spp., using a cut-off of 40% optical density.

The 30 pooled rectal swabs were duly marked and transported within 24 h at 4–5°C to the Veterinary and Zootechnical Laboratory of ICMT, which was in charge of processing and analyzing the samples. Upon arrival at the laboratory, the samples were inoculated into peptonized water at an adjusted ratio of 1:10 weight/volume and incubated at 36 ± 1°C for 18 ± 2 h after which 1 ml was incubated in selenite cystine broth and incubated at 36 ± 1°C for 24 ± 2 h. On day three, 0.1 ml of the broth was inoculated onto Xylose Lysine Deoxycholate (XDL; ICMT, Colombia) agar and Hecktoen agar (ICMT, Colombia) and incubated at 36 ± 1°C for 18 ± 2 h. Suspected *Salmonella* spp. colonies were selected from both agar media and re-streaked onto MacConkey agar (ICMT, Colombia) to obtain pure colonies after incubation at 36 ± 1°C for 18 ± 2 h. Subsequently, suspected isolates were tested by urea and sulfide-indole-motility tests as well as Gram staining. Finally, suspected isolates were subjected to PCR to confirm the serogroup and serotype, using the primers and conditions previously described by Cardona-Castro et al. (33).

2.5. Statistical analysis

Data from all pigs were used. The serological samples of the animals that died during the follow-up period were filled according

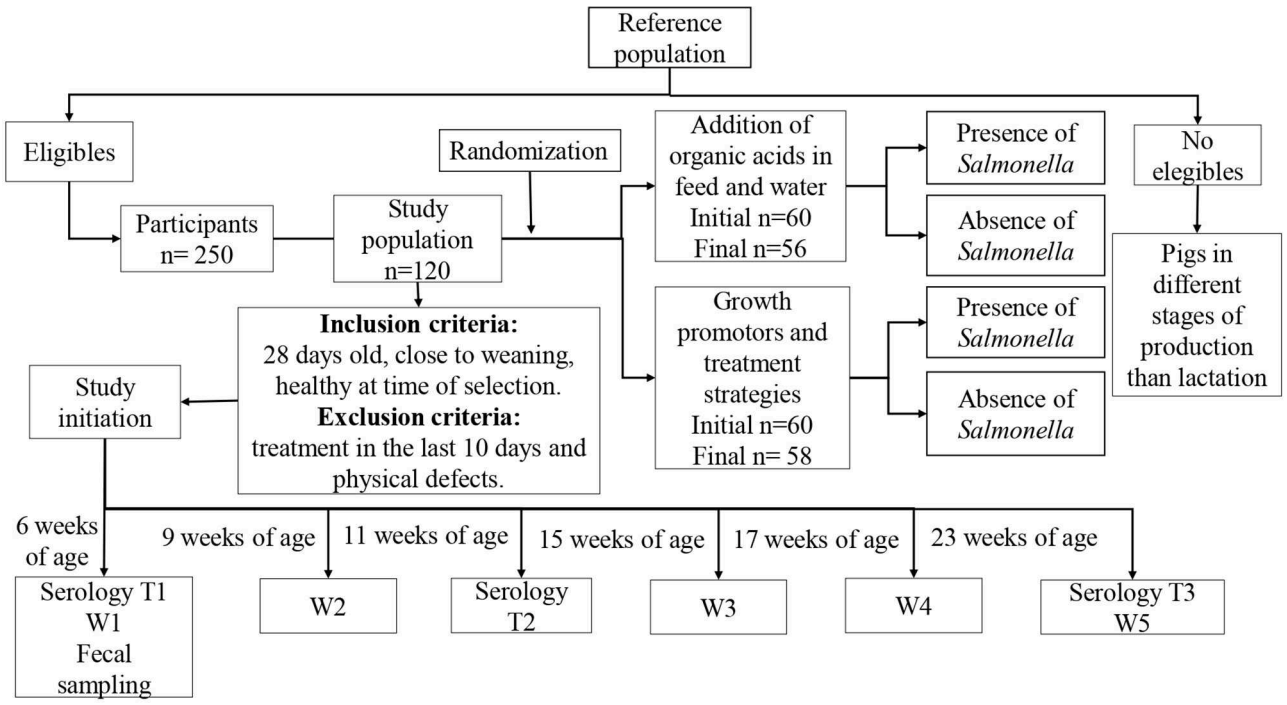


FIGURE 1
Study design. W1–5, weight 1 to weight 5.

to the mode of the results. For the statistical analyses, SPSS® version 21 CES university license, Microsoft Office Excel 2003 (Microsoft Corporation, Redmond, USA), JAMOVI version 1.8.4 of free distribution and EPIDAT 3.1 of free distribution was used.

A univariate analysis was carried out to describe the distribution of pigs included in the study according to their sex, age, weight, *Salmonella* seroprevalence and the line (breeder or finisher). To check for normality of the distribution of quantitative variables, Shapiro-Wilk normality test was performed. Next, bivariate analyses were undertaken investigating the association between the different variables, with a focus on the effect of treatment. Parametric tests were used for dependent quantitative variables that were normally distributed (T-student test), whereas non-parametric tests were used for the non-normally distributed variables (Mann-Whitney U test). Chi-square test was used for the count data variables, and the Fisher exact test was used when one or more of the expected cell values were <5. For all analyses, the *P*-value was reported using a significance value of $\alpha = 0.05$ (34, 35). Due to the limited number of samples, no attempts were made to model the seroprevalence over time using repeated measurements models.

3. Results

3.1. Salmonella baseline

In August 2020, the baseline seroprevalence of *Salmonella* was 59.0% in the pig herd. Only one *Salmonella*-positive sample was found and confirmed by PCR among the 100 fecal samples analyzed. The 130 fecal samples obtained between September 2020 and April 2021 were all negative for *Salmonella*. In 2021, the results of the second baseline sampling analyzing 100 blood samples yielded a seroprevalence of 47.0% (Table 1).

3.2. Clinical trial

The distribution of the pigs according to sex, age, line, *Salmonella* prevalence and weight is presented in Table 2 and shows no statistical difference between the two groups. During the observation period, six animals died among including four pigs from the intervention group and two pigs in the control group (Supplementary Figure S1). Based on a necropsy examination, the pigs died due to infarction, hemorrhage, meningitis, intestinal torsion and pneumonia. Hence, the causes of death were not related to the water and feed additions and this level of mortality was normal at the farm.

At T1, when the pigs were 6 weeks of age a *Salmonella* seroprevalence of 1.7% was found in both groups. At T2, when the pigs were 11 weeks of age a *Salmonella* seroprevalence of 18.3% was observed in the intervention group vs. 47.7% in the control group, showing a statistically significant difference between groups ($P < 0.001$). Finally, at T3 where the pigs were 23 weeks of age a *Salmonella* non-significant seroprevalence of 61.7% was observed in the intervention group vs. 76.7% in the control group ($P = 0.075$) (Supplementary Figure S2).

The median and the interquartile range (IQR) of the weight of the pigs at the different times of measurements are shown in

TABLE 1 *Salmonella* seroprevalence among the 100 pigs included in the base line study.

Date	Type of pig	No. of animals	Age	*Salmonella* serology positive samples (%)	Average seroprevalence
10/8/20	Lactating sows	10	1 year	6 (60.0%)	59.0%
10/8/20	Weaned piglets	30	6 weeks	9 (30.0%)	
17/8/20	Growing pigs	30	13 weeks	21 (70.0%)	
17/8/20	Finishing pigs	30	22 weeks	23 (76.7%)	
26/10/21	Lactating sows	10	1 year	7 (70.0%)	47.0%
26/10/21	Weaned piglets	30	9 weeks	3 (10.0%)	
21/10/21	Growing pigs	30	13 weeks	18 (60.0%)	
21/10/21	Finishing pigs	30	22 weeks	19 (63.3%)	

TABLE 2 Descriptive analysis of characteristics and *Salmonella* seroprevalence of 120 weaned piglets included in the clinical trial with organic acids.

Variable	Control group		Intervention group		*P*-value for group difference
	frequency	Relative frequency	Absolute frequency	Relative frequency	
Sex					
Female	35	58.3%	31	51.7%	0.22
Castrated	14	23.3%	10	16.7%	
Male	11	18.3%	19	31.7%	
Line					
Breeder	17	28.3%	12	20.0%	0.29
Finisher	43	71.7%	48	80.0%	
***Salmonella* seroprevalence**					
Positive at T1[a]	1	1.7%	1	1.7%	1
Positive at T2	28	47.7%	11	18.3%	<0.001
Positive at T3	46	76.7%	37	61.7%	0.075
Variable	Median	IQR	Median	IQR	*P*-value for group difference
Age (days) at T1	42	3	42	2	0.97
Weight (kg) at T1	15	3	14	3	0.11
Total	60	100%	60	100%	

[a] T1–T3 is the three times that *Salmonella* seroprevalence was measured during the trial where T1 was at the beginning of the trial, when the pigs were 6 weeks old, T2 at 11 weeks of age, and T3 at 23 weeks of age.

Figure 2. There was a statistically significant difference between the groups at W4 ($P < 0.001$) where the pigs were 17 weeks old with a better performance in the intervention group, where the median weight was 65.0 kg per pig (IQR = 10.0 kg) vs. 61.0 kg in the control group (IQR = 9.5 kg). Likewise, at W5 where the pigs were 23 weeks old, the growth performance was significantly higher ($P = 0.024$) in the intervention group, where the median weight was 101.0 kg per pig (IQR 12.5 kg) vs. 97.0 kg in the control group (IQR 11.0 kg).

For DWG3, there was a statistically significant difference between treatment groups ($P < 0.001$), showing higher values in the intervention group, which had a median of 722 g/pig/day (IQR 22 g/pig/day) vs. a median of 611 g/day (IQR 78 g/pig/day) in the control group (Figure 3). There was no difference between groups for DWG1, DWG2, and DWG4. However, the median of the cumulative DWG was 743 g/pig/day (IQR 12 g/pig/day) for the intervention group vs. 666 g/pig/day (IQR 10 g/pig/day) for the control group, showing a statistically significant difference ($P < 0.001$).

Regarding FCR, a statistically significant difference ($P = 0.025$) was observed at FCR3 where a median of 2.4 kg of feed per kg of weight gained (IQR 1.8 kg) was estimated for the intervention group vs. 2.8 kg (IQR 0.9 kg) in the control group. For FCR4, a statistically significant difference ($P = 0.009$) was observed where a median of 3.1 kg of feed per kg of weight gained (IQR 0.7 kg) was estimated for the intervention group vs. 2.8 kg (IQR 0.4 kg) in the control group (Figure 4). However, there was no significant difference ($P = 0.14$) when the median cumulative FCR was compared between groups, as the pigs in the intervention group

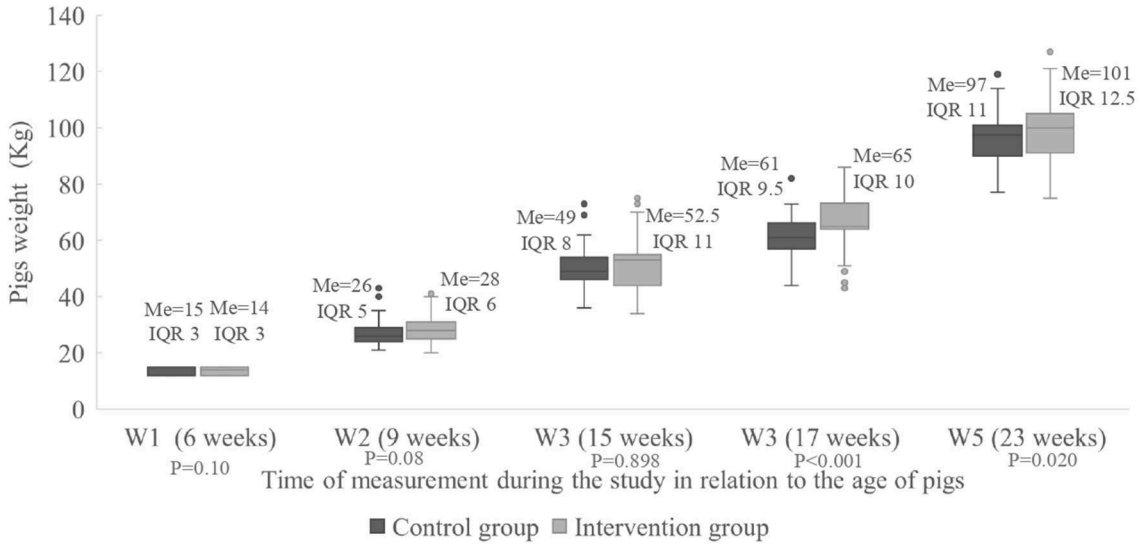


FIGURE 2
Weight of the individual pigs (kg) for the intervention and control groups measured five times during the study. Me, median; IQR, interquartile range; W1–5, Weight all time 1–5.

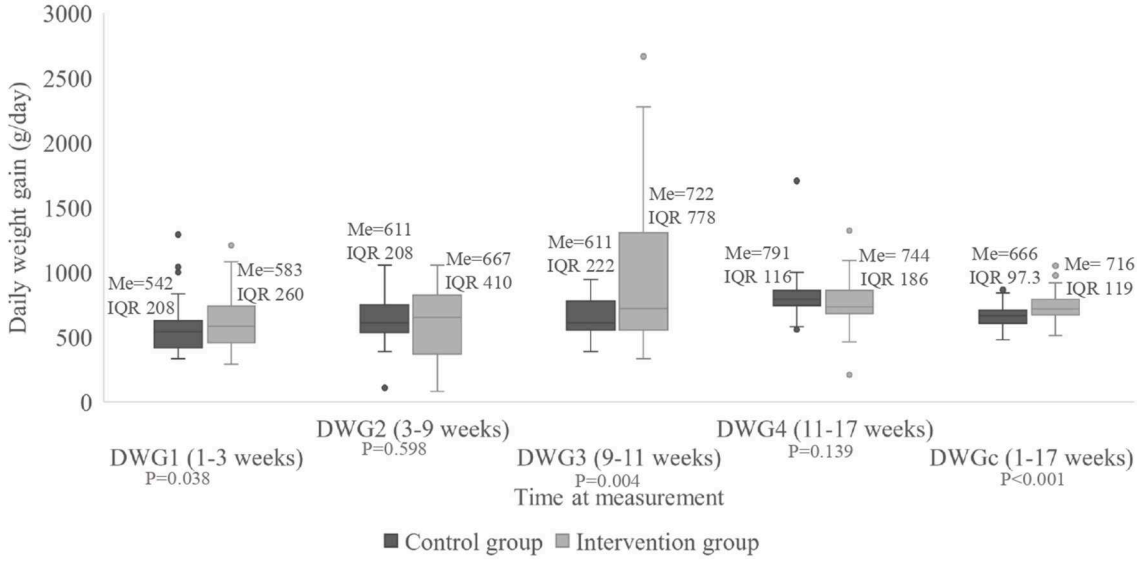


FIGURE 3
Daily weight gain of the pigs (g/day) divided according to group, measured at four times during the study as well as for the entire period. Me, median; IQR, interquartile range; DWGc, cumulative daily weight gain.

used 2.8 kg of feed per kg weight gained (IQR 0.6 kg) vs. 2.7 kg of feed (IQR 0.4 kg) the control group.

In the intervention group, the total feed consumption was 13,120 kg vs. 12,680 kg in the control group ($P = 0.61$). This corresponded to an average feed consumption of 2 kg per animal per day for the intervention group and 1.9 kg for the control group ($P = 0.87$). Furthermore, the total water consumption for the intervention group was 78,144 L and for the control group 70,310 L. The water consumption variable was not normally distributed; the median consumption was 620 L/day (IQR 460) for the intervention group and 560 L of water/day (IQR 410) for the control group. The difference in water consumption was not statistically significant ($P = 0.09$).

4. Discussion

The baseline results showed a high *Salmonella* seroprevalence of 59.0%, which did not concord with the low proportion of pigs excreting the bacteria as shown by the culture-based detection method (1.0%) (36). To investigate this further, several methods

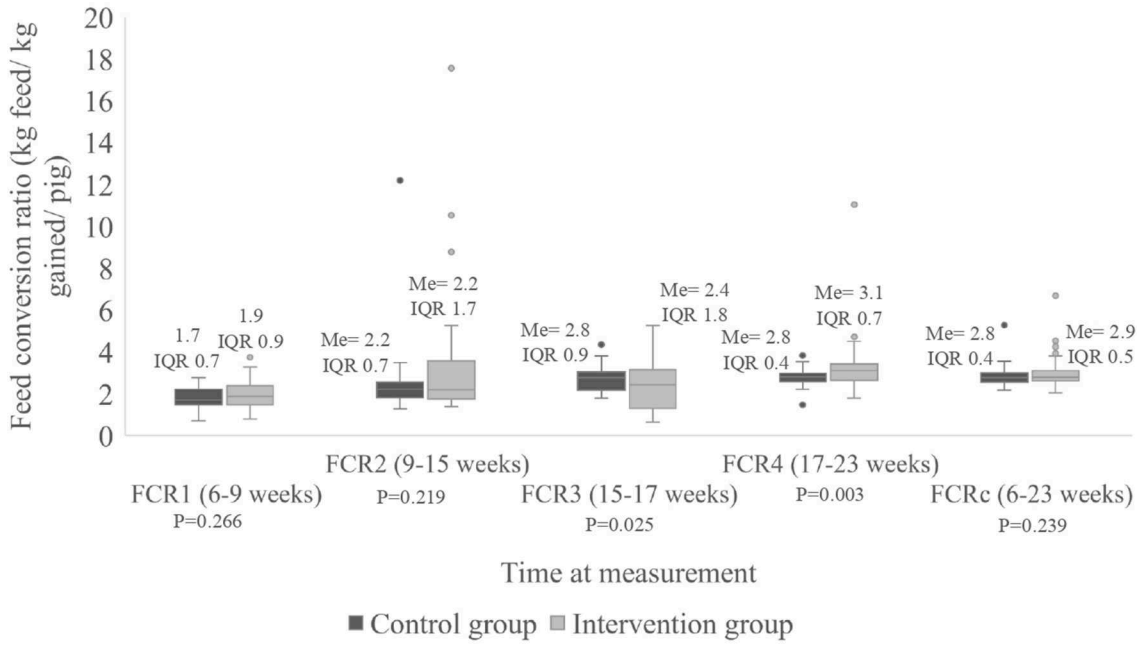


FIGURE 4
Feed conversion ratio (FCR) per pig (in kg/kg gained/pig) divided according to group, measured at four times during the study as well as for the entire period. Me, median; IQR, interquartile range; FCRc, cumulative feed conversion ratio.

and growth media were used to increase the sensitivity (37). However, these efforts were not successful in increasing the number of *Salmonella*-positive samples. This may be because of the known low sensitivity of culturing *Salmonella* spp. in fecal samples from pigs (8, 32, 38–40). Moreover, the regular administration of growth promoters to piglets on the farm could have reduced the *Salmonella* spp. excretion (40). Therefore, it was decided to measure only *Salmonella* seroprevalence during the study as an indication of the *Salmonella* prevalence.

There was a significantly lower *Salmonella* seroprevalence in the group of pigs provided organic acids (OA) (18.3%) compared with the control group (47.7%) at T2 (11 weeks). Contrary, at T1 (6 weeks) and T3 (15 weeks), there was no statistical difference in seroprevalence. OA favor the growth of lactobacilli, which contributes to a low pH, limit bacterial growth in the intestines and stimulates the immune system in a non-specific way; all of which decrease the probability of *Salmonella* colonization (3, 22, 41, 42). For this reason, the use of OA may have delayed the excretion and spread of *Salmonella* during the post-weaning period. However, as the observation period progressed, the majority of the pigs were eventually exposed to *Salmonella* spp. at some point. These findings are in agreement with the literature (3, 41, 43). Pigs develop partial immunity to *Salmonella* when the spread and exposure to the pathogen is reduced. Such partial immunity development is the core of a *Salmonella* reduction strategy as pigs at the time of slaughter will have a lower probability excreting *Salmonella* (36, 44). It is well-known that *Salmonella* cannot be eradicated without culling the farm (36, 44). There is a positive association between herd serology and the prevalence of *Salmonella* on the carcass as a low seroprevalence is associated with less prevalence on the carcass, less excretion and less overall contamination with *Salmonella* at the abattoir (45).

In Colombia, it is customary practice to use growth promoters like tylosin and zinc bacitracin. However, this is not in line with the principles of prudent use as it will lead to development of AMR and growth promoters are now banned in many countries (46, 47). Growth promoters are used as they are believed to support increased growth and reduce the severity of post-weaning diarrhea. In our study, pigs provided OA had a better cumulative DWG and weight productively than pigs administered growth promoters. The OA are feed additives that are metabolized by the animal, allowing their use without the risk of residues accumulating in the meat. The use of OA is already increasing as a response to strengthened regulations and consumer concerns on the use of antimicrobials in many countries (48). The mode of action of OA includes modulating stimulus that benefits the development of the mucosa, the length of microvilli, intestinal cell growth and therefore the absorptive capacity of the intestine is improved (3, 19).

The weight measured at time points W4 (17 weeks) and W5 (23 weeks) and the cumulative DWG during the study showed a better growth performance of pigs administered OA compared with the control group, which supports findings in other studies (49–56). van der Heijden et al. concluded that Selko pH® added to water at a concentration of 0.2% significantly reduced the seroprevalence of *Salmonella* and improved the productive performance of pigs (57). Likewise, formic, citric and benzoic acids can lead to improved growth when added to feed provided to weaned and growing pigs (23). A better DWG translates into less time spent by the pig in the herd, as well as a more efficient use of feed nutrients that represent one of the main costs of production (58).

Although there was a statistically significant difference ($P = 0.025$) at FCR3 and FCR4 ($P = 0.009$), favoring the intervention group and control group respectively, there was no significant difference in the cumulative FCR between the two groups. This may because the staff in charge of supplying the feed to the pigs did not fully take into account the pigs that died during the trial when calculating the feed to be administered. Therefore, the number of pigs used to calculate the feed provided was likely a little too high which may explain that no difference was found in the cumulative FCR between the groups (59).

The cleaning of the water pipes on the farm with citric acid before and during the study improved the water quality, which likely also resulted in healthier pigs (60, 61). Water is a potential source of various pig pathogens causing diseases that affect weight gain and feed conversion (62). For this reason, it is recommended to clean the water pipes regularly. The combination of cleaning of the pipes and the use of OA may be responsible for the higher overall productivity and apparently slower spread of *Salmonella* in the group administered OA. At the abattoir, such pigs are expected to have a lower probability of excreting *Salmonella* (44).

Salmonella antibodies can remain at measurable levels up to 3 months in the pig, which means that positives animals can be found even when they no longer are infected or excreting *Salmonella* spp. (36). Pigs included in the clinical trial may have experienced exposure to the pathogen without excreting *Salmonella* during sampling. Additionally, presence of antibodies in the individual animal may not be directly related to a carrier stage or probability of shedding *Salmonella* spp. (63). Hence, it is a limitation of our study that no other diagnostic tests were applied that could confirm whether pigs were excreting *Salmonella* (13). Post-harvest sampling of lymph nodes and ileocecal contents of the pigs may have increased the likelihood of detecting *Salmonella* if present, and thereby allowing a better assessment of how OA impacted the *Salmonella* levels in the pigs (37).

Selacid® was supplied at different concentrations during the study. It is known that different concentrations of OA can affect the *Salmonella* seroprevalence in pigs as shown by Calveyra et al. who concluded that at a concentration of 0.1%, OA had no significant effect on the *Salmonella* level whereas it did have a significant effect on improving daily weight gain in the pigs (64).

The dosage of Selko pH® we administered to drinking water (0.8 ml/L) was slightly lower than the dosage recommended by the technical data sheet (1–2 ml/L) from the manufacturer (65). The total estimated cost of the growth promoters added to the administered feed was 32 US$ as compared to 57 US$ for OA added to water and feed. The relative low dosage of OA may have had a reduced effect on *Salmonella* in the intervention group (25, 30, 66). On the other hand, the additional supplement of OA in the feed probably compensated for the lower concentration of Selko pH® used in the water. The types and concentrations of different OA products—as well as their costs—should be further investigated for their effect on *Salmonella* and overall productivity as the effect of the acids varies significantly depending on the components present in the feed (23, 59, 63). Moreover, attention should be given to palatability of the OA to ensure that the pigs do not consume less water or feed. Contrary to traditional organic acids, Selko pH® has the advantage that it is safe to use as it can be given to pigs in relative high concentrations without risking that the pigs stop drinking because of palatability issues.

For future research, it is recommended to include pig farms with known high prevalence of *Salmonella* spp., serial sampling and analyses of 25-g of fecal samples to increase the sensitivity.

5. Conclusion

This pilot study indicates that administration of OA in combination with regular cleaning of water pipes can improve productivity and delay exposure to *Salmonella* spp. when compared with commonly used antimicrobial growth promoters. A substitution of antimicrobial growth promoters with OA will lower antimicrobial use and resistance, while ensuring productivity. However, the study should be repeated before firmer conclusions can be drawn regarding productivity and the *Salmonella* spp. reduction potential of OA.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Ethics statement

This study was endorsed by the Institutional Committee Cuidado y Uso de los Animales (CICUA) at the CES University, Medellin, Colombia (Code No. 206/Act No. 38).

Author contributions

AD, LA, and MR-H were responsible for the conception of the study, experimental design, and manuscript writing. MR-H was responsible of collection of samples in the farm, data analysis, and interpretation. NC-C, LR-R, and LV-A were responsible for conception of the study and experimental design. All authors contributed to the article and approved the submitted version.

Funding

This work was part of the project entitled Salmonella Control in the Colombian Pig Industry that received financial support by the Ministry of Foreign Affairs (Danida) through the grant number 18-M07-KU.

Acknowledgments

The authors are grateful to ICMT veterinary laboratory (ICTM, Medellin, Colombia) and staff for help in processing and performing the serological and microbiological analyses. The Danish Agriculture & Food Council and University of Copenhagen

are thanked for opening their doors for the Colombian co-authors to learn from their expertise and knowledge. Finally, we thank Janeth Perez (Department of Veterinary and Animal Sciences, CES University, Medellin, Colombia) for her help with the statistical analysis.

Conflict of interest

LA works for an organization that gives advice to livestock producers and meat producing companies.

The remaining 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.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fvets.2023.1123137/full#supplementary-material

References

1. Catering E, Díaz-Guevara P, Moreno J, Bautista A, Monta L, Realpe ME, et al. Laboratory surveillance of *Salmonella* enterica from human clinical cases in Colombia 2005 – 2011. *Enferm Infecc Microbiol Clin.* (2017) 35:417–25. doi: 10.1016/j.eimc.2016.02.023

2. Mogollón Vergara D, Rodríguez Gutiérrez V, Verjan García N. Prevalence and molecular identification of *Salmonella* spp. isolated from commercialized eggs at Ibague, Colombia. *Revista de Salúd Animal.* (2016) 38:164–72.

3. Food and Agriculture Organization, World Health Organization. *Interventions for the Control of Non-typhoidal Salmonella spp. in Beef and Pork: Meeting Report and Systematic Review*. (2016). Available online at: https://apps.who.int/iris/handle/10665/249529 (accessed December 12, 2022).

4. Donado-Godoy P, Clavijo V, León M, Arevalo A, Castellanos R, Bernal J, et al. Counts, serovars, and antimicrobial resistance phenotypes of *Salmonella* on raw chicken meat at retail in Colombia. *J Food Prot.* (2014) 77:227. doi: 10.4315/0362-028X.JFP-13-276

5. National Food Institute, Technical University of Denmark. *Annual Report on Zoonoses in Denmark 2021*. National Food Institute (2022). Available online at: https://www.food.dtu.dk/ (accessed December 12, 2022).

6. Carvajal-Restrepo H, Sánchez-Jiménez MM, Díaz-Rodríguez S, Cardona-Castro N. Detection of *Salmonella* human carriers in Colombian outbreak areas. *J Infect Dev Ctries.* (2017) 11:228. doi: 10.3855/jidc.8023

7. Instituto Nacional de Salud, Dirección Redes en Salud Pública. *Características de los Aislamientos de Salmonella spp. Colombia Resultados de la Vigilancia 2000-2013*. (2014). Available online at: https://www.ins.gov.co/BibliotecaDigital/informe-vigilancia-por-laboratorio-de-salmonella-spp-2000-2013.pdf (accessed December 12, 2022).

8. Rondón-Barragán IS, Arcos EC, Mora-Cardona L, Fandiño C. Characterization of *Salmonella* species from pork meat in Tolima, Colombia. *Revista Colombiana de Ciencias Pecuarias.* (2015) 28:74–82.

9. Ayala-Romero C, Ballen-Parada C, Rico-Gaitan M, Chamorro-Tobar I, Zambrano-Moreno D, Poutou-Piñales R. Prevalencia de *Salmonella* spp. en ganglios mesentéricos de porcinos en plantas de beneficio colombianas. *RevMVZ Córdoba.* (2018) 23:6474. doi: 10.21897/rmvz.1242

10. European Food Safety Authority. The European Union one health 2020 zoonoses report. *EFSA J.* (2021) 19:6971. doi: 10.2903/j.efsa.2021.6406

11. Alban L, Stärk KDC. Where should the effort be put to reduce the *Salmonella* prevalence in the slaughtered swine carcass effectively? *Prevent Vet Med.* (2005) 68:63. doi: 10.1016/j.prevetmed.2005.01.001

12. Betancur Vélez E. Control de *Salmonella* en cerdos con uso de aditivos y sin el uso de antibióticos en el alimento, en cinco granjas porcícolas del municipio de Santa Rosa de Osos (Antioquia) (Master's thesis). Corporación Universitaria Lasallista, Caldas, Antioquia (2018).

13. Ruggeri J, Foresti F, Pavesi R, Terrini A, Giudici F, Padoan D, et al. The synergistic effect of organic acids, phytochemicals and a permeabilizing complex reduces *Salmonella* Typhimurium 1,4,[5],12:i- shedding in pigs. *Vet Res Commun.* (2018) 42:209. doi: 10.1007/s11259-018-9723-3

14. Bonardi S. *Salmonella* in the pork production chain and its impact on human health in the European Union. *Epidemiol Infect.* (2017) 145:1513. doi: 10.1017/S095026881700036X

15. Arguello H, Álvarez-Ordoñez A, Carvajal A, Rubio P, Prieto M. Role of slaughtering in *Salmonella* spreading and control in pork production. *J Food Prot.* (2013) 76:899. doi: 10.4315/0362-028X.JFP-12-404

16. Giraldo-Cardona JP, Gualdrón-Ramírez D, Chamorro-Tobar I, Pulido-Villamarín A, Santamaría-Durán N, Castañeda-Salazar R, et al. *Salmonella* spp. prevalence, antimicrobial resistance and risk factor determination in Colombian swine farms. *Pesquisa Veterinaria Brasileira.* (2019) 39:816. doi: 10.1590/1678-5150-pvb-6156

17. Pulido-Villamarín AP, Santamaría-Durán AN, Castañeda-Salazar R, Chamorro-Tobar I, Carrascal-Camacho AK, Aranda-Silva M, et al. Evaluación de anticuerpos frente a tres bacterias zoonóticas y factores de riesgo asociados en explotaciones porcinas de Colombia. *Rev Sci Tech.* (2020) 39:923. doi: 10.20506/rst.39.3.3188

18. Miller GY, Xuanli L, McNamara P, Barber DA. Influence of *Salmonella* in pigs preharvest and during pork processing on human health costs and risks from pork. *J Food Protect.* (2005) 68:1788–98. doi: 10.4315/0362-028X-68.9.1788

19. Mroz Z. Organic acids as potential alternatives to antibiotic growth promoters for pigs. *Adv Pork Prod.* (2005) 16:169–82.

20. van der Wolf PJ, van Schie FW, Elbers ARW, Engel B, Van der Heijden HMJF, Huneman WA, et al. Epidemiology: administration of acidified drinking water to finishing pigs in order to prevent *Salmonella* infections. *Vet Q.* (2011) 2176:20–5. doi: 10.1080/01652176.2001.9695097

21. Gómez-García M, Sol C, Nova PJG de, Puyalto M, Mesas L, Puente H, et al. Antimicrobial activity of a selection of organic acids, their salts and essential oils against swine enteropathogenic bacteria. *Porcine Health Manag.* (2019) 4:1. doi: 10.1186/s40813-019-0139-4

22. Michiels J, Missotten J, Rasschaert G, Dierick N, Heyndrickx M, Smet SDE. Effect of organic acids on *Salmonella* colonization and shedding in weaned piglets in a seeder model. *J Food Protect.* (2012) 75:1974. doi: 10.4315/0362-028X.JFP-12-210

23. Liu Y, Espinosa CD, Abelilla JJ, Casas GA, Lagos LV, Lee SA, et al. Non-antibiotic feed additives in diets for pigs: a review. *Anim Nutr.* (2018) 4:113. doi: 10.1016/j.aninu.2018.01.007

24. van Immerseel F, Russell JB, Flythe MD, Gantois I, Timbermont L, Pasmans F, et al. The use of organic acids to combat *Salmonella* in poultry: a mechanistic explanation of the efficacy. *Avian Pathol.* (2006) 35:182. doi: 10.1080/03079450600711045

25. Creus E, Pérez JF, Peralta B, Baucells F, Mateu E. Effect of acidified feed on the prevalence of *Salmonella* in market-age pigs. *Zoonoses Public Health.* (2007) 54:314. doi: 10.1111/j.1863-2378.2007.01069.x

26. Rasschaert G, Michiels J, Tagliabue M, Missotten J, de Smet S, Heyndrickx M. Effect of organic acids on *Salmonella* shedding and colonization in pigs on a farm with high *Salmonella* prevalence. *J Food Prot.* (2016) 79:51. doi: 10.4315/0362-028X.JFP-15-183

27. Taube VA, Neu ME, Hassan Y, Verspohl J, Beyerbach M, Kamphues J. Effects of dietary additives (potassium diformate/organic acids) as well as influences of grinding intensity (coarse/fine) of diets for weaned piglets experimentally infected

with *Salmonella* Derby or *Escherichia coli*. *J Anim Physiol Anim Nutr.* (2009) 93:350. doi: 10.1111/j.1439-0396.2008.00894.x

28. Walia K, Argüello H, Lynch H, Leonard FC, Grant J, Yearsley D, et al. Effect of feeding sodium butyrate in the late finishing period on *Salmonella* carriage, seroprevalence, and growth of finishing pigs. *Prev Vet Med.* (2016) 131:79. doi: 10.1016/j.prevetmed.2016.07.009

29. de Busser EV, Dewulf J, Nollet N, Houf K, Schwarzer K, de Sadeleer L. Effect of organic acids in drinking water during the last 2 weeks prior to slaughter on *Salmonella* shedding by slaughter pigs and contamination of carcasses. *Zoonoses Public Health.* (2009) 32:129. doi: 10.1111/j.1863-2378.2008.01172.x

30. Letellier A, Messier S, Lessard L, Quessy S. Assessment of various treatments to reduce carriage of *Salmonella* in swine. *Can J Vet Res.* (2000) 3:27–31. doi: 10.31274/safepork-180809-1037

31. Walsh M, Sholly D, Kelly D, Cobb M, Trapp S. The effects of supplementing weanling pig diets with organic and inorganic acids on growth performance and microbial shedding. *Swine Research Report.* (2003). p. 89–98.

32. Lynch H, Leonard FC, Walia K, Lawlor PG, Duffy G, Fanning S, et al. Investigation of in-feed organic acids as a low-cost strategy to combat *Salmonella* in grower pigs. *Prev Vet Med.* (2017) 139:50. doi: 10.1016/j.prevetmed.2017.02.008

33. Cardona-Castro N, Sánchez-Jiménez M, Lavalett L, Múñoz N, Moreno J. Development and evaluation of a multiplex polymerase chain reaction assay to identify *Salmonella* serogroups and serotypes. *Diagn Microbiol Infect Dis.* (2009) 65:327. doi: 10.1016/j.diagmicrobio.2009.07.003

34. Dani Daniel WW. *Bioestadística: Base Para el Análisis de las Ciencias de la Salud*. México: Limusa Wiley (2010). 736 p.

35. Martínez-González MA, Sánchez-Villegas A, Faulín Fajardo FJ. *Bioestadística Amigable*. Madrid: Díaz de Santos (2009). 919 p.

36. Kranker S, Alban L, Boes J, Dahl J. Longitudinal study of *Salmonella* enterica serotype typhimurium infection in three Danish farrow-to-finish swine herds. *J Clin Microbiol.* (2003) 4:2282. doi: 10.1128/JCM.41.6.2282-2288.2003

37. World Health Organization, Food and Agriculture Administration. *Codex Alimentarius. Anteproyecto de directrices para el control de Salmonella spp. no tifoidea en la carne de bovino y cerdo*. (2016). Available online at: http://www.who.int/foodsafety/consumer/5keys/en/ (accessed December 12, 2022).

38. Organización Mundial de Sanidad Animal. *Código Sanitario para los Animales Terrestres*. Salmonelosis (2022). Available online at: https://www.woah.org/fileadmin/Home/esp/Health_standards/tahm/3.10.07_SALMONELLOSIS.pdf (accessed December 12, 2022).

39. Mejía Silva WJ. *Epidemiología de la salmonelosis porcina en granjas de Cataluña y determinación de los factores de riesgo de la infección* (Master's thesis). Universidad Autónoma de Barcelona, Barcelona, España (2003).

40. Wells JE, Kalchayanand N, Berry ED, Oliver W. Effects of antimicrobials fed as dietary growth promoters on faecal shedding of Campylobacter, *Salmonella* and shiga-toxin producing *Escherichia coli* in swine. *J Appl Microbiol.* (2013) 114:318. doi: 10.1111/jam.12065

41. Dibner JJ, Buttin P. Use of organic acids as a model to study the impact of gut microflora on nutrition and metabolism. *J Appl Poultry Res.* (2002) 11:453. doi: 10.1093/japr/11.4.453

42. Boyen F, Haesebrouck F, Vanparys A, Volf J, Mahu M. Coated fatty acids alter virulence properties of *Salmonella* Typhimurium and decrease intestinal colonization of pigs. *Vet Microbiol.* (2008) 132:319. doi: 10.1016/j.vetmic.2008.05.008

43. Boyen F, Haesebrouck F, Maes D, van Immerseel F, Ducatelle R, Pasmans F. Non-typhoidal *Salmonella* infections in pigs: a closer look at epidemiology, pathogenesis and control. *Vet Microbiol.* (2008) 130:1. doi: 10.1016/j.vetmic.2007.12.017

44. Alban L, Baptista FM, Møgelmose V, Sørensen LL, Christensen H, Aabo S, et al. *Salmonella* surveillance and control for finisher pigs and pork in Denmark - a case study. *Food Res Int.* (2012) 45:656. doi: 10.1016/j.foodres.2011.02.050

45. Sørensen LL, Alban L, Nielsen B, Dahl J. The correlation between *Salmonella* serology and isolation of *Salmonella* in Danish pigs at slaughter. *Vet Microbiol.* (2004) 101:131. doi: 10.1016/j.vetmic.2004.02.016

46. Porkcolombia. *Normas Colombianas*. Revista Porkcolombia (2019). Available online at: https://porkcolombia.co/wp-content/uploads/2019/10/ED-249-PORKCOLOMBIA-DIGITAL.pdf (accessed January 25, 2023).

47. Dirección de Medicamentos y Tecnologías en Salud. *Plan Nacional de Respuesta a la Resistencia a los Antimicrobianos Plan Estratégico*. Bogotá: Dirección de Medicamentos y Tecnologías en Salud (2018).

48. Pacheco Nuñez GL, Palma Berrios JM. *Efecto del uso de acidificantes (CitroZimTM y CitroZim-NaTM) en dietas de lechones en etapa de inicio* (Master's thesis). Universidad de Zamorano, Zamorano (2015).

49. Luise D, Motta V, Salvarani C, Chiappelli M, Fusco L, Bertocchi M, et al. Long-term administration of formic acid to weaners: influence on intestinal microbiota, immunity parameters and growth performance. *Anim Feed Sci Technol.* (2017) 232:160. doi: 10.1016/j.anifeedsci.2017.06.015

50. Guggenbuhl P, Séon A, Quintana AP, Nunes CS. Effects of dietary supplementation with benzoic acid (VevoVitall®) on the zootechnical performance, the gastrointestinal microflora and the ileal digestibility of the young pig. *Livest Sci.* (2007) 108:218. doi: 10.1016/j.livsci.2007.01.068

51. Papatsiros V, Tassis P, Tzika E, Papaioannou D, Petridou E, Alexopoulos C, et al. Effect of benzoic acid and combination of benzoic acid with a probiotic containing Bacillus Cereus var. toyoi in weaned pig nutrition. *Pol J Vet Sci.* (2011) 14:117. doi: 10.2478/v10181-011-0017-8

52. Halas D, Hansen CF, Hampson DJ, Mullan BP, Kim JC, Wilson RH, et al. Dietary supplementation with benzoic acid improves apparent ileal digestibility of total nitrogen and increases villous height and caecal microbial diversity in weaner pigs. *Anim Feed Sci Technol.* (2010) 160:137. doi: 10.1016/j.anifeedsci.2010.07.001

53. Diao H, Gao Z, Yu B, Zheng P, He J, Yu J, et al. Effects of benzoic acid (VevoVitall®) on the performance and jejunal digestive physiology in young pigs. *J Anim Sci Biotechnol.* (2016) 7:32. doi: 10.1186/s40104-016-0091-y

54. Upadhaya SD, Lee KY, Kim IH. Protected organic acid blends as an alternative to antibiotics in finishing pigs. *Asian Australas J Anim Sci.* (2014) 27:1600. doi: 10.5713/ajas.2014.14356

55. Kuang Y, Wang Y, Zhang Y, Song Y, Zhang X, Lin Y, et al. Effects of dietary combinations of organic acids and medium chain fatty acids as a replacement of zinc oxide on growth, digestibility and immunity of weaned pigs. *Anim Feed Sci Technol.* (2015) 208:145. doi: 10.1016/j.anifeedsci.2015.07.010

56. Long SF, Xu YT, Pan L, Wang QQ, Wang CL, Wu JY, et al. Mixed organic acids as antibiotic substitutes improve performance, serum immunity, intestinal morphology and microbiota for weaned piglets. *Anim Feed Sci Technol.* (2018) 235:23. doi: 10.1016/j.anifeedsci.2017.08.018

57. van der Heijden M, van Dam H, Niewerth D, Frankena K. Effectiveness of *Salmonella* control strategies in fattening pigs. In: *Safe Pork.* (2005).

58. Pfuderer S, Bennett RM, Brown A, Collins LM. A flexible tool for the assessment of the economic cost of pig disease in growers and finishers at farm level. *Prev Vet Med.* (2022) 208:105757. doi: 10.1016/j.prevetmed.2022.105757

59. Garzón Rivero D, Espitia Forero ÉT. *Crisis de los contenedores una mirada desde el contexto global y sus implicaciones en Colombia* (Master's thesis). Institución Universitaria Colegios de Colombia, Bogotá (2022).

60. de Sousa C, Colmenares MC, Correia A. Contaminación bacteriológica en los sistemas de distribución de agua potable: revisión de las estrategias de control. *Bol Mal Salud Amb.* (2008) 48:17.

61. Quintero Barrera LD, Pulido-Villamarín A del P, Chamorro-Tobar I. *Determinación de la presencia de Salmonella spp. en el agua de granjas porcícolas de diferentes regiones de Colombia* (Master's thesis). Universidad Pontificia Javeriana, Bogotá] (2020).

62. Rivas-Nichorzon M, Alfaro-Escalona M, Silva-Acuña R, Gomez-Piñeres E. Calidad bacteriológica y pH del agua en una unidad de producción porcina ubicada en el Rincón de Monagas, estado Monagas, Venezuela. *Zootec Trop.* (2016) 34:127–33.

63. Parker S, Waddell L, Wilhelm B, Raji A, Sanchez J, Fazil A, et al. Assessment of the efficacy and quality of evidence for five on-farm interventions for *Salmonella* reduction in grow-finish swine: a systematic review and meta-analysis. *Prev Vet Med.* (2012) 107:1. doi: 10.1016/j.prevetmed.2012.07.011

64. Calveyra JC, Nogueira MG, Kich JD, Biesus LL, Vizzotto R, Berno L, et al. Effect of organic acids and mannanoligosaccharide on excretion of *Salmonella* typhimurium in experimentally infected growing pigs. *Res Vet Sci.* (2012) 93:46–7. doi: 10.1016/j.rvsc.2011.08.018

65. Trouw Nutrition. Selko ®-pH (2018). Available online at: www.selko.com (accessed December 12, 2022).

66. de Ridder L, Maes D, Dewulf J, Pasmans F, Boyen F, Haesebrouck F, et al. Evaluation of three intervention strategies to reduce the transmission *of Salmonella* Typhimurium in pigs. *Vet J.* (2013) 197:613. doi: 10.1016/j.tvjl.2013.03.026

frontiers | Frontiers in Veterinary Science

TYPE Original Research
PUBLISHED 10 March 2023
DOI 10.3389/fvets.2023.1092302

OPEN ACCESS

EDITED BY
Faham Khamesipour,
Shahid Beheshti University of Medical Sciences, Iran

REVIEWED BY
Lucy Brunton,
Royal Veterinary College (RVC), United Kingdom
Kebede Amenu,
Addis Ababa University, Ethiopia
Folorunso Oludayo Fasina,
University of Pretoria, South Africa

*CORRESPONDENCE
David C. Speksnijder
✉ d.c.speksnijder@uu.nl

SPECIALTY SECTION
This article was submitted to Veterinary Infectious Diseases, a section of the journal Frontiers in Veterinary Science

RECEIVED 07 November 2022
ACCEPTED 02 February 2023
PUBLISHED 10 March 2023

CITATION
Anwar Sani R, Wagenaar JA, Dinar TEHA, Sunandar S, Nurbiyanti N, Suandy I, Pertela G, Jahja EJ, Purwanto B, CORNERSTONE group, Geijlswijk IMv and Speksnijder DC (2023) The comparison and use of tools for quantification of antimicrobial use in Indonesian broiler farms. *Front. Vet. Sci.* 10:1092302. doi: 10.3389/fvets.2023.1092302

The comparison and use of tools for quantification of antimicrobial use in Indonesian broiler farms

Rianna Anwar Sani[1], Jaap A. Wagenaar[1,2,3], Tagrid E. H. A. Dinar[1], Sunandar Sunandar[4], Nofita Nurbiyanti[4], Imron Suandy[5], Gian Pertela[6], Elvina J. Jahja[7], Budi Purwanto[8], CORNERSTONE group, Ingeborg M. van Geijlswijk[9] and David C. Speksnijder[1,3,10]*

[1]Division of Infectious Diseases and Immunology, Faculty of Veterinary Medicine, Utrecht University, Utrecht, Netherlands, [2]Wageningen Bioveterinary Research, Lelystad, Netherlands, [3]WHO Collaborating Center for Reference and Research on Campylobacter and Antimicrobial Resistance from a One Health Perspective/WOAH Reference Laboratory for Campylobacteriosis, Utrecht, Netherlands, [4]Center for Indonesian Veterinary Analytical Studies (CIVAS), Bogor, Indonesia, [5]Ministry of Agriculture of the Republic of Indonesia, Jakarta, Indonesia, [6]Laboratory Services and Surveillance Department, PT Medion Farma Jaya, Bandung, Indonesia, [7]Animal Health Department, PT Medion Farma Jaya, Bandung, Indonesia, [8]Technical Education and Consultation Department, PT Medion Ardhika Bhakti, Bandung, Indonesia, [9]Department of Population Health Sciences, Faculty of Veterinary Medicine, Utrecht University, Utrecht, Netherlands, [10]University Farm Animal Clinic, Harmelen, Netherlands

Introduction: Indonesia has a large broiler industry with extensive antimicrobial use (AMU) according to empirical evidence. However, there are no quantitative data of on-farm AMU. Quantification of AMU at farm level is crucial to guide interventions on antimicrobial stewardship (AMS). The objective of this study was to compare on-farm AMU monitoring methods, to assess which monitoring method is best suited to gain insight in the quantitative AMU at farm level in medium-scale Indonesian broiler farms.

Method: AMU was calculated using four different indicators—mg/PCU (mass-based), $TF_{UDDindo}$ (Treatment Frequency of Used Daily Dose, dose-based), TF_{DDDvet} (Treatment Frequency of Defined Daily Dose, dose-based), and $TF_{count-based}$ (count-based)—for the total AMU of 98 production cycles with an average length of 30 days.

Results: Broilers were exposed to an average of 10 days of antimicrobial treatments per production cycle, whereas 60.8% of the antimicrobials belonged to the Highest Priority Critically Important Antimicrobials (HPCIAs). For each pair of indicators, the Spearman rank correlation coefficient was calculated to assess if the production cycles were ranked consistently in increasing AMU across the different indicators. The correlation varied between 0.4 and 0.8.

Discussion: This study illustrates the considerable difference in the ranking of AMU between the different indicators. In a setting comparable to medium-scale broiler farms in Indonesia, where resources are scarce and there is no professional oversight, the $TF_{count-based}$ method is best suitable. Before implementing an AMU monitoring method, careful consideration of the use-indicators is paramount to achieve fair benchmarking.

KEYWORDS

antimicrobial resistance, antimicrobial stewardship, veterinary antimicrobial use monitoring, poultry, Indonesia

1. Introduction

The increase of antimicrobial resistance (AMR) is seen as a major health threat for humans and animals worldwide. It is estimated that 1.27 million human deaths are attributable to bacterial AMR in 2019, and if no action is taken, AMR could become one of the biggest causes of human death by 2050 (1, 2). Multiple studies have illustrated that antimicrobial use (AMU) in livestock results in increased occurrence and dissemination of cross sectoral AMR. A reduction in AMU will reduce selection for AMR, which could eventually result in a decrease of AMR (3–7). A concern regarding AMR development in livestock is the frequent use of antimicrobials categorized by the World Health Organization (WHO) as Highest Priority Critically Important Antimicrobials (HPCIAs) for human medicine, such as 3rd and 4th generation cephalosporins, colistin and fluoroquinolones (8).

It is estimated that the majority of globally used antimicrobials (73%) are used in animals reared for food production, and the total amount used in animals is projected to increase by 11.5% by 2030, primarily in Asia (6, 9). This increase is most likely due to the intensification of the livestock industry to meet the growing demand for animal protein, particularly in Low- and Middle-Income Countries (LMICs) (5, 10). In many of these countries, professional veterinary oversight is lacking and antimicrobials can be purchased without a prescription, increasing the risk of the development of AMR due to indiscriminate use in livestock (11, 12).

With a population of 280 million people in 2022, Indonesia is the fourth highest populated country in the world. The Indonesian broiler sector accounts for 87% of the consumed meat, and empirical studies indicate that the broiler industry accounts for around 60% of the antimicrobial use in livestock (13, 14). Although a pilot surveillance study in 2019 has collected qualitative data on AMU, quantitative data on AMU at farm-level in the Indonesian broiler sector is lacking (15). There is no structural professional veterinary oversight over AMU (14). Availability of reliable AMU data at farm level is vital for antimicrobial stewardship (AMS) initiatives, targeting imprudent use, encouraging improvements in animal husbandry, biosecurity, and enabling detailed risk and trend analyses (16).

Setting up AMU monitoring systems involves various challenges, a major one being the choice of indicators for quantifying and reporting results. The indicator is a technical unit used to quantify an animal's exposure to antimicrobials. In the numerator, the indicator contains a unit of measurement (UM) that expresses the amount of antimicrobials used. Depending on the context and objective of the AMU monitoring system, a dose-based, mass-based or count-based UM can be used. A dose-based UM uses the number of standardized dosages (usually in mg/kg) in the numerator, a mass-based UM the total mass of the antimicrobials applied (usually in milligrams) in the numerator, and a count-based UM the number of administrations of an antimicrobial product. All UMs are applied during a defined period (e.g., production cycle, year). The denominator contains the animal population that is exposed to antimicrobials in a specific time period (16). By dividing the UM by the animal population that is exposed in the same time period, a treatment frequency (TF) can be calculated (quantity of AMU per time period). A major challenge is developing an AMU monitoring tool that is both easy to use in the local context and reliable in the collection, analysis and reporting of AMU data.

The objective of our study was to compare on-farm AMU monitoring methods for Indonesian broiler farms, to assess which monitoring method is best suited to gain insight in the quantitative AMU at farm level in medium-scale Indonesian broiler farms.

2. Materials and methods

Usage data from the CORNERSTONE project was used (17). This project is a longitudinal study which was initiated and coordinated by researchers from the Faculty of Veterinary Medicine of Utrecht University, in cooperation with the Center for Indonesian Veterinary Analytical Studies (CIVAS), Medion (Indonesian veterinary pharmaceutical company with direct relationships with poultry farmers) and FAO Indonesia, taking place from 2018 to 2023. In this project, a sample of nineteen medium scale broiler farms located in the western part of Java Island, Indonesia was selected for baseline data collection and an intervention study with the objective of increasing prudent AMU. The study is performed on medium-scale farms as this group forms the largest number of commercial farms in Indonesia. The farms were selected using a convenience sampling method from the client database provided by Medion and have either open- or semi-open housing systems. All farms were independent medium-scale commercial broiler farms with 5,000–20,000 broilers, utilizing developed housing and equipment, applying low to moderate biosecurity measures and usually marketing the birds commercially. During the recruitment process, farmers were explained the objective of the CORNERSTONE project was to gain insights in on-farm AMU in order to develop recommendations to optimize AMU. The implementation of these recommendations is voluntary, and farmers can quit the study at any point in time. All farmers signed an informed consent form prior to data collection. All traceable data was anonymized.

2.1. Selecting AMU indicators

Existing on-farm AMU monitoring systems were explored and the guideline "Quantification of veterinary antimicrobial usage at herd level and analysis, communication and benchmarking to improve responsible usage" (AACTING) was selected as the basis

Abbreviations: AMR, antimicrobial resistance; AMS, antimicrobial stewardship; AMU, antimicrobial use; CIA, critically important antimicrobials; CIVAS, Indonesian center for veterinary analytical studies; DDDvet, defined daily dose for veterinary products; FAO, food and agriculture organization of the United Nations; HIA, highly important antimicrobials; HPCIA, highest priority critically important antimicrobials; LMIC, low- and middle income country; Mg/PCU, milligrams per population correction unit; TF, treatment frequency; UDD, used daily dose; UM, unit of measurement; VMP, veterinary medicinal product; WHO, World Health Organization; WOAH, World Organization for animal health.

to develop an on-farm AMU monitoring system for medium-scale broiler farms in Indonesia (18). The different steps of the AACTING guideline were followed, which addresses the requirements for developing an AMU monitoring system regarding (1) data collection, (2) data analysis (including how to quantify AMU), (3) benchmarking, and (4) reporting (18).

In Step 2 (data analysis), the different options for unit of measurement (UM; the numerator of an indicator) to quantify AMU at farm level were considered. A UM of each of the three different categories—mass-based, dose-based, and count-based AMU metrics—was used for this study. Most farmers or farm managers on medium-scale poultry farms lack knowledge of prudent AMU and do not consult a veterinary professional when administering antimicrobials. This leads to a high variety of dosages. Using a standardized dose in the denominator on these farms could lead to an over- or underestimation of the actual AMU. The seriousness of this error was assessed by calculating the actual dose used. For each production cycle analyzed, we calculated two dose-based (Used Daily Dose (UDD) and Defined Daily Dose (DDDvet)), one mass-based (mg/kg) and one count-based (number of single-day treatments) UM. DDDvet as defined by the European Medicines Agency (EMA) uses a standardized dose derived from European data, whereas UDD is calculated using the measured use data from the studied farms (18). By calculating AMU both with UDD and DDDvet the applicability of the European standard DDDvet in the context of Indonesian medium-scale broiler farms is examined.

2.2. Data collection

AMU data was collected from at least four successive production cycles of one broiler house per participating farm. During each production cycle, an extension worker from CIVAS visited the farms three times (at the start, in the middle and just before harvest) to assemble and check the quality of the collected data. These extension workers instructed the farmers at the start of the project on what data they needed to collect. AMU data was collected using daily treatment records filled in by the farmers along with drug collection bins. The records contained the date and age of the broilers at application, the (brand) name of the veterinary medicinal product (VMP), purpose of use, the amount of the products used and the route of application. The drug collection bins were provided during the first visit from extension workers and emptied at the end of each cycle. The farmers were requested to place all used packages of administered products (except for feed packets) into the drug bins. During a cycle, the farmers were requested to send a picture or copy of the daily records every week. The farmers recorded the number of chicks at the start of the cycle, daily mortality rate, number of broilers sent to slaughter, and harvest weight. For some production cycles mortality rates were missing; in these cases, the number of broilers at the start of the cycle was used to calculate the denominator.

As the farmers did not record the average daily bodyweight of the broilers, the "standard" Indonesian growth curve for the Cobb strain was used to estimate the bodyweight of the broilers on each day of the production cycle (19). A standardized mean bodyweight throughout the cycle of 1.0 kg (as used in EMA guideline) was used for the mass-based indicator (20).

All collected data were entered and analyzed in Microsoft Excel 365 (Microsoft Corp., Redmond, USA). Quality check of the data was performed manually by checking the input. The exact (antimicrobial) contents of the VMPs that the farmers had applied were obtained through the Index for Veterinary Medicines Indonesia and cross-checked (Index list with used products) by an Indonesian veterinarian from CIVAS (21).

2.3. Calculation of the four different AMU monitoring tools

The first indicator calculated is mass-based and expressed in milligrams (mg) of active substance per Population Correction Unit (PCU). This indicator is calculated as (20):

$$\textit{mass-based mg/PCU} = \frac{\textit{Total amount of active substance administered during a cycle (mg)}}{\textit{standardized bodyweight (1.0 kg) * N broilers (present at treatment)}}$$

The second indicator is the dose-based Treatment Frequency of used daily dose ($TF_{UDDindo}$). The UM for this indicator was calculated for broilers specifically on the included production cycles of the study farms and was therefore named UDD_{indo}. UDD_{indo} is defined as "the actual administered dose (as active substance in mg) per standardized bodyweight (kg) of an animal at treatment." The UM UDD_{indo} needed to be established per treatment before the indicator $TF_{UDDindo}$ can be calculated. UDD_{indo} was calculated per treatment as:

$$\textit{dose-based } UDD_{indo}\ (mg/kg) = \frac{\textit{amount of active substance administered per treatment (mg)}}{\textit{N treated * standardized bodyweight at treatment (kg)}}$$

When the UDD_{indo} was calculated for each specific treatment during a cycle, the average UDD_{indo} for each active substance in all studied production cycles was calculated by dividing the sum of UDD_{indo} for a specific active substance by the number of treatments that contained the same antimicrobial active substance (Table 1).

Once the UDD_{indo} for each active substance was determined, the $TF_{UDDindo}$ was calculated by:

$$\textit{dose-based } TF_{UDDindo}\ (\textit{days of treatment/production cycle (30 days)}) = \frac{\Sigma\ \textit{amount of active substance administered (mg)}}{\textit{N treated * standardized bodyweight at treatment (kg) * UDDindo * 30 days}}$$

The third indicator is comparable to $TF_{UDDindo}$ but uses defined daily dosages instead of used daily dosages. This indicator is TF Defined Daily Dose (TF_{DDDvet}). The DDDvet values were obtained according to the calculations by the European Medicines Agency (20). As the bodyweight plays a significant role in

TABLE 1 Overview of defined DDDvet and calculated UDDindo values used to calculate TF_{DDDvet} and $TF_{UDDindo}$ respectively per production cycle.

Antimicrobial group	Content	DDDvet mg/kg	UDD_{indo} calculated mg/kg
Polymyxins (HPCIA)	Colistin	5.1	12.5
Fluoroquinolones (HPCIA)	Ciprofloxacin	Not available	30.0
Fluoroquinolones (HPCIA)	Enrofloxacin	10.0	31.4
Fluoroquinolones (HPCIA)	Flumequine	14.0	5.1
Macrolides (HPCIA)	Tylosin	81.0	20.3
Macrolides (HPCIA)	Erythromycin	20.0	19.3
Macrolides (HPCIA)	Spiramycin	73.0	7.8
Fosfomycin (CIA)	Fosfomycin	Not available	20.9
Aminoglycosides (CIA)	Spectinomycin	124.0	14.6
Aminoglycosides (CIA)	Neomycin	24.0	5.4
Penicillins (CIA)	Amoxicillin	16.0	23.1
Sulfonamides (HIA)	Sulfadiazine, trimethoprim	34.0	30.6
Sulfonamides (HIA)	Sulfaquinoxaline, natrium, pyrimethamin	60.0	16.5
Lincosamides (HIA)	Lincomycin	8.6	13.7
Tetracyclines (HIA)	Doxycycline	15.0	9.5
Tetracyclines (HIA)	Oxytetracycline	39.0	14.8

The Antimicrobial groups are: Highest Priority Critically Important Antimicrobials (HPCIA's), Critically Important Antimicrobials (CIA's) and Highly Important Antimicrobials (HIA's).

calculating AMU in broilers, the same standardized bodyweight at day of treatment was used as in $TF_{UDDindo}$.

$$\text{Dose-based } TF_{DDDvet}\ (\text{days of treatment/production cycle (30 days)}) = \frac{\Sigma\ \text{amount of active substance administered (mg)}}{N\ \text{treated} * \text{standardized bodyweight at treatment (kg)} * \text{DDDvet} * 30\ \text{days}}$$

The fourth indicator $TF_{count-based}$ is count-based and expressed as the number of days under treatment per production cycle. If a VMP contained two antimicrobial active substances, it was counted as two separate treatments:

$$\text{Count-based } TF_{count-based}\ (\text{days of treatment/production cycle (30 days)}) = \frac{n\ \text{days of treatments of active substance per cycle}}{30\ (\text{average length of a production cycle})}$$

The treatment frequencies therefore portray the proportion of days the broilers were under antimicrobial treatment during a standardized production cycle of 30 days.

2.4. Benchmarking and statistical analysis

An arbitrary benchmark analogous to the Dutch system was placed on the upper quartile in the ranking of each of the four indicators (22). The cycles within the highest AMU quartile ($n = 25$) were defined as "high AMU."

For each of the four aforementioned indicators the AMU per production cycle was ordered from the lowest to the highest value. To test if these rankings for each specific indicator were correlated Spearman rank correlation coefficients (ρ) were calculated for each pair of indicators. The Spearman rank correlation coefficient measures the agreement between ranking methods and ranges from −1 (perfect negative agreement) to 0 (no agreement) to +1 (perfect positive agreement). The statistical significance test for a Spearman correlation assumes independent observations. The production cycles that were observed in this study were clustered in nineteen participating farms (four to six production cycles per farm). In the statistical analysis the intraclass correlation (ICC) was therefore calculated to check this assumption of independent observations. The Bonferoni adjusted p-value was calculated to compensate for the family wise error. For each pair of indicators, the number of production cycles ranked in the upper quartile for only one of the indicators but not for the other indicators was calculated. Additionally it was calculated how many cycles were ranked in the upper quartile in all four indicators.

3. Results

3.1. Application of the four different AMU monitoring tools

The checklist for each step provided by the AACTING guideline was filled out as part of collecting primary data for the context of the included medium scale broiler farms (Table 2).

Per farm, four to six production cycles were monitored (in total 98 production cycles across 19 farms), on average 5.2 per farm (Annex 1). In 97 production cycles, the broilers belonged to the Cobb strain, 1 production cycle used broilers from the Ross strain. Antimicrobials were used in 97 of the 98 (99%) production cycles. In total, 150 different VMPs were used, 53 of which contained antimicrobials. The daily recording forms were primarily used to analyze AMU per production cycle. The packages collected in drug collection bins were counted to cross-check the daily recording forms. All daily recording forms corresponded with the collected packages. The antimicrobials used belong to nine different antimicrobial classes, three of which are classified by the WHO as HPCIAs, three as Critically Important Antimicrobials (CIAs) and three as Highly Important Antimicrobials (HIAs) (23). Twenty-three VMPs contained a combination of two different antimicrobial substances.

The mean number of broilers that were present in the included study houses during a production cycle was 9,442 (ranging from

TABLE 2 Checklist for developing an AMU monitoring system in the context of Indonesian medium-scale broiler farms.

Requirements data collection according to AACTING guideline	Available options in the context of medium-scale broiler farms in Indonesia
1. Identify Data sources	Data at farm level through: - Extension worker visits - Drug bins collecting all medicinal products used - Questionnaires filled in by farmers
2. Required information needed to calculate the quantity of each antimicrobial active substance used (numerator)	Use data required: - Unique ID/name of the antimicrobial containing VMP (through daily recordings) - Number of packages or amounts used (daily recordings) - Amount of active antimicrobial substances in all VMPs used [using Indonesian Index of Veterinary Medicine (IOHI) provided by ASOHI (association for Veterinary Medicine in Indonesia)] - Age at treatment (daily recordings)
3. Required information to calculate the size of population at risk of treatment (denominator)	- Flock size (recorded at farm level through daily recordings) - Flock size at time of treatment (recorded at farm level through daily recording) - Assumed biomass (bodyweight) per animal (standard weight set through Cobb growth curve provided by Medion)
4. Define data collection time windows as well as data lock points	- Window data collection: mean period of one production cycle of 30 days on medium-scale broiler farms in Indonesia
5. Determine how data should be provided	- Manual input in written daily recordings
6. Determine who should provide data	- Farmer (for this study with guidance of an extension worker)
7. Determine who can change the data	- Extension workers (validation of data input by farmer required) - System operators in case of errors
Requirements Data Analysis according to AACTING guideline	**Answers in context of medium-scale broiler farms in Indonesia**
1. Determine numerator for analysing the datas	Numerator for Mass-based (mg/PCU) and Dose-based ($TF_{UDDindo}$, TF_{DDDvet}) was expressed in milligrams of administered active substance. Numerator for Count-based indicator ($TF_{count-based}$) was expressed in number of treatments (treatment being defined as a single-day treatment with one active substance)
2. Determine the denominator quantifying the size of population of animals at risk	Denominator for each indicator was expressed as: - Mass-based (mg/PCU): Standardized bodyweight (1.0 kg) multiplied by number of broilers present at treatment - Dose-based ($TF_{UDDindo}$): standardized bodyweight (according to Cobb growth curve provided by Medion), multiplied by number of broilers present at treatment, multiplied by the calculated Used Daily Dose, multiplied by time period of 30 days - Dose-based (TF_{DDDvet}): standardized bodyweight (according to Cobb growth curve provided by Medion), multiplied by number of broilers present at treatment, multiplied by the standardized Defined Daily Dose vet (calculated by EMA), multiplied by time period of 30 days - Count-based ($TF_{count-based}$): time period of 30 (days)
3. Determine which AMU indicator best fits with the goal of the entire system and the AMU monitoring objectives	Based on - Data collection capacity without the aid of extension workers on the farms within this study - Objective of quantifying AMU at farm level and benchmarking in a fair manner The AMU indicator best suitable for these study farms would be Count-based ($TF_{count-based}$)

1,715–27,500, SD: 6,905). All four indicators ranked Cycle 3 on Farm 1 to have the highest AMU per standardized cycle (of 30 days). Leaving out the single production cycle in which no antimicrobials were used, all four indicators also identified the same production cycle (Cycle 3 on Farm 2) as having the lowest AMU. The mean AMU per standardized production cycle (n = 98) expressed in a mass-based indicator was 46.9 mg/PCU (SD: 58.3 mg/PCU). For the dose-based indicators, the mean $TF_{UDDindo}$

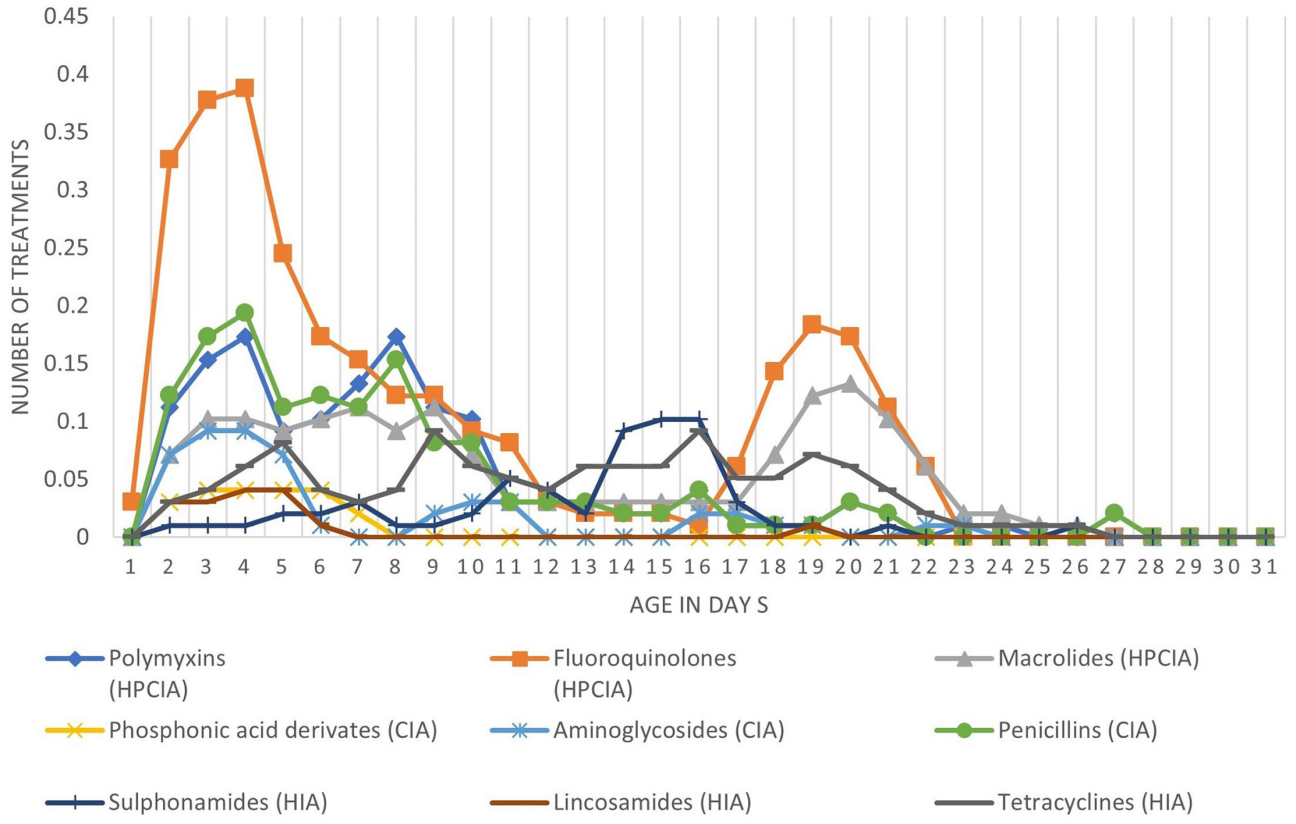


FIGURE 1
Average number of antimicrobial treatments per broiler per day of age divided in the different antimicrobial classes.

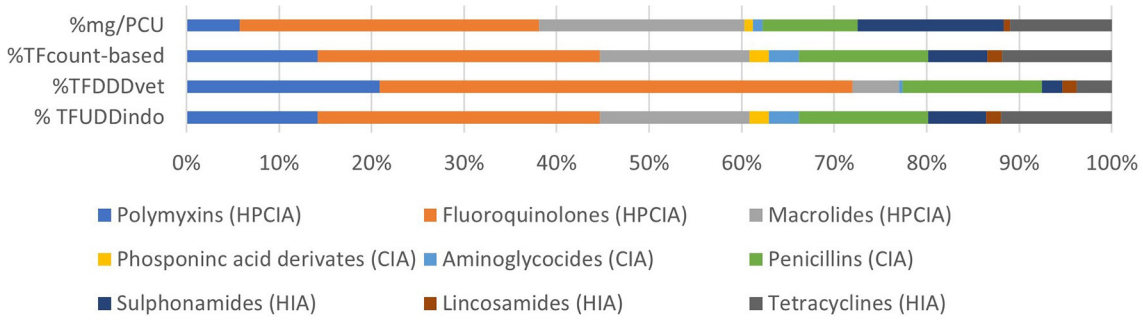


FIGURE 2
Proportion of antimicrobial classes used in all monitored cycles using the four different AMU indicators.

was 0.3 (SD: 0.3) and TF_{DDDvet} was 0.6 (SD: 0.6). The mean $TF_{count-based}$ was 0.3 (SD 0.2).

Figure 1 shows the number of treatments per antimicrobial class per day of age. On average, there were 10.2 antimicrobial treatment days per cycle. During the first 6 days of age, there is a high treatment incidence of fluoroquinolones (HPCIA) (e.g., in 39% of the monitored cycles, broilers were under fluoroquinolone treatment on Day 4 of the cycle), and a second period of high fluoroquinolone and macrolide (both HPCIA) treatment incidence from Days 17 to 23. Other antimicrobial classes show different dynamics of use during the first 23 days of the production cycle. Figure 2 shows the proportion of antimicrobial classes used in all monitored cycles using the different indicators. The proportions calculated as $TF_{count-based}$, and $TF_{UDDindo}$ show similar patterns, whereas the proportions for antimicrobial classes used calculated as mass-based (mg/PCU) and the TF_{DDDvet} indicator are different from the first two. Although overall $TF_{count-based}$ and $TF_{UDDindo}$ show similar patterns when calculated over all cycles, the variation becomes clear when individual cycles are analyzed (Figures 3A–D). For example, in Cycle 2 on Farm 12 (12.2) or Cycle 5 on Farm 13 (13.5), the proportion HPCIAs versus CIAs that were used differ

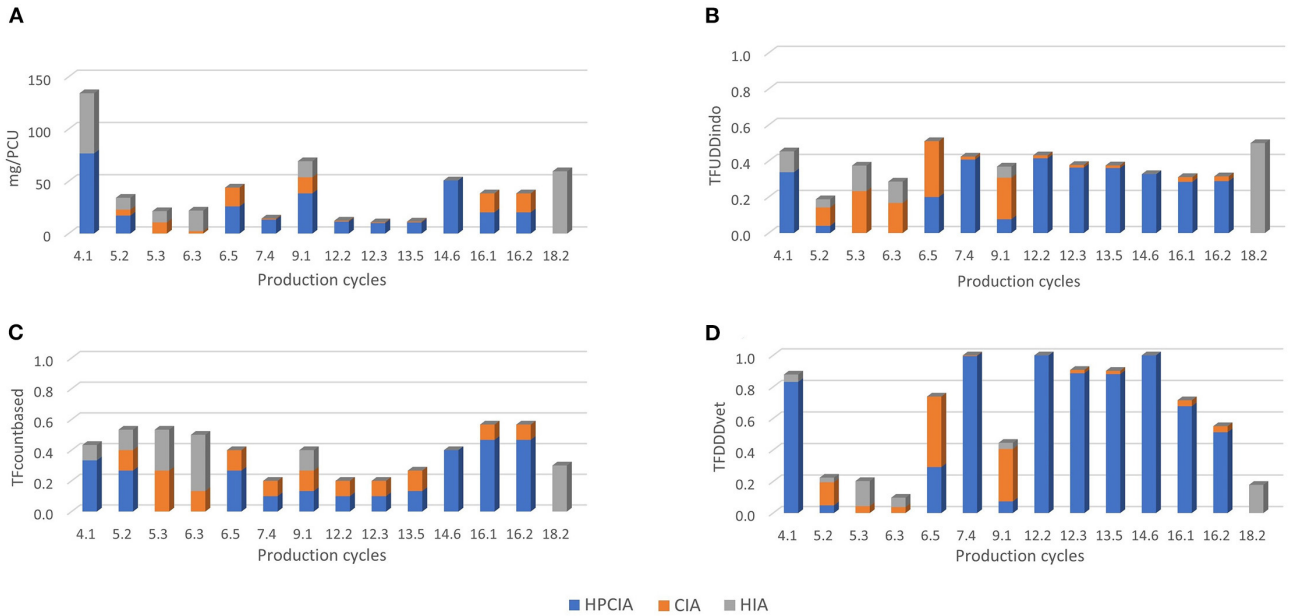


FIGURE 3
Distribution of AMU amongst the different priority antimicrobial classes as defined by WHO (HPCIA, CIA and HIA) of the 14 production cycles that were ranked as "high AMU" within only one indicator. Individual production cycles are labeled as [farm.cycle]; 4.1 means cycle 1 on farm 4. **(A)** Distribution of AMU defined as mg/PCU. **(B)** Distribution of AMU defined as $TF_{UDDindo}$. **(C)** Distribution of AMU defined as $TF_{count-based}$. **(D)** Distribution of AMU defined as TF_{DDDvet}.

considerably depending on whether $TF_{UDD-indo}$ or $TF_{count-based}$ was used.

Most AMU across all the monitored cycles belong to the HPCIAs, most of which were fluoroquinolones, irrespective of the indicator used (Figure 2). The percentage HPCIA use differs between indicators from 60.3 (mg/PCU) to 77.2% (TF_{DDDvet}) (Figure 2). Depending on the indicator used, various production cycles were classified as "high AMU," defined as having an AMU in the upper quartile within a specific indicator (Table 3).

The ICC was negligible (<0.1) meaning that observations within each cluster were behaving as independent observations and the Spearman rank correlation test could be applied.

The lowest correlation found between two indicators was 0.4 (TF_{DDDvet} and $TF_{count-based}$) and the highest correlation was 0.8 (mg/PCU and $TF_{UDDindo}$) (Table 3, Figures 4A–F). The Bonferoni adjusted *p*-value for each of the six pairwise comparisons between indicators was <0.05. Seven of the 25 production cycles in the upper quartile were classified as "High AMU" by all four indicators. Fourteen out of the 25 production cycles in the upper quartile were only marked as "High AMU" by just one indicator.

TABLE 3 Pairwise comparison of AMU indicators using spearman rank correlation.

	mg/PCU	TFUDD	TFDDDvet	Tfcount-based
mg/PCU	1.00	0.78	0.69	0.69
	$N = 0$	$N = 6$	$N = 8$	$N = 8$
TFUDD		1.00	0.69	0.62
		$N = 0$	$N = 8$	$N = 10$
TFDDDvet			1.00	0.39
			$N = 0$	$N = 15$
TFcount-based				1.00
				$N = 0$

The values within the cell indicate the rho (ρ) coefficient and the number of farms ranked as "High AMU" [threshold upper quartile of AMU (N = 25)] with one indicator but below the threshold in the other indicatior in the pairwise comparison. The p-value for all Spearman rank correlation calculations was <0.05. The color shades indicate the level of correlation found in this study: dark green represents perfect correlation and deep yellow represents no correlation.

4. Discussion

In this study, we applied existing AMU indicators following the AACTING guidelines to gain insight into AMU at farm level on medium-scale broiler farms in Indonesia (18). Quantitative AMU data as well as data on the number of broilers present throughout the production cycle was used. Antimicrobials were used in 99% of the production cycles, although large variations between production cycles could be observed. Regardless of the unit of measurement (UM) used, the majority of antimicrobials used belonged to the HPCIA category. All UMs identified the same cycles as the cycle with the highest and lowest AMU, respectively. The UMs differed in the ranking of production cycles with increasing AMU. Nineteen production cycles were categorized as "high AMU" (upper quartile of AMU) for both the dose-Based UM TF_{DDDvet} and the mass-based UM $TF_{count-based}$ together Only ten cycles were categorized as "high AMU" when calculated for both the mass-based UM mg/PCU and the dose-Based UM $TF_{UDDindo}$ together.

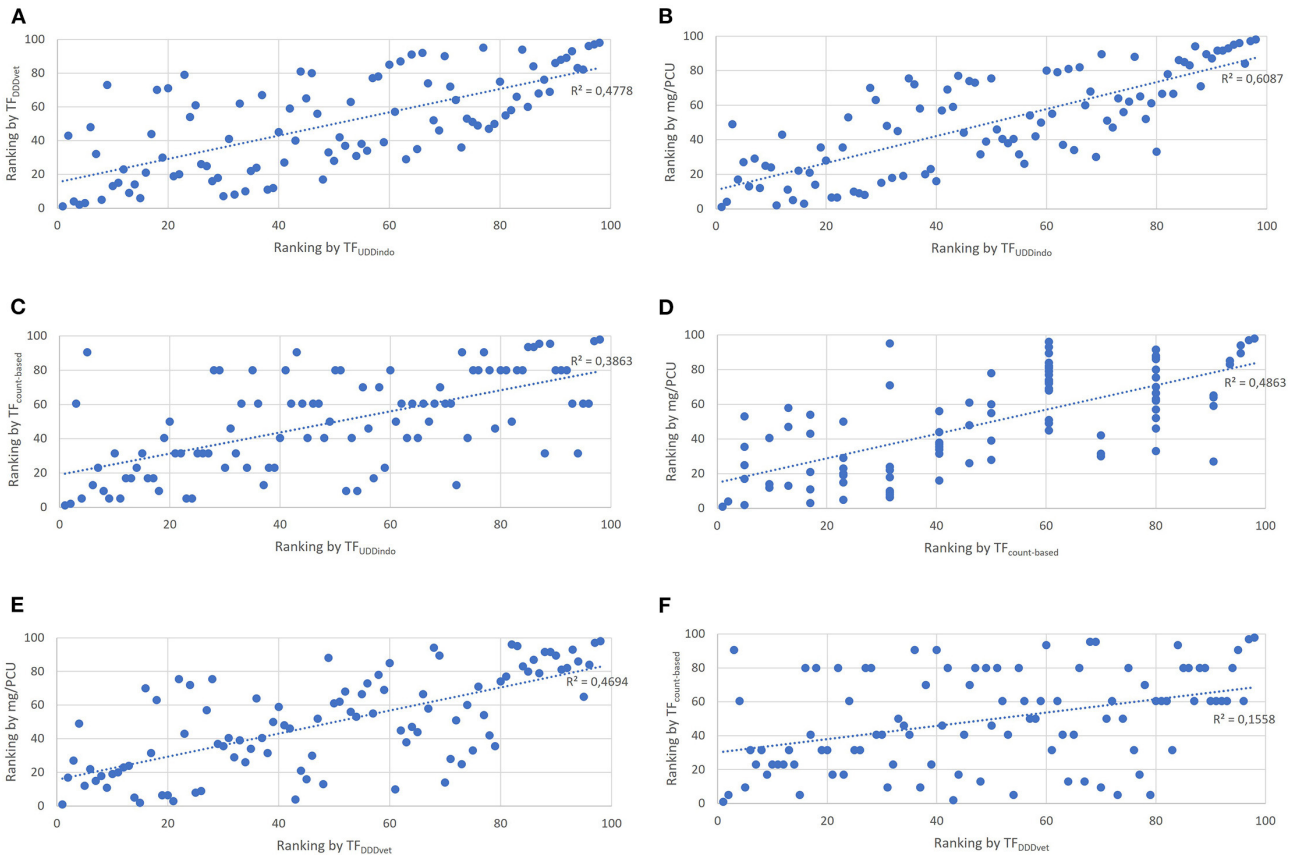


FIGURE 4
Scatter plots showing the correlation of individual production cycle AMU rankings between the 4 tested AMU indicators. **(A)** Correlation between TF_{DDDvet} and $TF_{UDDindo}$. **(B)** Correlation between mg/PCU and $TF_{UDDindo}$. **(C)** Correlation between $TF_{count-based}$ and $TF_{UDDindo}$. **(D)** Correlation between mg/PCU and $TF_{count-based}$. **(E)** Correlation between mg/PCU and TF_{DDDvet}. **(F)** Correlation between $TF_{count-based}$ and TF_{DDDvet}.

4.1. Data collection and quality control

An effective monitoring system for AMU requires a measure of the amount of antimicrobials consumed and a measure of the population of animals at risk of treatment (16). Furthermore, ongoing systematic data should be collected to measure AMU change over time.

AMU data collection can be performed at different levels of aggregation or detail, and for different purposes. Indonesia reports national veterinary AMU at the level of species and administration route, and this is paralleled by collection of AMR data in poultry which is an ongoing surveillance system in Indonesia [personal communication Dr. Desmayanti; (24, 25)]. Data collection at the farm level, however, is important to understand why and how such large quantities of antimicrobials are used, identify high users, and provide the basis for developing AMS programs on farms (26). This study is the first that collected longitudinal and quantitative data on a sample of Indonesian broiler farms. This gave the opportunity to compare data-analysis systems for reporting and benchmarking, build experience in collecting data on broiler farms, and add quantitative data to the qualitative AMU studies that have been performed in the recent past in Indonesia. From our study, it is clear that an intensive follow-up is needed to collect reliable data from medium scale broiler farms in Indonesia. Even with the intensive follow-up there was no one guarantee that the AMU data was exact. Based on anecdotal reports from extension workers, farmers were not used to registering treatments precisely and appeared to find it difficult to make registration part of their daily routine. Intensive follow-up with frequent farm visits are a prerequisite for reliable data in situations where other data quality controls like intensive veterinary oversight are lacking. However, collecting on-farm data from a sample of farms as proxy for use, and extrapolate this to regional or even country level, will be a very time- and labor-consuming approach given the number of farms needed and the dispersed locations of farms (27). This is important to realize when deciding which AMU indicator will be used. A more detailed indicator (such as a dose-based indicator) where extensive data collection is required could be less suitable in this setting. Large-scale (>20,000 broilers) commercial farms, usually with developed housing and equipment, were not included in our study due to limited access to data (9). Due to a stricter farm management on large-scale farms, we can speculate that this might facilitate more thorough data collection. However, when data are collected to inform national policy, data from both large-scale and medium-scale farms should be included as there might be clear differences in usage. In the CORNERSTONE project, data collection is performed so that an intervention can be designed as part of an antimicrobial stewardship program.

4.2. Data analysis

Which UM is chosen often depends on the context (e.g., data availability, resources, objective of the monitoring system). A different choice of numerator (and thus indicator) can influence the interpretation of AMU at both national level (28, 29) and farm level (30, 31).

As antimicrobials classified as HPCIA are crucial in human medicine, it is paramount in AMU monitoring systems that the use of HPCIAs is not masked. When using a mass-based UM, the risk exists that the AMU can falsely appear to have decreased through switching to antimicrobial classes with a higher potency and so a lower required dose active substance, even though the duration of animal exposure to antimicrobials may not have changed. Remarkably, for the mass-based indicator mg/PCU in this study, the class of antimicrobials calculated to have been most used was the highly potent class of fluoroquinolones (HPCIA). This seemingly contradictory result can be explained by the three times higher dose of fluoroquinolones that was used on our study farms compared to the DDDvet, leading to a higher amount of milligrams being used than expected. This could also be the explanation for the (counter-intuitive) highest correlation between the UMs $TF_{UDDindo}$ and mg/PCU.

A dose-based UM can be used to correct for the dosage. The challenge for dose-based UMs in a setting often lacking professional veterinary oversight, is that the recommended dose according to the SPC may not always reflect drug use in practice, as farmers frequently deviate from the recommended dosage (22, 26, 32). These variations were clear in this study, where the dosage of enrofloxacin used in the different cycles varied from 0.0017 to 203 mg/kg (the standardized dose according to EMA is 10 mg/kg). For fluoroquinolones this is due to huge variation in applied doses per farm. As a result of this variation in dosage per individual farm, even the standardized UDD_{indo}, derived from the collected farm-level data leads to a varying over- or underestimation for each individual production cycle. This, in turn, leads to an incorrect ranking. Furthermore, comparing UDD_{indo} and DDD_{vet} shows that in this dataset the actual used dose (UDD_{indo}) for colistin and enrofloxacin, both HPCIAs, was a 3-fold higher than the standardized DDD_{vet} as calculated by EMA (Table 1). In contrast, all other UDD_{indo} values were much lower than the DDD_{vet} values (Table 1). Considering the importance of HPCIA and the substantial difference in actual dose used and the DDDvet in this dataset, and varying under- and overestimation per individual farm by UDD_{indo}, dose-based indicators have their restrictions in measuring AMU on medium-scale Indonesian broiler farms.

If a count-based UM is used, it is not necessary to have data available on the actual amount of antimicrobials used. Using a count-based UM thus requires less data, creating a lower burden for farmers to record data, but is less accurate compared to a dose-based UM if the goal is to examine the actual AMU at farm level. This is because it does not take into account the actual dosage applied, but counts every treatment with the same value (this value is "1"). However, the underestimation of use of potent antimicrobials, as would happen if a mass-based UM was used, is avoided when a count-based UM is used, because every treatment is weighted the same. However, it does not provide insight into under- or overdosing of a VMP, what appeared to happen frequently in our study population. It only counts the days that animals are under antimicrobial treatment in a predefined period, without weighing the quality of the AM treatment.

Besides choosing the numerator of the indicator for AMU, the AMU needs to be divided by a proxy for the targeted animal population to have comparable results (16). The weight of broilers increases by a factor of almost 40 (from 40 g to 1,500 g under Indonesian conditions) during their short life, which could imply a high risk of under- or overestimation of AMU when a single standardized animal weight is used (33). A mass-based UM for AMU usually uses slaughter weight, underestimating the effective exposure to antimicrobials per kilogram bodyweight, as most treatments often take place in the first week of production at low bodyweight. Due to varying management conditions of the farms in this study, there was also considerable variety in the actual bodyweight of the broilers at specific age on different farms, not always following the Cobb growth curve. A study by Kassabova in 2019 showed that using different weights to calculate dose-based AMU also significantly influences the outcome of the measurement (22). When available data on growth curves is limited, it could therefore be the best option to use a count-based UM, where the weight of the animals is not needed.

In summary, there are pros and cons for each UM for AMU. In the current setting of medium-scale broiler farms in Indonesia, the count-based UM seems most suitable (and realistic) to achieve a fair benchmarking of farms.

4.3. Benchmarking

Benchmarking AMU refers to comparing a farm's AMU with the AMU of the reference population (18). A prerequisite is that AMU for all entities in this population is quantified in a comparable manner. Using a different indicator can lead to a change in the way farms are ranked, which was clearly visible in this study (26, 34). Although some studies performed in broilers (34) and pigs (26) showed a correlation between the mass- and dose-based indicator, the correlation in this study was considerably lower [∼0.6 (this study) compared to 0.8 (26)]. An explanation for this could be that the other studies were performed using data from countries where the administered dosages were more according to the SPC than in this study. A consistent over- or underestimation of the dosage would still result in a similar ranking of antimicrobial users, even though the exact values differ. However, if the over- or underestimation varies strongly, like in this study, the correlation automatically decreases.

Due to the limited number of participating farms and variation in the use of antimicrobials between production cycles, it was decided to benchmark per cycle instead of per farm in this study. This method is feasible in studies such as this one, where extensive supervision is possible. For this study, there was no preliminary data and benchmarking was only performed after data for all production cycles had been collected. Since farms have varying empty periods (in which no production cycle is running), quite some time can elapse between data collection of different cycles. For future studies, a timely benchmark is advised. This way, as soon

as data is collected from one production cycle, it can immediately be reported back to the farmer whether or not their farm ranks as "high AMU." Considering the duration of data collection, data analysis and efforts required to draft a report, benchmarking per farm is probably more realistic than per production cycle. Regardless of whether benchmarking is done within a smaller study or at a national level, similar farms should be used as a reference population. In this context, medium-scale farms should be compared with medium-scale farms and Large-scale farms with Large-scale farms.

4.4. Reporting

Reporting on the outcome of AMU quantifications is necessary for the improvement of AMS initiatives. Ongoing, systematic monitoring of on-farm AMU can guide targeted improvements of AMU as part of stewardship programs (16, 18). It is important to ensure the report is adjusted to the person it is addressed to (16). In our study, we reported back to farmers who mostly lack knowledge of prudent AMU and AMR. The report language should be understandable for this type of farmer and offer a clear overview of the AMU on their own farm compared to others within the reference population. In this study, practical suggestions on how to reduce AMU (particularly of HPCIAs) at farm level were added. If data is also reported to the government or at an international level, it is important to clearly define the reference population and add a time period to the AMU data (18). Anonymization of the participating farms is a prerequisite for each type of reporting and should be agreed upon when farms are included in studies.

Previous surveillance questionnaires concluded that AMU is widespread in the Indonesian broiler sector and that 80% is used preventively (15). Enrofloxacin is the most frequently used antimicrobial (15). With 10.2 average days under antimicrobial treatment per cycle and 82.6% of all treatments with the purpose of prevention or growth booster (2%) (data not shown), our results are comparable and there is an evident need to improve responsible antimicrobial use on medium scale broiler farms. An AMU monitoring system at farm level could be an effective tool to create insight for farmers in their use, which can in turn, assist in monitoring of the desired decreasing AMU.

5. Limitations of the study

Data were collected from only 19 farms, with close to 100 cycles. The cycles are not independent and might be clustered per farm for certain issues (e.g., dosing). The farms are selected by Medion based on their willingness to participate and are therefore not a representative sample of the AMU in medium-scale farms in their region. They might be more motivated to register treatments and open to advice. Due to the COVID-19 pandemic, data collection took longer than expected (it took place from 2019 to 2022). This might have influenced AMU habits, as during a 3-year time period, the antimicrobial treatment management could change.

6. Conclusion

Based on data from this study on 19 medium-scale broiler farms, the most feasible and fair method to benchmark medium scale farms is to use the UM $TF_{count-based}$. One reason is that farmers from this sector are not yet used to extensive AMU data collection, as would be needed for the other indicators. Another important factor is the highly variable dosing practice found in this sector, which contrasts with the rigid legislation and veterinary oversight in European countries, for example. Consequently, a dose-based UM will not represent actual use and result in unfair benchmarking.

This study was the first to create insight into quantitative and qualitative AMU data at farm level in medium-scale broiler farms in Indonesia. The next step would be to use these tools on a larger sample of farms, and to use the outcomes for implementing interventions. Collecting AMU data at farm level in a database can subsequently help in monitoring AMU trends and aid policy makers in designing targeted AMS interventions. The easier count-based indicator $TF_{count-based}$ would be best suitable for the current state of medium scale broiler farms in Indonesia. With this indicator the level of HPCIA use is not underestimated. Depending on future resources and possibilities to steer dosing practices, a dose-based indicator could be used as the successor of the count-based indicator.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary material, further inquiries can be directed to the corresponding author.

Ethics statement

We conducted an observational study on the amount and type of antimicrobials used by farmers on regular commercial broiler farms in Indonesia. As we did not apply any intervention on humans nor animals, but only observed current antimicrobial use practices in commercial broiler farms based on written records and disposed drug packages, ethical review and approval was not required for the study. For the use and analysis of farm data (including antimicrobial use), we obtained written consent from the participating farmers. Furthermore, we anonymized all personal data so that no information can be traced back to individual farms.

CORNERSTONE group

Tri S. Purwanto, Muhammad A. Bagaskara, Annisa Rachmawati, and Rangga Putra, Center for Indonesian Veterinary Analytical Studies (CIVAS), Bogor, Indonesia; Hannan Daradjat, Animal Health Department, PT Medion Farma Jaya, Bandung, Indonesia; Patricia Noreva, Ministry of Agriculture of the Republic of Indonesia, Jakarta, Indonesia; Riana A. Arief and Erianto Nugroho, Food and Agriculture Organization (FAO) Country Office of Indonesia, Jakarta, Indonesia.

Author contributions

RA and TD coordinated data collection. The Cornerstone group under supervision of SS was responsible for the data collection and validation. RA and IG performed data analysis. RA prepared the manuscript. All authors reviewed the written manuscript and were involved in designing and conducting the study.

Funding

This study was funded by two research grants from NWO (Dutch Research Council): "Containment of antimicrobial resistance, toward a sustainable poultry production chain in Indonesia" (grant number W 07.50.1827) and "Diagnostics for diseases in Indonesian poultry production to support antimicrobial stewardship interventions" (grant number VidW.1154.19.017).

Acknowledgments

The authors of this paper would like to express their appreciation for the efforts applied in data collection by CIVAS extension workers and Medion and the cooperation of the farmers that allowed us to collect data on their farms. Furthermore, the authors would like to thank Dr. Liys Desmayanti from the Indonesian Ministry of Agriculture of the Republic of Indonesia for her valuable advice.

Conflict of interest

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.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fvets.2023.1092302/full#supplementary-material

References

1. O'Neill J. *Antimicrobial Resistance: Tackling a Crisis for the Health and Wealth of Nations*. Geneva: Review on Antimicrobial Resistance (2014).

2. Antimicrobial Resistance Collaborators. Global burden of bacterial antimicrobial resistance in 2019: a systematic analysis. *Lancet.* (2022) 399:629–55.

3. Chantziaras I, Boyen F, Callens B, Dewulf J. Correlation between veterinary antimicrobial use and antimicrobial resistance in food-producing animals: a report on seven countries. *J Antimicrob Chemother.* (2014) 69:827–34. doi: 10.1093/jac/dkt443

4. Woolhouse M, Ward M, van Bunnik B, Farrar J. Antimicrobial resistance in humans, livestock and the wider environment. *Philos Trans R Soc Lond B Biol Sci.* (2015) 370:20140083. doi: 10.1098/rstb.2014.0083

5. Van Boeckel TP, Brower C, Gilbert M, Grenfell BT, Levin SA, Robinson TP, et al. Global trends in antimicrobial use in food animals. *Proc Natl Acad Sci U S A.* (2015) 112:5649–54. doi: 10.1073/pnas.1503141112

6. Magnusson U, Moodley A, Osbjer K. Antimicrobial resistance at the livestock-human interface: implications for Veterinary Services. *Rev Sci Tech.* (2021) 40:511–21. doi: 10.20506/rst.40.2.3241

7. Hesp A, Veldman K, van der Goot J, Mevius D, van Schaik G. Monitoring antimicrobial resistance trends in commensal *Escherichia coli* from livestock, the Netherlands, 1998 to 2016. *Euro Surveill.* (2019) 24:1800438. doi: 10.2807/1560-7917.ES.2019.24.25.1800438

8. McEwen SA, Collignon PJ. Antimicrobial resistance: a one health perspective. *Microbiol Spectr.* (2018) 6:17. doi: 10.1128/microbiolspec.ARBA-0009-2017

9. Tiseo K, Huber L, Gilbert M, Robinson TP, Van Boeckel TP. Global trends in antimicrobial use in food animals from 2017 to 2030. *Antibiotics.* (2020) 9:918. doi: 10.3390/antibiotics9120918

10. Wahyono ND, Utami MMD. A review of the poultry meat production industry for food safety in Indonesia. *J Phys Conf Ser.* (2018) 953:012125. doi: 10.1088/1742-6596/953/1/012125

11. Collignon PJ, McEwen SA. One health-its importance in helping to better control antimicrobial resistance. *Trop Med Infect Dis.* (2019) 4:22. doi: 10.3390/tropicalmed4010022

12. Aidara-Kane A, Angulo FJ, Conly J, Minato Y, Silbergeld EK, McEwen SA, et al. World Health Organization (WHO) guidelines on use of medically important anti-microbials in food-producing animals. *Antimicrob Resist Infect Control.* (2018) 7:294. doi: 10.1186/s13756-017-0294-9

13. Wright T, Darmawan B. Voluntary Poultry Report Indonesia. *USDA For Agric Serv.* (2017) 1701:1–4. Available online at: https://www.fas.usda.gov/data/indonesia-voluntary-poultry-report

14. Coyne L, Patrick I, Arief R, Benigno C, Kalpravidh W, McGrane J, et al. The costs, benefits and human behaviours for antimicrobial use in small commercial broiler chicken systems in Indonesia. *Antibiotics.* (2020) 9:154. doi: 10.3390/antibiotics9040154

15. Suandy I. AMU-AMR surveillance system in Indonesia on livestock and animal health sector: approach and findings. In: *OIE Sub-Regional Conference on Antimicrobial Resistance (AMR) Related Research and Information.* Bangkok, Thailand (2022). Available online at: https://rr-asia.woah.org/wp-content/uploads/2020/01/11-amu-amr-surveillance-indonesia_saundy.pdf

16. Sanders P, Vanderhaeghen W, Fertner M, Fuchs K, Obritzhauser W, Agunos A, et al. Monitoring of farm-level antimicrobial use to guide stewardship: overview of existing systems and analysis of key components and processes. *Front Vet Sci.* (2020) 7:540. doi: 10.3389/fvets.2020.00540

17. NWO. *Containment of Antimicrobial Resistance: Towards a Sustainable Poultry Production Chain in Indonesia*. NWO (2022). Available online at: https://www.nwo.nl/projecten/w-07501827-0 (accessed October 24, 2022).

18. The AACTING-Network. *Guidelines for Collection, Analysis and Reporting of Farm-Level Antimicrobial Use, in the Scope of Antimicrobial Stewardship*. New York, NY: The AACTING-Network (2019). Available online at: https://aacting.org/0.2019;version~1.2_2019 (accessed October 20, 2022).

19. Cobb-Vantress. *Broiler performance and Nutrition Supplement Cobb 500 Males.* (2022). Available online at: https://www.cobb-vantress.com/assets/Cobb-Files/product-guides/5502e86566/2022-Cobb500-Broiler-Performance-Nutrition-Supplement.pdf (accessed October 25, 2022).

20. EMA. Principles on assignment of defined daily dose for animals (DDDA) and defined course dose for animals (DCDA) table of contents. *Eur Med Agency.* (2015) 44:1–64.

21. Direktorat Jenderal Produksi Peternakan Departemen Pertanian Dengan Asosiasi Obat Hewan Indonesia. Indeks Obat Hewan Indonesia. *J Chem Inform Model.* (2014) 11:864.

22. Kasabova S, Hartmann M, Werner N, Käsbohrer A, Kreienbrock L. Used daily dose vs. defined daily dose-contrasting two different methods

to measure antibiotic consumption at the farm level. *Front Vet Sci.* (2019) 6:116. doi: 10.3389/fvets.2019.00116

23. World Health Organization (WHO). *Critically Important Antimicrobials for Human Medicine 6th Revision 2018 Ranking of Medically Important Antimicrobials for Risk Management of Antimicrobial Resistance Due to Non-Human Use*. Geneva: WHO (2018). Available online at: https://www.who.int/publications-detail-redirect/9789241515528 (accessed October 25, 2022).

24. World Organisation for Animal Health (WOAH). *Annual Report on Antimicrobial Agents Intended for Use in Animals*. 6th edn. Paris: WOAH (2022). Available online at: https://www.woah.org/app/uploads/2022/06/a-sixth-annual-report-amu-final.pdf (accessed October 25, 2022).

25. World Organisation for Animal Health (WOAH). *OIE Template: World Organisation for Animal Health*. Paris: WOAH (2022). Available online at: https://www.oie.int/app/uploads/2021/08/eng-amuse-template-final.xls (accessed October 25, 2022).

26. O'Neill L, Rodrigues da Costa M, Leonard F, Gibbons J, Calderón Díaz JA, McCutcheon G, et al. Does the use of different indicators to benchmark antimicrobial use affect farm ranking? *Front Vet Sci.* (2020) 7:558793. doi: 10.3389/fvets.2020.558793

27. National Antimicrobial Resistance Coordination Committee of Indonesia NARCC (NARCC) G. *National Action Plan on Antimicrobial Resistance Indonesia 2017–2019*. Indonesia: NARCC (2017). Available online at: https://www.who.int/publications/m/item/indonesia-national-action-plan-on-antimicrobial-resistance-indonesia-2017-2019

28. Dupont N, Fertner M, Kristensen CS, Toft N, Stege H. Reporting the national antimicrobial consumption in Danish pigs: influence of assigned daily dosage values and population measurement. *Acta Vet Scand.* (2016) 58:27. doi: 10.1186/s13028-016-0208-5

29. Taverne FJ, Jacobs JH, Heederik D, Mouton JW, Wagenaar JA, van Geijlswijk IM. Netherlands Veterinary Medicines Authority (SDa). Influence of applying different units of measurement on reporting antimicrobial consumption data for pig farms. *BMC Vet Res.* (2015) 11:250. doi: 10.1186/s12917-015-0566-7

30. Echtermann T, Muentener C, Sidler X, Kümmerlen D. Antimicrobial drug consumption on Swiss pig farms: a comparison of Swiss and European defined daily and course doses in the field. *Front Vet Sci.* (2019) 6:240. doi: 10.3389/fvets.2019.00240

31. Waret-Szkuta A, Coelho V, Collineau L, Hémonic A, Buy C, Treff M, et al. how input parameters and calculation rules influence on-farm antimicrobial use indicators in animals. *Front Vet Sci.* (2019) 6:438. doi: 10.3389/fvets.2019.00438

32. Postma M, Sjölund M, Collineau L, Lösken S, Stärk KD, Dewulf J, et al. Assigning defined daily doses animal: a European multi-country experience for antimicrobial products authorized for usage in pigs. *J Antimicrob Chemother.* (2015) 70:294–302. doi: 10.1093/jac/dku347

33. Food and Agriculture Organization of the United Nations (FAO). *The FAO Action Plan on Antimicrobial Resistance 2021–2025*. Rome: FAO. Available online at: https://www.fao.org/3/cb5545en/cb5545en.pdf (accessed October 25, 2022).

34. Chauvin C, Querrec M, Perot A, Guillemot D, Sanders P. Impact of antimicrobial drug usage measures on the identification of heavy users, patterns of usage of the different antimicrobial classes and time-trends evolution. *J Vet Pharmacol Therap.* (2008) 31:960. doi: 10.1111/j.1365-2885.2008.00960.x

frontiers | Frontiers in Veterinary Science

TYPE Original Research
PUBLISHED 18 April 2023
DOI 10.3389/fvets.2023.1117975

OPEN ACCESS

EDITED BY
João Pedro Rueda Furlan,
University of São Paulo, Brazil

REVIEWED BY
G. Douglas Inglis,
Agriculture and Agri-Food Canada (AAFC), Canada
Hesamaddin Shirzad-Aski,
Golestan University of Medical Sciences, Iran

*CORRESPONDENCE
Orhan Sahin
✉ osahin@iastate.edu

SPECIALTY SECTION
This article was submitted to Veterinary Epidemiology and Economics, a section of the journal Frontiers in Veterinary Science

RECEIVED 15 December 2022
ACCEPTED 31 March 2023
PUBLISHED 18 April 2023

CITATION
Goulart DB, Zhang Q and Sahin O (2023) Growth kinetics and fitness of fluoroquinolone resistant and susceptible *Campylobacter jejuni* strains of cattle origin.
Front. Vet. Sci. 10:1117975.
doi: 10.3389/fvets.2023.1117975

Growth kinetics and fitness of fluoroquinolone resistant and susceptible *Campylobacter jejuni* strains of cattle origin

Debora Brito Goulart[1], Qijing Zhang[1] and Orhan Sahin[2]*

[1]Department of Veterinary Microbiology and Preventive Medicine, Iowa State University, Ames, IA, United States, [2]Department of Veterinary Diagnostic and Production Animal Medicine, Iowa State University, Ames, IA, United States

Human enterocolitis is frequently caused by the Gram-negative microaerobic bacterium *Campylobacter jejuni*. Macrolides (e.g., erythromycin) and fluoroquinolones (FQs) (e.g., ciprofloxacin) are the preferred antibiotics for the treatment of human campylobacteriosis. Rapid emergence of FQ-resistant (FQ-R) *Campylobacter* during treatment with FQ antimicrobials is well known to occur in poultry. Cattle is also an important reservoir of *Campylobacter* for humans, and FQ-R *Campylobacter* from cattle has become highly prevalent in recent years. Even though the selection pressure may have contributed to the expansion of FQ-R *Campylobacter*, the actual impact of this factor appears to be rather low. In this study, we examined the hypothesis that the fitness of FQ-R *Campylobacter* may have also played a role in the rise seen in FQ-R *Campylobacter* isolates by employing a series of *in vitro* experiments in MH broth and bovine fecal extract. First, it was shown that FQ-R and FQ-susceptible (FQ-S) *C. jejuni* strains of cattle origin had comparable growth rates when individually cultured in both MH broth and the fecal extract with no antibiotic present. Interestingly, FQ-R strains had small but statistically significant increases over FQ-S strains in growth in competition experiments performed in mixed cultures with no antibiotic present. Lastly, it was observed that FQ-S *C. jejuni* strains developed resistance to ciprofloxacin more readily at high initial bacterial cell density (10^7CFU/mL) and when exposed to low levels of the antibiotic (2–4μg/mL) compared with that at a low level of initial bacterial cell density (10^5CFU/mL) and exposure to a high level of ciprofloxacin (20μg/mL) in both MH broth and the fecal extract. Altogether, these findings indicate that even though FQ-R *C. jejuni* of cattle origin may have a slightly higher fitness advantage over the FQ-S population, the emergence of FQ-R mutants from susceptible strains is primarily dictated by the bacterial cell density and the antibiotic concentration exposed under *in vitro* condition. These observation may also provide plausible explanations for the high prevalence of FQ-R *C. jejuni* in cattle production due to its overall fit nature in the absence of antibiotic selection pressure and for the paucity of development of FQ-R *C. jejuni* in the cattle intestine in response to FQ-treatment, as observed in our recent studies.

KEYWORDS

antimicrobial resistance, fluoroquinolone, *Campylobacter*, cattle, mutant selection window, growth rate, bacterial fitness

1. Introduction

Campylobacter is one of the most prevalent causes of bacterial foodborne gastroenteritis worldwide (1, 2). In the United States, *Campylobacter* causes an estimated 1.3 million illnesses and costs ~$1.7 billion yearly for medical treatment and lost productivity (3, 4). Human *Campylobacter* infections are primarily caused by the consumption of contaminated poultry meat (5, 6). In addition to chickens, *Campylobacter* is prevalent in both beef and dairy cattle (7–9). Humans can acquire *Campylobacter* from cattle through direct contact, ingestion of unpasteurized milk, and water contamination (10–15). Although most individuals infected with *Campylobacter* may not require antibiotic treatment, severe and systemic infections necessitate antimicrobial therapy, including macrolides (e.g., erythromycin) and fluoroquinolones (FQs) (e.g., ciprofloxacin) (16–19). Unfortunately, both classes of antibiotics are becoming less effective in treating campylobacteriosis due to increasing rates of resistance to these drugs in *Campylobacter* (20–22). The fact that *Campylobacter* is a zoonotic pathogen exposed to FQs used in both animal production (e.g., beef cattle and non-lactating dairy cattle) and human medicine may contribute to the development of FQ-resistant (FQ-R) *Campylobacter.* In counties like the United States, Australia, and Canada, FQ antibiotics such as enrofloxacin and danofloxacin have indications for subcutaneous use in both sick (therapeutic treatment) and healthy cattle (metaphylaxis) at high risk of bovine respiratory disease (BRD) development (23–29).

Fluoroquinolone-resistant mutant can spontaneously develop in *Campylobacter* (30, 31), and the use of FQ antibiotics selects and enriches these mutants (32). In *Campylobacter*, FQ resistance is mostly caused by point mutations in the quinolone resistance-determining regions (QRDR) of DNA gyrase (*gyrA*) (33, 34), most commonly with the Thr-86-Ile amino acid substitution (C257T mutation), in conjunction with the function multidrug efflux pump CmeABC (34–37). Interestingly, FQ resistance caused by *gyrA* mutations can be maintained in *Campylobacter* without antibiotic selection pressure, suggesting that FQ-R mutants do not carry a fitness burden (38, 39). For example, a previous study conducted by our group revealed a significant fitness advantage of FQ-R over FQ-susceptible (FQ-S) *Campylobacter jejuni* without antibiotic selection pressure when co-inoculated into chickens (40). Interestingly, the fitness change in FQ-R *C. jejuni* could not be attributed to compensatory mutations because no mutations other than the resistance-conferring C257T mutation were found in the *gyrA* and *gyrB* genes of the resistant strains (40).

Because FQ-R *Campylobacter* may still maintain fitness in the absence of antibiotic selection pressure, the reduced or discontinued antimicrobial use in food-producing animals may not necessarily result in an immediate decline in the frequency of FQ-R *Campylobacter*. For example, FQ-R *Campylobacter* was found in 40% of chicken products in two United States companies that had not used FQs for at least 1 year (41). Likewise, FQ-R *Campylobacter* remained for many rotations on Danish broiler farms that had stopped using FQ antibiotics for 4 years (42). In a recent study conducted by our group, it was found that the vast majority of dairy calves (26/30; 87%) were colonized by FQ-R *C. jejuni* even though they had no known previous exposure to FQ antibiotics (32). Similar findings were noted in a study with beef calves in which more than 60% of the *Campylobacter* isolates were resistant to at least one FQ antibiotic (e.g., nalidixic acid or ciprofloxacin) before treatment (43). A study conducted at commercial beef cattle confined feeding operations in Alberta, Canada found a relatively low level of resistance to FQs (~5–7%, ciprofloxacin and nalidixic acid) in *C. jejuni* isolates upon feedlot arrival, but the resistance rate significantly increased (to ~10–15%) after 60 days of maintenance period at some operations that did not use any FQ antibiotics (44). Interestingly, the same study showed a correlation between FQ resistance and genotype as certain subtypes of *C. jejuni* had higher rates of resistant isolates (44). Intriguingly, a longitudinal research on the incidence of antimicrobial-resistant *Campylobacter* in swine raised without antibiotics discovered a ciprofloxacin resistance rate of 17.1% in *Campylobacter coli* (45). These studies suggest that the fitness of FQ-R *Campylobacter* may contribute to the persistence of FQ resistance in the farm environment of various food-producing animals regardless of antimicrobial usage.

Very recently, we conducted a study with commercial dairy calves to evaluate the effect of subcutaneous (s.c.) administration of a single dose danofloxacin on the development of FQ resistance in *C. jejuni* in both healthy and BRD-induced calves (32). Data from that study showed that most of the calves were naturally colonized by a mixture of FQ-R and FQ-S *C. jejuni* strains (~50% of each population) even though these animals were known not to be exposed to FQs previously per the farm records, suggesting that FQ-R strains may have a fitness advantage over FQ-S strains that allowed them to thrive in the gastrointestinal tract of cattle in the absence antibiotic selection pressure. To test this hypothesis, here we performed a series of *in vitro* experiments using both Mueller-Hinton (MH) broth and bovine fecal extract (in an attempt to mimic cattle intestinal tract), including the growth kinetics and competition as well as resistance development, using the FQ-R and FQ-S *C. jejuni* strains collected from the same study (32). It should be noted that natural carriage of *Campylobacter* in the intestine of healthy cattle is common and the organism is usually not associated with any overt disease in cattle (32).

2. Methods

2.1. Bovine fecal extract

Campylobacter-free rectal feces collected freshly and saved at −80°C during our previous investigation (46) was used as a bovine fecal extract in the current study. To confirm the *Campylobacter*-free status, fecal samples were plated on Mueller-Hinton (MH) agar (Difco, BD, Sparks, MD) plates containing *Campylobacter* growth supplement (SR084E; Oxoid, Basingstoke, England) and Preston *Campylobacter* selective supplement (SR117E; Oxoid). Plates were incubated at 42°C for 48 h under microaerobic conditions (10% CO_2, 5% O_2, 85% N_2). Enrichment culture was also performed as described elsewhere (32) to ensure the free *Campylobacter* status of the fecal samples, as this method is more sensitive than direct culture when the number of *Campylobacter* in cattle feces is low (47). Once the free status was confirmed by enrichment, the fecal extract was prepared using the *Campylobacter*-free bovine feces resuspended in MH broth (1:1 in equal volume), and the resuspension was sterilized by a step-wise filtering process (0.80 µm and 0.20 µm pore sized filters; Corning® syringe filters, Millipore Sigma, United States) as described in one of our previous investigations (48). To check for sterility, the

filtered feces were plated on MH agar and blood agar plates (5% sheep blood agar) and incubated at 37°C under aerobic and microaerobic conditions for 72 h. Once sterility was confirmed (no growth of any bacterial colony), the filtered bovine fecal extract was stored in 50 mL sterile centrifuge tubes (10 mL per tube) at −80°C until further use.

2.2. Bacterial strains and culture conditions

The FQ-S and FQ-R *C. jejuni* strains used in this study are listed in Table 1. The majority of *C. jejuni* strains ($n = 4$; origin: Iowa) were isolated from the feces of healhty calves in our very recent study on FQ-resistance development in experimental cattle (32). These four strains were selected because they belonged to the most common MLST sequence types (ST) colonizing the calves and had different FQ susceptibility phenotypes (32). One (ST-93) of strains was originally isolated from the feces of healhy feedlot cattle (Missouri) in our previous study (7) and was one of the inoculum strains used to inoculate the experimental calves in our recent study (32). This strain (ST-93) was re-isolated from the experimentally inoculated calves in that study (32), and was selected to be included (the re-isolated strain) for use in the current study. The strain NCTC 11168 (49) was originally isolated from a diarrheaic human stool and is a commonly used reference strain by many investigators around the world. All the cattle strains were previously identified to the species level by MALDI-TOF mass spectrometry following the manufacturer's (Bruker Daltonik, Billerica, MA, United States) instructions and standard operating procedures at the Veterinary Diagnostic Laboratory at Iowa State University (32). Minimum inhibitory concentrations (MICs) of ciprofloxacin for all of the strains were determined using commercial Sensititre CAMPY2 plates (Thermo Fisher Scientific) in our previous study (32); no further standard MIC testing was performed in the current study. Instead, ability to grow in MH agar containing 4 μg/mL ciprofloxacin (clinical resistance breakpoint per CLSI) was used as an indication of FQ-resistance in the present study. This method was used in many of our previous studies and shown to correlate well with the standard MIC-based resistance determination (32, 34, 40, 46, 50, 51). *Campylobacter jejuni* strains (in glycerol stocks saved in freezers) were grown on MH agar at 42°C for 48 h under microaerobic conditions. The ID of all isolates once again confirmed by MALDI-TOF. Then, each culture was transferred to another fresh MH agar and incubated for ~20 h at 42°C. The cells were collected and resuspended in MH broth for inoculation for further *in vitro* analysis.

2.3. Growth kinetics of FQ-susceptible and FQ-resistant *Campylobacter jejuni*

A fresh culture of each *C. jejuni* strain was first adjusted to $OD_{600} = 0.1$ (which corresponds to ~10^8 CFU/mL, as determined previously), diluted 1:100 in MH broth, and 100 μL of the diluted culture was separately inoculated into 3 tubes (with filtered lids to allow air exchange during incubation; Ibis Scientific, NV, United States) with 10 mL of the bovine fecal extract and another set of 3 tubes (the same type as above) with 10 mL of plain MH broth for comparison, yielding an initial bacterial cell density of ~10^4 CFU/mL (confirmed by viable CFU counts from appropriate serial dilutions inoculated on agar plates for incubation and colony counting). The cultures were incubated together at 39°C under microaerobic conditions to emulate bovine physiological body temperature. To assess differences during the bacterial growth, aliquots of the cultures (100 μL from each of the 3 replicate tubes) were collected at 12, 24, 36, and 48 h of incubation, serially diluted in MH broth as appropriate, and plated onto MH plates for enumeration of bacterial colonies from each replicate tubes separately (3 technical replicates) as described elsewhere (52). Growth curves of the strains were obtained separately in mono-cultures. Two independent experiments (biological replicates) were conducted using the same strains and conditions (6 replicates total per strain per growth medium). No strain genotyping was performed for further confirmatory purposes at this step.

2.4. Pairwise competition experiments between FQ-susceptible and -resistant *Campylobacter jejuni* strains

Each of the pairs used in the competition assay contained a FQ-R and a FQ-S *C. jejuni* strain in equal starting concentration. In the first experiment, susceptible and resistant strains were harvested separately in MH broth and adjusted to the same OD_{600} value. Equal volumes of each strain (100 μL) were inoculated together into 3 tubes (the same type as above with filtered lids) with 10 mL of bovine fecal extract and another set of 3 tubes with 10 mL of MH broth for comparison to give an approximate final cell density of 10^7 CFU/mL for each strain. The cultures were incubated together at 39°C under microaerobic conditions for 24 h and then passaged by transfer of 100 μL of each culture to 10 mL of fresh a medium of corresponding type. To assess the growth differences between the strains, the passages were continued up to 10 times (with 24 h intervals) as described elsewhere (52). Total (susceptible + resistant) *C. jejuni* colonies and FQ-R

TABLE 1 Characteristics of *Campylobacter jejuni* strains used in the current study.

Isolate	Source	Origin	Isolation date	Cipro MIC ug/mL[a]	CIP	Reference
ST-93	Feces of healthy cattle	Missouri	2013	0.12	S	(7, 32)
ST-61	Feces of healthy cattle	Iowa	2018	0.12	S	(32)
ST-929 s	Feces of healthy cattle	Iowa	2018	0.12	S	(32)
ST-929r	Feces of healthy cattle	Iowa	2018	4	R	(32)
ST-982	Feces of healthy cattle	Iowa	2018	8	R	(32)
NCTC 11168[b]	Human feces	United Kingdom	1977	0.12	S	(49)

[a]Ciprofloxacin susceptibility phenotype; R denotes resistant (MIC ≥ 4), S denotes susceptible (MIC ≤ 2).
[b]Strain used as control.

colonies in each mixture at the end of each passage were determined by serially diluting the mixture in MH broth and transferring 100 µL of the dilution from each tube of the 3 replicate tubes to plain MH plates (antibiotic-free) and ciprofloxacin-containing (4 µL/mL) MH plates, respectively. The number of ciprofloxacin-susceptible cells for each replicate was calculated by subtracting the number of colonies on MH plates with ciprofloxacin from the number of colonies on MH plates without ciprofloxacin. Results (average of 3 replicates) were expressed as the individual growth curves of resistant and susceptible strains. In the second experiment, the initial cell density was reduced to 10^3 CFU/mL (from 10^7 CFU/mL) for each strain to evaluate the effect of a lower initial bacterial cell density on the outcome. Two independent experiments (biological replicates) were conducted using the same strains and conditions for each study with different initial cell densities (6 replicates total per strain per initiall cell density per growth medium).

2.5. Assessment of FQ resistance development In FQ-susceptible *Campylobacter jejuni* under different cell density and selection pressure

A fresh culture of each of the four FQ-S *C. jejuni* strains (Table 1; ciprofloxacin MIC = 0.12 µg/mL) was separately inoculated into 3 tubes (the same type as above with filtered lids) with 10 mL of bovine fecal extract containing various concentrations of ciprofloxacin (2, 4 or 20 µg/mL) and another set of 3 tubes with 10 mL of MH broth containing the same ciprofloxacin concentrations for comparison. The experiments were conducted with high (107 CFU/mL) and low (105 CFU/mL) initial bacterial cell densities in the culture media. The cultures were incubated at 39°C under microaerobic conditions. Aliquots from each mixture (100–250 µL) were collected at different time points (0, 1, 2, and 3 days of incubation) for CFU counting. Total (susceptible + resistant) *C. jejuni* colonies and FQ-R colonies in each mixture at each time points were determined by using plain MH plates (antibiotic-free) and ciprofloxacin-containing (4 µL/mL) MH plates, respectively. Of note, the detection limit of this method was ~4 to 10 CFU/mL. The number of ciprofloxacin-susceptible cells was calculated by subtracting the number of colonies on MH plates with ciprofloxacin from the number of colonies on MH plates without ciprofloxacin. Two independent experiments were conducted using the same strains and conditions for each study with different starting bacterial cell densities and/or ciprofloxacin concentrations (three replicate tubes per experiment).

2.6. Statistical analysis

One-way analysis of variance (ANOVA) followed by Tukey's *post hoc* test was used to calculate the significant differences in growth levels (log-transformed) of each *C. jejuni* strain at each time point (growth kinetics study). Student *t*-test was used to calculate the significant differences in growth levels of FQ-R and FQ-S *C. jejuni* at each time point in the pairwise competition assay, and in the development of FQ resistance mutants from FQ-S *C. jejuni* assay. Differences between the mean values were considered significant at $p < 0.05$. The data was analyzed using GraphPad software (Prism, San Diego, CA, United States).

3. Results

3.1. FQ-resistant and FQ-susceptible *Campylobacter jejuni* have comparable growth kinetics when individually cultured

FQ-R (e.g., ST-982 and ST-929r) and FQ-S (e.g., ST-929s, ST-93, ST-61, and NCTC 11168) *C. jejuni* strains were separately cultured in antibiotic-free bovine fecal extract (Figure 1A) and plain MH broth (Figure 1B). Although significant differences (value of $p \leq 0.05$) in growth rates were observed between FQ-R and FQ-S *C. jejuni* strains starting from 24 h of incubation (especially in bovine fecal extract) until the completion of the experiment (Table 2), the strains had comparable growth kinetics overall in both media. There was no distinct growth kinetic pattern in FQ-R strains vs. FQ-S strains in bovine fecal extract, with a mixture of both phenotypes having a relatively faster (ST61-S, ST93-S, ST929-R) or slower (ST929-S, ST982-R) growth. The difference in the growth pattern of FQ-R strains vs. FQ-S strains was even less discernible in MH broth.

3.2. FQ-resistant and FQ-susceptible *Campylobacter jejuni* strains have comparable fitness

Results of the *in vitro* competition experiments using FQ-R and FQ-S *C. jejuni* strains are shown in Figures 2, 3 as $\log_{10}$ CFU/mL for each resistant and susceptible strain during the sequential passages of mixed cultures. Figure 2 shows experiments done using an initial bacterial cell concentration of 10^7 CFU/mL for each strain, while Figure 3 depicts the experiments done using an initial bacterial cell concentration of 10^3 CFU/mL for each strain. Interestingly, regardless of the initial bacterial cell concentration employed and different bacterial genotypes used, the growths of the FQ-R *C. jejuni* strains consistently reached higher concentration than those of the FQ-S *C. jejuni* strains throughout the entire experiment, both in bovine fecal extract and MH broth. Although the majority of differences observed were statistically significant, they were relatively of small scale and ranged only between 0.03–1.29 $\log_{10}$ CFU/mL in MH broth and 0.07–1.33 $\log_{10}$ CFU/mL in bovine fecal extract at high initial bacterial cell concentration (Figure 2), and between 0.015–1.72 $\log_{10}$ CFU/mL in MH broth and 0.015–1.9 $\log_{10}$ CFU/mL in bovine fecal extract at low initial bacterial cell concentration (Figure 3). Overall, these findings indicated that even though FQ-R *C. jejuni* may have a small fitness advantage over FQ-S *C. jejuni*, a highly comparable growth kinetics was evident between the susceptible and resistant strains during the *in vitro* competition experiments (Figures 2, 3).

3.3. Development of FQ resistance in FQ-susceptible *Campylobacter jejuni* strains depends on initial bacterial cell density

All four FQ-susceptible *C. jejuni* strains tested developed resistance to ciprofloxacin within 24 h of incubation in both bovine fecal extract and MH broth (both containing 4 µg/mL ciprofloxacin) when the initial bacterial cell density was relatively high (10^7 CFU/mL; Figure 4). In big contrast, no FQ-R *C. jejuni* colonies were detected at all throughout the experiment when a lower starting bacterial cell concentration

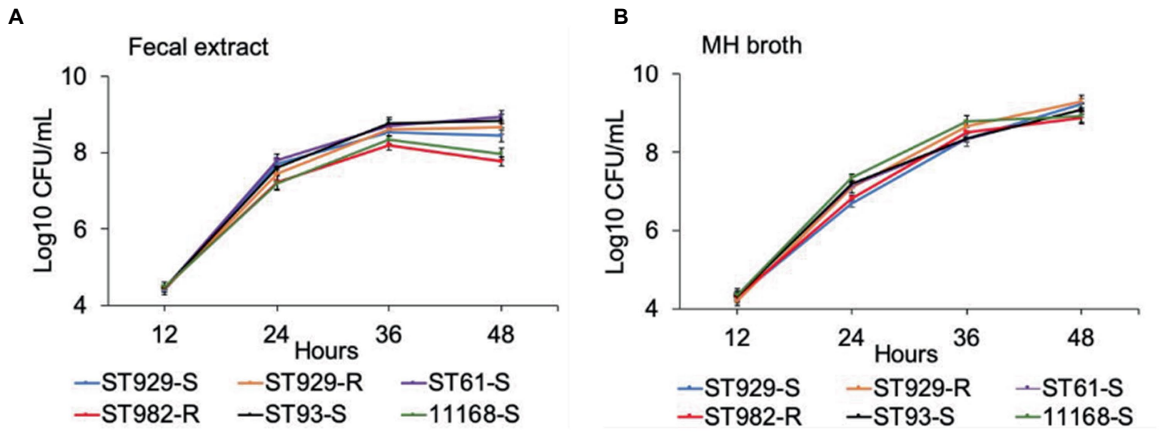


FIGURE 1
Growth kinetics of FQ-resistant and FQ-susceptible *Campylobacter jejuni* strains grown in bovine fecal extract **(A)** and MH broth **(B)**. FQ-resistant *C. jejuni* ST-982 and ST-929r are represented by the red and orange lines, respectively. FQ-susceptible *C. jejuni* ST-929s, ST-93, ST-61, and NCTC 11168 are represented by the blue, black, purple, and green lines, respectively. The number of the bacterial colonies was measured at 12, 24, 36, and 48h of incubation. The experiment was repeated twice, and the results of one representative experiment are shown.

TABLE 2 Comparison of the growth kinetics of FQ-resistant vs. FQ-susceptible *Campylobacter jejuni* strains grown individually in bovine fecal extract and MH broth.

	Fecal extract				MH broth			
	12h[a]	24h	36h	48h	12h	24h	36h	48h
ST-929 (S)[b] vs. ST-982 (R)[c]	ns[d]	$p < 0.001$	$p < 0.001$	$p < 0.001$	ns	ns	ns	$p < 0.001$
ST-929 (S) vs. ST-929 (R)	ns	$p < 0.001$	$p = 0.044$	$p < 0.001$	ns	$p < 0.001$	$p = 0.006$	ns
ST-93 (S) vs. ST-982 (R)	ns	$p < 0.001$	$p < 0.001$	$p < 0.001$	ns	$p < 0.001$	ns	$p = 0.004$
ST-61 (S) vs. ST-982 (R)	ns	$p < 0.001$	$p < 0.001$	$p < 0.001$	ns	$p = 0.002$	ns	$p = 0.002$

A p-value ≤ 0.05 was considered significant. The blue-highlighted p-values represent a faster growth of FQ-S *C. jejuni* strains and the red-highlighted p-values represent a faster growth of FQ-resistant *C. jejuni* strains. The data was analyzed using GraphPad software (Prism, San Diego, CA, United States).
[a]Period of time (hours) after the start of incubation.
[b]S denotes susceptible (ciprofloxacin MIC ≤ 2).
[c]R denotes resistant (ciprofloxacin MIC ≥ 4).
[d]ns denotes non-significant (p-value > 0.05).

(10^5 CFU/mL) was used in either growth medium containing the same ciprofloxacin concentration (data not shown). As typically expected, the initial inoculum (10^7 CFU/mL) of none of the four *C. jejuni* isolates tested had any detectible level of (spontaneous) FQ-R mutants at the start of the experiment (day 0, Figure 4). However, FQ-R colonies appeared as soon as 1 day after the initiation of incubation (day 1) and increased in numbers at the subsequent sampling points (day 2 and day 3, Figure 4). Interestingly, the FQ-R *C. jejuni* population represented virtually 100% of the total colonies detected at all post-incubation sampling points (days 1, 2, and 3) for all 4 strains tested in both bovine fecal extract and MH broth (Figure 4). These results indicated that the initial bacterial cell density significantly and broadly influenced the emergence of FQ-R mutants from FQ-S *C. jejuni* under antibiotic selection pressure (4 μg/mL of ciprofloxacin).

3.4. Magnitude of antibiotic selection pressure significantly influences the development of FQ resistance from FQ-susceptible *Campylobacter jejuni*

The development of ciprofloxacin resistance in FQ-S *C. jejuni* strains when exposed to 2 μg/mL (Figure 5) followed comparable pattern to that observed when the strains were exposed to 4 μg/mL of the antibiotic (Figure 4). At the beginning of the experiment (day 0) FQ-S strains (10^7 CFU/mL starting cell density) did not have any detectable FQ-R mutants, as expected (Figure 5). Within a day (day 1) of the exposure to a low dose (2 μg/mL) of ciprofloxacin, FQ-R colonies were emerged from both FQ-S *C. jejuni* strains tested (~2 $\log_{10}$ CFU/mL) and expanded substantially (~6–8 $\log_{10}$ CFU/mL) during the course of the experiment (days 2 and 3), with a highly similar pattern in both bovine fecal extract and MH broth (Figure 5). Notably, virtually 100% of the colonies detected were FQ-R at all sampling points after the addition of the antibiotic in the growth medium (days 1, 2, and 3), irrespective of the strain and culture media used (Figure 5). In stark contrast, when FQ-S *C. jejuni* strains (10^7 CFU/mL starting cell density) were exposed to a higher concentration (20 μg/mL) of ciprofloxacin (Figure 6), only a small fraction (<2 $\log_{10}$ CFU/mL) of the original inoculum was able to survive and develop FQ resistance on all of the sampling days (days 1, 2 and 3), regardless of the strains tested and growth medium used. However, similar to what was observed with a lower ciprofloxacin concentration (2 μg/mL; Figure 5), virtually all of the detected colonies were FQ-R (Figure 6).

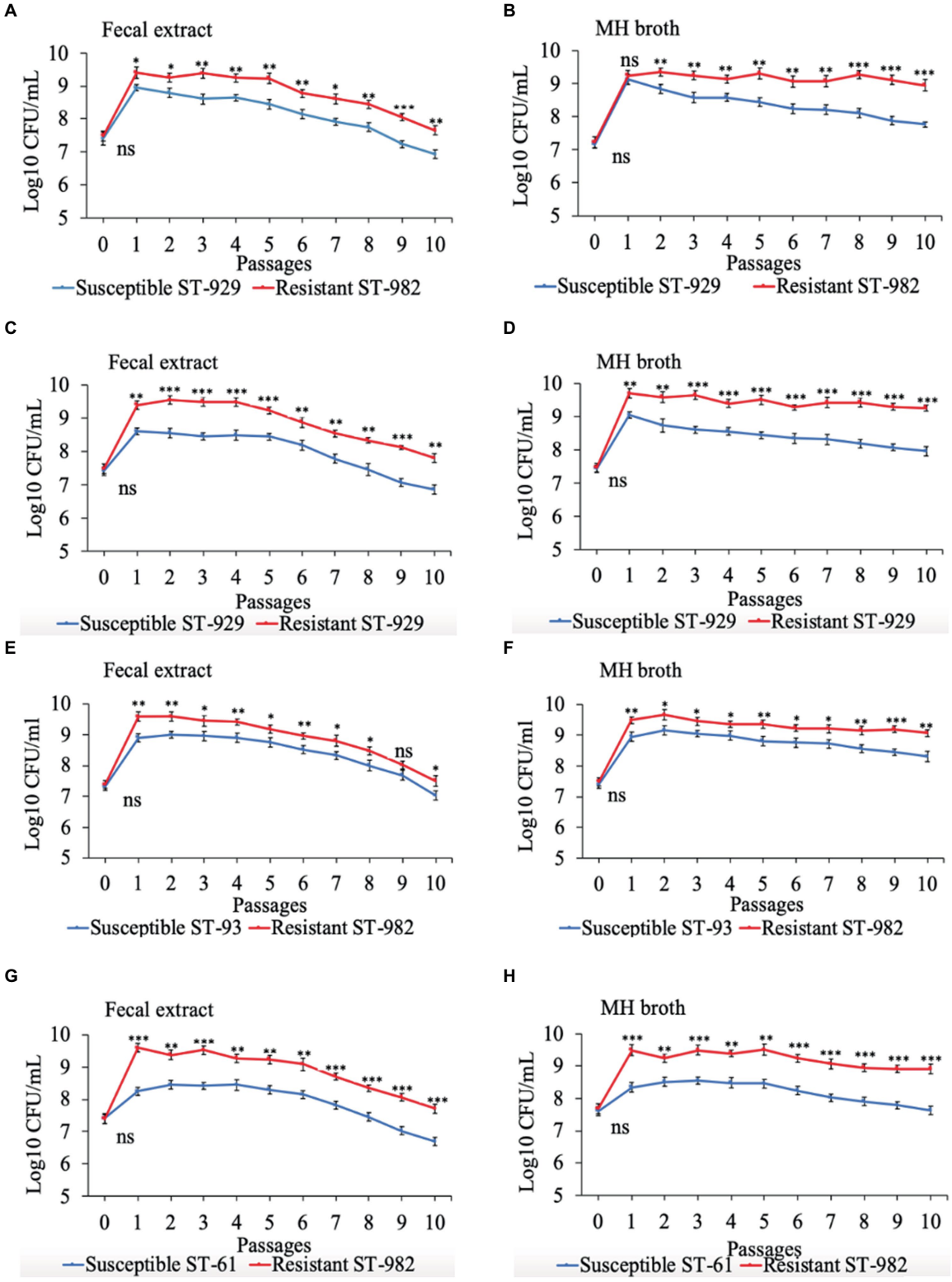


FIGURE 2
Growth kinetics of FQ-resistant *Campylobacter jejuni* (shown as red lines; resistant ST-982 and resistant ST-929) and FQ-susceptible *C. jejuni* (shown as blue lines; susceptible ST-929, susceptible ST-93, and susceptible ST-61) strains of various genetic background as determined by pairwise competition experiments in mixed culture in bovine fecal extract **(A,C,E,G)** and MH broth **(B,D,F,H)**. The initial bacterial cell density was 10^7CFU/mL for each strain. The CFU of each strain at the baseline of each passage was calculated (24h interval). Significant differences between resistant and susceptible strains are indicated by asterisks: *p*-values less or equal to 0.001 are summarized with three asterisks, *p*-values less or equal to 0.01 are summarized with two asterisks, and *p*-values less or equal to 0.05 are summarized with one asterisk. The experiment was repeated twice, and the results of one representative experiment are shown.

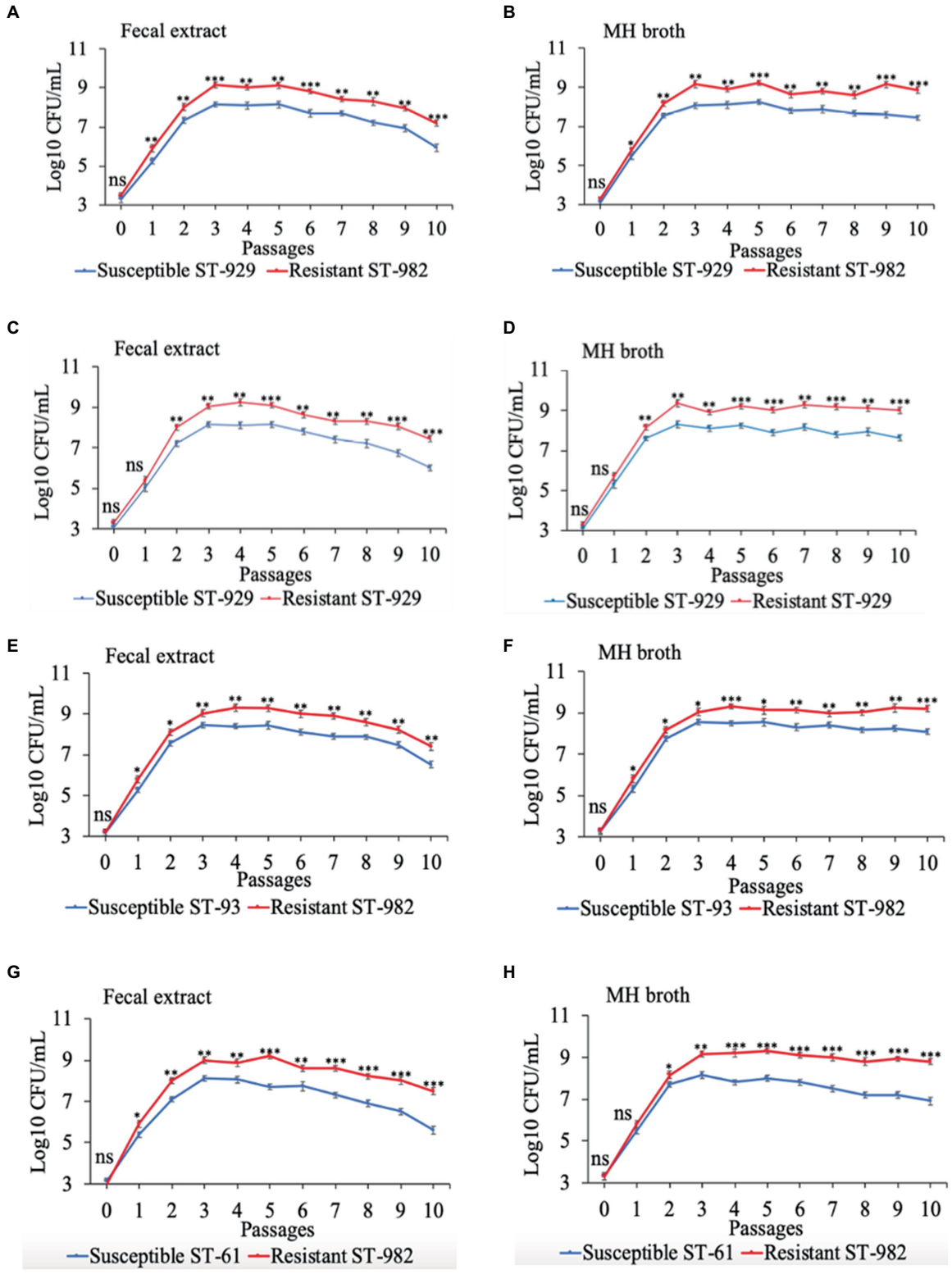


FIGURE 3
Growth kinetics of FQ-resistant *Campylobacter jejuni* (shown as red lines; resistant ST-982 and resistant ST-929) and FQ-susceptible *C. jejuni* (shown as blue lines; susceptible ST-929, susceptible ST-93, and susceptible ST-61) strains of various genetic background as determined by pairwise competition experiments in mixed culture in bovine fecal extract **(A,C,E,G)** and MH broth **(B,D,F,H)**. The initial bacterial cell density was 10^3CFU/mL for each strain. The CFU of each strain at the baseline of each passage was calculated (24h interval). Significant differences between resistant and susceptible strains are indicated by asterisks: *p*-values less or equal to 0.001 are summarized with three asterisks, *p*-values less or equal to 0.01 are summarized with two asterisks, and *p*-values less or equal to 0.05 are summarized with one asterisk. The experiment was repeated twice, and the results of one representative experiment are shown.

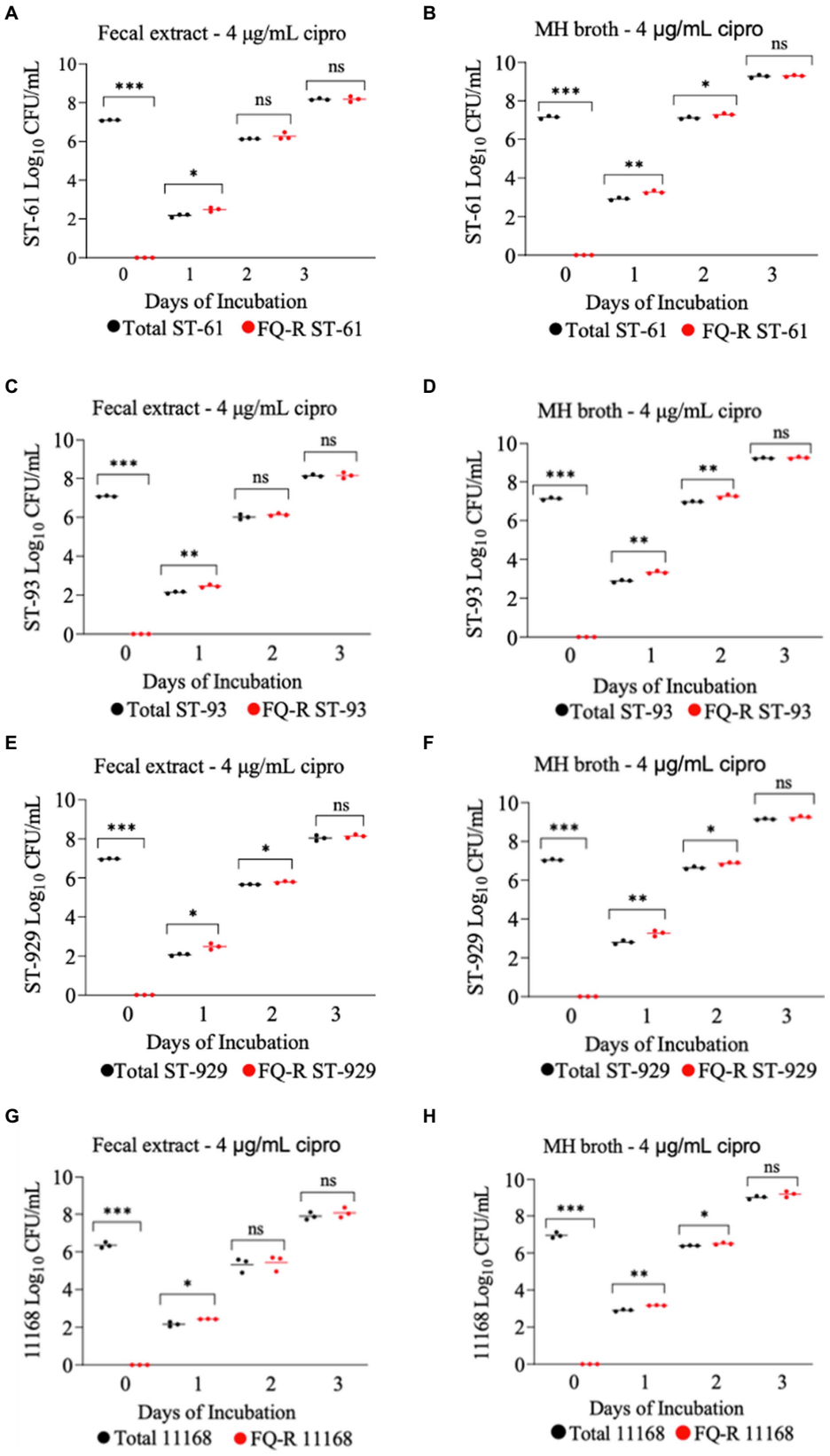


FIGURE 4
Development of FQ-resistant *Campylobacter jejuni* mutants (shown as red dots) from FQ-susceptible strains (ST-61, ST-93, ST-929, and NCTC 11168) grown in bovine fecal extract **(A,C,E,G)** and MH broth **(B,D,F,H)** supplemented with 4µg/mL of ciprofloxacin. The initial bacterial cell density (day 0) of each inoculum was 10^7CFU/mL. Black dots denote total (susceptible+resistant) colonies. Each dot represents the $\log_{10}$ CFU/mL of each strain at a given time point (horizontal bars represent the mean $\log_{10}$ CFU/mL of three replicates). The number of bacterial colonies was measured on days 0, 1, 2, and 3 of incubation. The detection limit of the culture was ~10CFU/mL medium. The experiment was repeated twice, and the results of one representative experiment are shown.

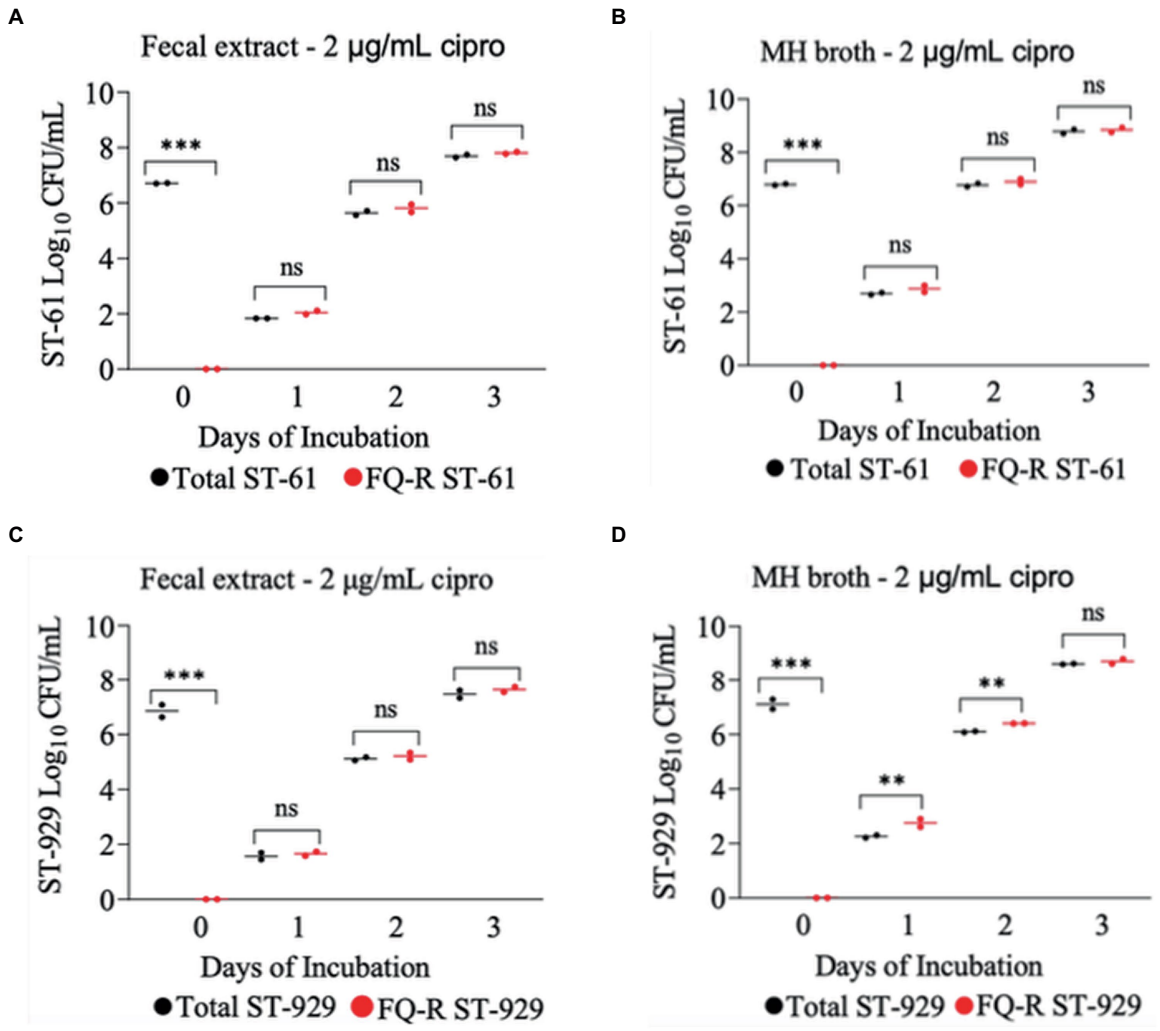


FIGURE 5
Development of FQ-resistant *C. jejuni* mutants (shown as red dots) from FQ-susceptible strains (ST-61, and ST-929) grown in bovine fecal extract **(A,C)** and MH broth **(B,D)** supplemented with 2µg/mL of ciprofloxacin. The initial bacterial cell density (day 0) of each inoculum was 10^7CFU/mL. Black dots denote total (susceptible+resistant) colonies. Each dot represents the $\log_{10}$ CFU/mL of each strain at a given time (horizontal bars represent the mean $\log_{10}$ CFU/mL of three replicates). The number of bacterial colonies was measured on days 0, 1, 2, and 3 of incubation. The detection limit of the culture was ¯4CFU/mL medium. The experiment was repeated twice, and the results of one representative experiment are shown.

4. Discussion

Over the past decades, *Campylobacter* has developed perpetual resistance to clinically important antibiotics that are used for the treatment of severe cases of human infections, in particular to FQs, posing a threat to treatment efficacy in clinical cases (21, 38, 53). The global predominance of FQ-R *Campylobacter* may have been directly influenced by the frequency with which resistant mutants emerged in response to the selection pressure imposed by the use of antibiotics in both human medicine and veterinary settings (34, 54–59). Notoriously, the transmission and spread of antibiotic-resistant pathogens is not only affected by the emergence of resistant mutants in response to the selection pressure, but also influenced by the relative fitness of the drug-resistant organisms in the absence of selection pressure (39, 40, 60, 61). Cattle are a significant source of human *Campylobacter* infections, and there is a clear trend that FQ-R *Campylobacter* from cattle has become highly prevalent in recent years (7, 15, 62, 63). Even though the selection pressure (use of FQs in cattle) may have contributed to the expansion of FQ-R *Campylobacter*, the actual impact of this factor appears to be rather low (32, 44, 46, 64). In the current study, we examined the hypothesis that the fitness of FQ-R *Campylobacter* may have also played a role in the rise seen in FQ-R *Campylobacter* isolates of cattle origin. By using the FQ-R and FQ-S *C. jejuni* strains collected from calves from our recent study (32), we determined (a) *in vitro* growth kinetics of FQ-R and FQ-S strains in mono-cultures, (b) fitness of FQ-R *C. jejuni* without antibiotic selection pressure, and (c) examined the FQ resistance development in FQ-S *C. jejuni* by using different ciprofloxacin concentrations and initial bacterial cell densities.

Quinolone resistance typically develops at an average rate of 5×10^{-9} in *Campylobacter,* with this rate being as high as 5×10^{-7} in some strains (65, 66). When *Campylobacter* is exposed to FQs, ciprofloxacin-resistant mutants will likely arise if the cell population is large enough (>10^6 CFU) (38), suggesting that *Campylobacter*

possess a high mutation rate to FQ resistance. Our results are in line with an *in vitro* study conducted previously by our research group (50), in which FQ-resistance emerged readily from FQ-S *C. jejuni* at high (10^7 and 10^6 CFU/mL) initial bacterial cell densities when cultured in broth medium containing 4 µg/mL ciprofloxacin though no resistance developed when the initial concentration was 10^3 CFU/mL. Similar findings were also observed in the present study, as FQ-S *C. jejuni* developed resistance to ciprofloxacin (4 µg/mL) within 24 h of *in vitro* exposure at a relatively high initial bacterial cell density (10^7 CFU/mL; Figure 4), while no colonies of resistant *C. jejuni* strain was detected at a low initial bacterial cell density (10^5 CFU/mL). In agreement with these *in vitro* data, observations made in animals also indicate the importance of bacterial cell density in the development of FQ resistance in *Campylobacter*. For example, FQ resistance in *Campylobacter* emerges rapidly in chickens but not in cattle under FQ selection pressure, which can, at least in part, be explained by the fact that the organism typically colonizes the chicken intestine at a much higher magnitude (10^{8-9} CFU/g feces) (32, 67) than it does the cattle intestinal tract (10^{3-5} CFU/g feces) (67, 68). In chickens, as soon as 24 h after treatment with FQ antibiotics (enrofloxacin, sarafloxacin, or difloxacin; typically given in drinking water for 5 days), FQ-R *Campylobacter* mutants were found in the feces of treated birds and gradually colonized the intestinal tract at high densities (34, 56, 57). In big contrast, our recent study with calves showed that a single dose s.c. enrofloxacin treatment (7.5 or 12.5 mg/kg) did not result in any detectable level of FQ resistance development from FQ-S *C. jejuni* inhabiting the intestine (~10^{4-5} CFU/g feces) of calves (46). Similarly, therapeutic administration of neither oral (20 mg/kg daily for 7 days) nor subcutaneous (20 mg/kg daily for 1–7 days) enrofloxacin resulted in development FQ-resistance in *C. jejuni* NCTC 11168 following experimental inoculation of mice via oral gavage (69).

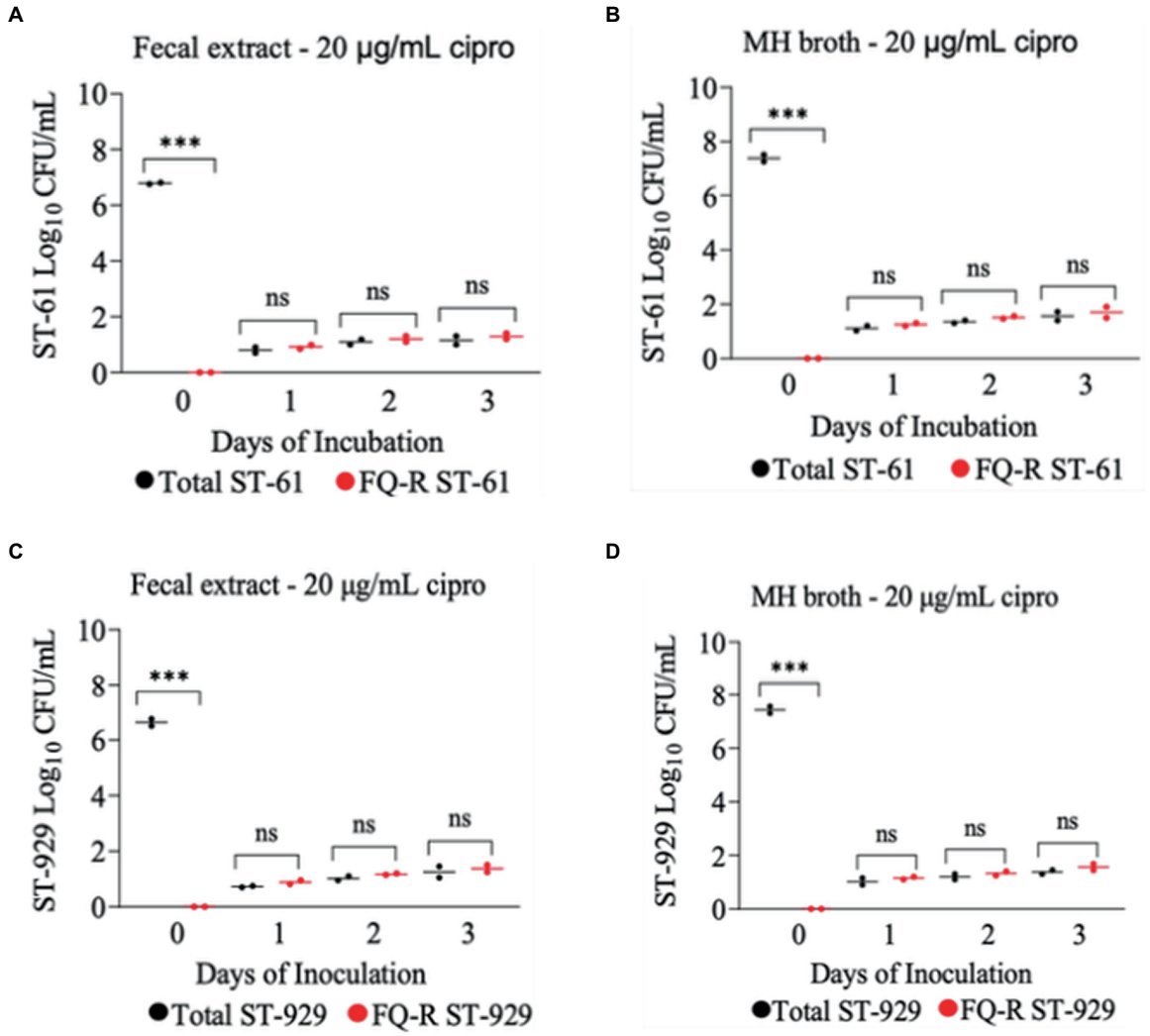


FIGURE 6
Development of FQ-resistant *C. jejuni* mutants (shown as red dots) from FQ-susceptible strains (ST-61, and ST-929) grown in bovine fecal extract **(A,C)** and MH broth **(B,D)** supplemented with 20µg/mL of ciprofloxacin. The initial bacterial cell density (day 0) of each inoculum was 10^7CFU/mL. Black dots denote total (susceptible+resistant) colonies. Each dot represents the $\log_{10}$ CFU/mL of each strain at a given time (horizontal bars represent the mean $\log_{10}$ CFU/mL of three replicates). The number of bacterial colonies was measured on days 0, 1, 2, and 3 of incubation. The detection limit of the culture was ~4CFU/mL medium. The experiment was repeated twice, and the results of one representative experiment are shown.

In the current study, FQ-S *C. jejuni* strains developed resistance to ciprofloxacin more readily when exposed to low levels of ciprofloxacin (2 and 4 μg/mL) compared with exposure to a high level of ciprofloxacin (20 μg/mL). Our data suggest that a high dose of ciprofloxacin is lethal to *Campylobacter*, whereas a low dose may favor the emergence of FQ-R *C. jejuni* from the susceptible strains. Even though it can be rather speculative and cannot be stated with a high degree of certainity, the notion of the mutant selection window (MSW) theory could provide a reasonable explanation for this observation. The range of antimicrobial concentrations known as the MSW ranges from the lowest concentration required to block the growth of wild-type bacteria (MIC) to the highest concentration needed to inhibit the growth of the least susceptible mutant (70). The upper boundary is also known as the mutant prevention concentration (MPC) (71). According to previous publications, the typical MIC of ciprofloxacin in FQ-R *C. jejuni* ranges from 4 to 16 μg/mL (34, 38, 51, 56, 72). Under this theory, the antibiotic becomes lethal to bacteria at concentrations over the MSW, and could no longer select for resistant strains. In the present study, the high level of antibiotic selection pressure might have reached/exceeded the MSW, and thus greatly reducing the emergence of FQ-R mutants in bovine fecal extract and MH broth (Figure 6). In line with this finding, a recent study conducted by our group in which calves were treated with a single dose s.c. enrofloxacin (7.5 and 12.5 mg/kg) found that the drug concentration in the rectal feces of calves had a median of 38–54 μg/g feces for enrofloxacin and 18–21 μg/g feces for ciprofloxacin within 12 h of the injection (73). Notably, in the same study, no FQ-R *C. jejuni* was detected in any of the calves that received enrofloxacin independent of the drug dose used (46). Similarly, we also showed that single dose s.c. danofloxacin treatment in calves colonized with both FQ-R and FQ-S *C. jejuni* resulted in high drug concentration in the rectal feces (median of 382–236 μg/g feces), but did not appear to lead to the development of *de novo* FQ resistance from susceptible strains (32). In contrast to cattle, in a study conducted with broiler chickens, the peak concentration of enrofloxacin was only around 2–4 μg/mL in the intestines of the birds during a standard multi-dose enrofloxacin water treatment, in which FQ-R *C. jejuni* developed soon after the treatment (72). Altogether, these results suggest that the low ciprofloxacin concentrations used in the current study and observed in the intestine of chickens (67, 68) may well have been within the MSW, while the high ciprofloxacin concentrations employed in this study and detected in calf feces (32, 46, 74) may have reached very close to or even exceeded the MPC.

The persistence of antibiotic-resistant *Campylobacter* is influenced by its ability to compete with antibiotic-susceptible strains; this competition dictates whether antibiotic-resistant *Campylobacter* prevails or declines in the absence of antibiotic selection pressure (38). In our study, FQ-R and FQ-S *C. jejuni* had comparable growth rates when individually cultured in either bovine fecal extract or MH broth (Figure 1). Next, we performed pairwise competition experiments to assess the fitness of FQ-R *Campylobacter* by co-culturing several FQ-R *C. jejuni* and FQ-S *C. jejuni* strains of cattle origin in either bovine fecal extract or MH broth containing no antibiotic. Interestingly, FQ-R strains did not have any fitness defect in mixed cultures in the absence of antibiotic selection pressure, but rather displayed a small, albeit significant, growth advantage over the FQ-S strains (Figures 2, 3). Importantly, similar observations were made in calves (from which the *C. jejuni* isolates used here were derived) in our recent study (32), where FQ-R resistant strains were found to coexist with FQ-S strains approximately in equal proportions in the intestinal tract with no antibiotic selection pressure present. Collectively, the findings from both *in vivo* and *in vitro* studies clearly indicate the overall fit nature of FQ-R *C. jejuni* of cattle origin and provide a plausible explanation, at least in part, for the rising trend seen in the prevalence of FQ-R *Campylobacter* in cattle over the past decade.

Our study has some limitations. For example, bovine fecal samples collected from calves in our previous study (46) were stored at −80°C for about 3 years before being used as a bovine fecal extract in the present study. Thus, the storage may have impacted the composition and microbiological properties of the fecal samples. Moreover, the freeze-thawing process (fecal samples were thawed to prepare the fecal extract and then frozen back until further use) may have caused some degree of degradation of the bovine fecal extract. Finaly, even though the bovine fecal extract may be a relevant growth medium to be employed in the experiments performed in the current study, it is important to emphasize that the degree to which it actually mimicked the gastrointestinal tract of cattle is likely to be quite small. Use of digesta instead of fecal extract could have offered more relevant results as it would better mimic the anaerobic conditions in the intestinal lumen. It also should be underlined that caution must be used when extrapolating from *in vitro* results to *in vivo* results and attempting to explain the data with unproven scientific concepts (e.g., the MSW theory).

5. Conclusion

Findings from the current study indicate that FQ-R and FQ-S *C. jejuni* strains of cattle origin had comparable growth kinetics and fitness in mono- and co-cultures, respectively. Moreover, FQ-S *C. jejuni* were shown to develop resistance to FQs more readily when exposed to low levels of ciprofloxacin and at a high initial bacterial cell density compared with exposure to a high level of ciprofloxacin and at a low level of initial bacterial cell density. The latter finding suggests that emergence of FQ-R *C. jejuni* mutants from susceptible strains in cattle is likely hampered by both the relatively low level (CFU/g feces) of bacterial colonization and the high level of antibiotic selection pressure in the intestinal tract following the FQ treatment. Altogether, FQ-R *C. jejuni* derived from cattle is found to compete well with FQ-S *C. jejuni* and does not display any fitness defect in the absence of antibiotic selection pressure, providing a plausible explanation for the high prevalence of FQ-R *Campylobacter* in cattle production.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Ethics statement

The studies involving animals were reviewed and approved by the Institutional Animal Care and Use Committee (IACUC-18-372) at Iowa State University. Written informed consent from the owners for the participation of their animals in this study was not required in accordance with the national legislation and the institutional requirements.

Author contributions

DG: methodology, data collection, statistical analysis, preparation of the manuscript. QZ: methodology, funding acquisition, supervision, and preparation of the manuscript. OS: methodology, funding acquisition, supervision, and preparation of the manuscript. All authors contributed to the article and approved the submitted version.

Funding

This work was supported by the Agriculture and Food Research Initiative's Competitive Grant 2017-68003-26499 from the USDA National Institute of Food and Agriculture.

Conflict of interest

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.

Publisher's note

References

1. Murray CJL, Vos T, Lozano R, Naghavi M, Flaxman AD, Michaud C, et al. Disability-adjusted life years (DALYs) for 291 diseases and injuries in 21 regions, 1990-2010: a systematic analysis for the global burden of disease study 2010. *Lancet*. (2012) 380:2197–223. doi: 10.1016/S0140-6736(12)61689-4

2. Costa D, Iraola G. Pathogenomics of emerging *Campylobacter* species. *Clin Microbiol Rev*. (2019) 32:32. doi: 10.1128/CMR.00072-18

3. Scallan E, Hoekstra RM, Angulo FJ, Tauxe RV, Widdowson MA, Roy SL, et al. Foodborne illness acquired in the United States-major pathogens. *Emerg Infect Dis*. (2011) 17:7–15. doi: 10.3201/eid1701.P11101

4. Buzby JC, Allos BM, Roberts T. The economic burden of *campylobacter*-associated Guillain-Barré syndrome. *J Infect Dis*. (1997) 176, Suppl 2:S192–7. doi: 10.1086/513785

5. Skarp CPA, Hänninen ML, Rautelin HIK. Campylobacteriosis: the role of poultry meat. *Clin Microbiol Infect*. (2016) 22:103–9. doi: 10.1016/j.cmi.2015.11.019

6. Pérez-Boto D, García-Fernández A, Abad-Moreno JC, Echeita MA, Mainar-Jaime RC. Molecular analysis of *campylobacter jejuni* strains causing outbreaks in humans and poultry in Spain between 2011 and 2016. *Microorganisms*. (2020) 8:900.

7. Tang Y, Sahin O, Pavlovic N, Lejeune J, Carlson J, Wu Z, et al. Rising fluoroquinolone resistance in *campylobacter* isolated from feedlot cattle in the United States. *Sci Rep*. (2017) 7:1–8. doi: 10.1038/s41598-017-00584-z

8. Stanley K, Jones K. Cattle and sheep farms as reservoirs of *campylobacter*. *J Appl Microbiol*. (2003) 94:104–13. doi: 10.1046/j.1365-2672.94.s1.12.x

9. Wagenaar JA, French NP, Havelaar AH. Preventing *campylobacter* in the food chain: the current status and the way forward. *Annu Rev Food Sci Technol*. (2021) 12:315–33.

10. Newkirk R, Hedberg C, Bender J. Establishing a milkborne disease outbreak profile: potential food defense implications. *Foodborne Pathog Dis*. (2011) 8:433–7. doi: 10.1089/fpd.2010.0731

11. Heuvelink AE, van Heerwaarden C, Zwartkruis-Nahuis A, Tilburg JJHC, Bos MH, Heilmann FGC, et al. Two outbreaks of campylobacteriosis associated with the consumption of raw cows' milk. *Int J Food Microbiol*. (2009) 34:70–4. doi: 10.1016/j.ijfoodmicro.2008.12.026

12. Jaakkonen A, Kivistö R, Aarnio M, Kalekivi J, Hakkinen M. Persistent contamination of raw milk by *campylobacter jejuni* ST-883. *PLoS One*. (2020) 15:1–22. doi: 10.1371/journal.pone.0231810

13. Fernandes AM, Balasegaram S, Willis C, Wimalarathna HML, Maiden MC, McCarthy ND. Partial failure of milk pasteurization as a risk for the transmission of *campylobacter* from cattle to humans. *Clin Infect Dis*. (2015) 61:903–9. doi: 10.1093/cid/civ431

14. Clark CG, Price L, Ahmed R, Woodward DL, Melito PL, Rodgers FG, et al. Characterization of waterborne outbreak–associated *Campylobacter jejuni*, Walkerton, Ontario. *Emerg Infect Dis*. (2003) 9:1232–41. doi: 10.3201/eid0910.020584

15. Teixeira JS, Boras VF, Hetman BM, Taboada EN, Inglis GD. Molecular epidemiological evidence implicates cattle as a primary reservoir of *Campylobacter jejuni* infecting people via contaminated chickens. *Pathogens*. (2022) 11:1366. doi: 10.3390/pathogens11111366

16. Blaser M, Engberg J. Clinical aspects of *Campylobacter jejuni* and *Campylobacte coli* infections In: I Nachamkin, C Szymanski and M Blaser, editors. *Campylobacter*. Washington, DC: United States, ASM Press. (2008) 99–121.

17. Allos BM. *Campylobacter jejuni* infections: update on emerging issues and trends. *Clin Infect Dis*. (2001) 32:1201–6. doi: 10.1086/319760

18. Dai L, Sahin O, Grover M, Zhang Q. New and alternative strategies for the prevention, control, and treatment of antibiotic-resistant *Campylobacter*. *Transl Res*. (2020) 223:76–88. doi: 10.1016/j.trsl.2020.04.009

19. Eiland LS, Jenkins LS. Optimal treatment of *Campylobacter* dysentery. *J Pediatr Pharmacol Ther*. (2008) 13:170–4. doi: 10.5863/1551-6776-13.3.170

20. Xia Q, Muraoka WT, Shen Z, Sahin O, Wang H, Wu Z, et al. Adaptive mechanisms of *Campylobacter jejuni* to erythromycin treatment. *BMC Microbiol*. (2013) 13:133. doi: 10.1186/1471-2180-13-133

21. Sproston EL, Wimalarathna HML, Sheppard SK. Trends in fluoroquinolone resistance in *Campylobacter*. *Microb Genom*. (2018) 4:1–8. doi: 10.1099/mgen.0.000198

22. Shen Z, Wang Y, Zhang Q, Shen J. Antimicrobial resistance in *Campylobacter* spp. *Microbiol Spectr*. (2018) 6:1–13. doi: 10.1128/microbiolspec.ARBA-0013-2017

23. Giguère S, Dowling P. Fluoroquinolones In: S Giguere, J Prescott, J Baggot, R Walker and P Dowling, editors. *Antimicrobial therapy in veterinary medicine*. *5th* ed. Hoboken, NJ: John Wiley & Sons (2013). 295–314.

24. Ives SE, Richeson JT. Use of antimicrobial metaphylaxis for the control of bovine respiratory disease in high-risk cattle. *Vet Clin N Am – Food Anim Pr*. (2015) 31:341–50. doi: 10.1016/j.cvfa.2015.05.008

25. Nickell JS, White BJ. Metaphylactic antimicrobial therapy for bovine respiratory disease in stocker and feedlot cattle. *Vet Clin N Am – Food Anim Pr*. (2010) 26:285–301. doi: 10.1016/j.cvfa.2010.04.006

26. Apley MD, Coetzee JF. Antimicrobial therapy of respiratory disease in cattle. *Vet Clin North Am Food Anim Pract*. (2018) 34:165–86.

27. Zoetics United States. With a 4-day withdrawal, ADVOCIN®treats and controls BRD late in the feeding period. [internet]. (2021). Available at: https://www.zoetisus.com/products/beef/advocin/index.aspx (Accessed February 13, 2023).

28. Cheng AC, Turnidge J, Collignon P, Looke D, Barton M, Gottlieb T. Control of fluoroquinolone resistance through successful regulation, Australia. *Int Conf Emerg Infect Dis*. (2012) 18:1453–60. doi: 10.3201/eid1809.111515

29. Waldner CL, Parker S, Campbell JR. Antimicrobial usage in western Canadian cow-calf herds. *Can Vet J*. (2019) 60:414–22.

30. Hänninen ML, Hannula M. Spontaneous mutation frequency and emergence of ciprofloxacin resistance in *Campylobacter jejuni* and *Campylobacter coli*. *J Antimicrob Chemother*. (2007) 60:1251–7. doi: 10.1093/jac/dkm345

31. Wieczorek K, Osek J. Antimicrobial resistance mechanisms among *Campylobacter*. *Bio Med Res Int*. (2013) 2013:1–12. doi: 10.1155/2013/340605

32. Goulart DB, Beyi AF, Wu Z, Adiguzel MC, Schroeder A, Singh K, et al. Effect of danofloxacin treatment on the development of fluoroquinolone resistance in *Campylobacter jejuni* in calves. *Antibiotics*. (2022) 11:1–17. doi: 10.3390/antibiotics11040531

33. Engberg J, Aarestrup F, Taylor D, Gerner-Smidt P, Nachamkin I. Quinolone and macrolide resistance in *Campylobacter jejuni* and *C. coli*: resistance mechanisms and trends in human isolates. *Emerg Infect Dis*. (2001) 7:24–34. doi: 10.3201/eid0701.010104

34. Luo N, Sahin O, Lin J, Michel LO, Zhang Q. In vivo selection of *Campylobacter* isolates with high levels of fluoroquinolone resistance associated with *gyrA* mutations and the function of the CmeABC efflux pump. *Antimicrob Agents Chemother*. (2003) 47:390–4. doi: 10.1128/AAC.47.1.390-394.2003

35. Zhang Q, Lin J, Pereira S. Fluoroquinolone-resistant *Campylobacter* in animal reservoirs: dynamics of development, resistance mechanisms and ecological fitness. *Anim Health Res Rev*. (2003) 4:63–72. doi: 10.1079/AHR200356

36. Ge B, McDermott PF, White DG, Meng J. Role of efflux pumps and topoisomerase mutations in fluoroquinolone resistance in *Campylobacter jejuni* and *Campylobacter coli*. *Antimicrob Agents Chemother*. (2005) 49:3347–54. doi: 10.1128/AAC.49.8.3347-3354.2005

37. Yao H, Shen Z, Wang Y, Deng F, Liu D, Naren G, et al. Emergence of a potent multidrug efflux pump variant that enhances *Campylobacter* resistance to multiple antibiotics. *MBio*. (2016) 7:1–11. doi: 10.1128/mBio.01543-16

38. Luangtongkum T, Jeon B, Han J, Plummer P, Logue C, Zhang Q. Antibiotic resistance in *Campylobacter*: emergence, transmission and persistence. *Future Microbiol*. (2009) 4:189–200. doi: 10.2217/17460913.4.2.189

39. Zhang Q, Sahin O, McDermott PF, Payot S. Fitness of antimicrobial-resistant *Campylobacter* and *Salmonella*. *Microbes Infect*. (2006) 8:1972–8. doi: 10.1016/j.micinf.2005.12.031

40. Luo N, Pereira S, Sahin O, Lin J, Huanq S, Michel L, et al. Enhanced in vivo fitness of fluoroquinolone-resistant *Campylobacter jejuni* in the absence of antibiotic selection pressure. *Proc Natl Acad Sci U S A*. (2005) 102:541–6. doi: 10.1073/pnas.0408966102

41. Price LB, Johnson E, Vailes R, Silbergeld E. Fluoroquinolone-resistant *Campylobacter* isolates from conventional and antibiotic-free chicken products. *Environ Health Perspect*. (2005) 113:557–60. doi: 10.1289/ehp.7647

42. Pedersen K, Wedderkopp A. Resistance to quinolones in *Campylobacter jejuni* and *Campylobacter coli* from Danish broilers at farm level. *J Appl Microbiol*. (2003) 94:111–9. doi: 10.1046/j.1365-2672.2003.01811.x

43. Smith AB, Renter DG, Cernicchiaro N, Shi X, Nickell JS, Keil DJ, et al. A randomized trial to assess the effect of fluoroquinolone metaphylaxis on the fecal prevalence and quinolone susceptibilities of *Salmonella* and *Campylobacter* in feedlot cattle. *Foodborne Pathog Dis*. (2017) 14:600–7. doi: 10.1089/fpd.2017.2282

44. Webb AL, Selinger LB, Taboada EN, Inglis GD. Subtype-specific selection for resistance to fluoroquinolones but not to tetracyclines is evident in *Campylobacter jejuni* isolates from beef cattle in confined feeding operations in southern Alberta, Canada. *Appl Environ Microbiol*. (2018) 84:1–18. doi: 10.1128/AEM.02713-17

45. Quintana-Hayashi MP, Thakur S. Longitudinal study of the persistence of antimicrobial-resistant *Campylobacter* strains in distinct swine production systems on farms, at slaughter, and in the environment. *Appl Environ Microbiol*. (2012) 78:2698–705. doi: 10.1128/AEM.07723-11

46. Goulart D, Beyi A, Wu Z, Adiguzel M, Wilson S, Xu C, et al. Influence of single dose enrofloxacin injection on development of fluoroquinolone resistance in *Campylobacter jejuni* in calves. *Antibiotics*. (2022) 11:1407. doi: 10.3390/antibiotics11101407

47. Gharst G, Hanson D, Kathariou S. Effect of direct culture versus selective enrichment on the isolation of thermophilic *Campylobacter* from feces of mature cattle at harvest. *J Food Prot*. (2006) 69:1024–7. doi: 10.4315/0362-028X-69.5.1024

48. Lin J, Sahin O, Michel LO, Zhang Q. Critical role of multidrug efflux pump CmeABC in bile resistance and in vivo colonization of *Campylobacter jejuni*. *Infect Immun*. (2003) 71:4250–9. doi: 10.1128/IAI.71.8.4250-4259.2003

49. Parkhill J, Wren BW, Mungall K, Ketley JM, Churcher C, Basham D, et al. The genome sequence of the food-borne pathogen *Campylobacter jejuni* reveals hypervariable sequences. *Nature*. (2000) 403:665–8. doi: 10.1038/35001088

50. Han J, Sahin O, Barton YW, Zhang Q. Key role of Mfd in the development of fluoroquinolone resistance in *Campylobacter jejuni*. *PLoS Pathog*. (2008) 4:e1000083. doi: 10.1371/journal.ppat.1000083

51. Yan M, Sahin O, Lin J, Zhang Q. Role of the CmeABC efflux pump in the emergence of fluoroquinolone-resistant *Campylobacter* under selection pressure. *J Antimicrob Chemother*. (2006) 58:1154–9. doi: 10.1093/jac/dkl412

52. Hao H, Dai M, Wang Y, Peng D, Liu Z, Yuan Z. 23S rRNA mutation A2074C conferring high-level macrolide resistance and fitness cost in *Campylobacter jejuni*. *Microb Drug Resist*. (2009) 15:239–44. doi: 10.1089/mdr.2009.0008

53. Tacconelli E, Carrara E, Savoldi A, Harbarth S, Mendelson M, Monnet D, et al. Discovery, research, and development of new antibiotics: the WHO priority list of antibiotic-resistant bacteria and tuberculosis. *Lancet Infect Dis*. (2018) 18:318–27. doi: 10.1016/S1473-3099(17)30753-3

54. Pereira RV, Altier C, Siler JD, Mann S, Jordan D, Warnick LD. Longitudinal effects of enrofloxacin or tulathromycin use in preweaned calves at high risk of bovine respiratory disease on the shedding of antimicrobial-resistant fecal *Escherichia coli*. *J Dairy Sci*. (2020) 103:10547–59. doi: 10.3168/jds.2019-17989

55. Humphrey TJ, Jørgensen F, Frost JA, Wadda H, Domingue G, Elviss NC, et al. Prevalence and subtypes of ciprofloxacin-resistant *Campylobacter* spp. in commercial poultry flocks before, during, and after treatment with fluoroquinolones. *Antimicrob Agents Chemother*. (2005) 49:690–8. doi: 10.1128/AAC.49.2.690-698.2005

56. van Boven M, Veldman KT, de Jong MCM, Mevius DJ. Rapid selection of quinolone resistance in *Campylobacter jejuni* but not in *Escherichia coli* in individually housed broilers. *Antimicrob Agents Chemother*. (2003) 52:719–23. doi: 10.1093/jac/dkg402

57. McDermott PF, Bodeis SM, English LL, White DG, Walker RD, Zhao S, et al. Ciprofloxacin resistance in *Campylobacter jejuni* evolves rapidly in chickens treated with fluoroquinolones. *J Infect Dis*. (2002) 185:837–40. doi: 10.1086/339195

58. Griggs DJ, Johnson MM, Frost JA, Humphrey T, Jørgensen F, Piddock LJV. Incidence and mechanism of ciprofloxacin resistance in *Campylobacter* spp. isolated from commercial poultry flocks in the United Kingdom before, during, and after fluoroquinolone treatment. *Antimicrob Agents Chemother*. (2005) 49:699–707. doi: 10.1128/AAC.49.2.699-707.2005

59. Usui M, Sakemi Y, Uchida I, Tamura Y. Effects of fluoroquinolone treatment and group housing of pigs on the selection and spread of fluoroquinolone-resistant *Campylobacter*. *Vet Microbiol*. (2014) 170:438–41. doi: 10.1016/j.vetmic.2014.w01.036

60. Baker S, Duy PT, Nga TVT, Dung TTN, Phat VV, Chau TT, et al. Fitness benefits in fluoroquinolone-resistant *Salmonella typhi* in the absence of antimicrobial pressure. *eLife*. (2013) 2:1–17. doi: 10.7554/elife.01229

61. Gillespie SH. Antibiotic resistance in the absence of selective pressure. *Int J Antimicrob Agents*. (2001) 17:171–6. doi: 10.1016/S0924-8579(00)00340-X

62. Cha W, Mosci R, Wengert SL, Singh P, Newton DW, Salimnia H, et al. Antimicrobial susceptibility profiles of human *Campylobacter jejuni* isolates and association with phylogenetic lineages. *Front Microbiol*. (2016) 7:1–12. doi: 10.3389/fmicb.2016.00589

63. Boysen L, Rosenquist H, Larsson JT, Nielsen EM, Sorensen G, Nordentoft S, et al. Source attribution of human campylobacteriosis in Denmark. *Epidemiol Infect*. (2014) 142:1599–608. doi: 10.1017/S0950268813002719

64. Inglis GD, Taboada EN, Boras VF. Rates of fluoroquinolone resistance in domestically acquired *Campylobacter jejuni* are increasing in people living within a model study location in Canada. *Can J Microbiol*. (2021) 67:37–52. doi: 10.1139/cjm-2020-0146

65. Wang Y, Huang WM, Taylor DE. Cloning and nucleotide sequence of the *Campylobacter jejuni gyrA* gene and characterization of quinolone resistance mutations. *Antimicrob Agents Chemother*. (1993) 37:457–63. doi: 10.1128/AAC.37.3.457

66. Wang Y, Taylor DE. Natural transformation in *Campylobacter* species. *J Bacteriol*. (1990) 172:949–55. doi: 10.1128/jb.172.2.949-955.1990

67. Sahin A, Issmat I, Sahin O, Kassem AII, Shen BZ, Lin AJ, et al. *Campylobacter* in poultry: ecology and potential interventions. *Avian Dis*. (2015) 59:185–200. doi: 10.1637/11072-032315-Review

68. Stern N In: I N, M B, L T, editor. *Reservoirs for C. jejuni and approaches for intervention in poultry in Campylobacter jejuni: Current status and future trend*. Washington, DC: United States, ASM Press. (1992).

69. Inglis GD, Zaytsoff SJM, Selinger LB, Taboada EN, Uwiera RRE. Therapeutic administration of enrofloxacin in mice does not select for fluoroquinolone resistance in *Campylobacter jejuni*. *Can Sci Publ*. (2018) 64:681–94. doi: 10.1139/cjm-2017-0741

70. Drlica K. The mutant selection window and antimicrobial resistance. *J Antimicrob Chemother*. (2003) 52:11–7. doi: 10.1093/jac/dkg269

71. Smith HJ, Nichol KA, Hoban DJ, Zhanel GG. Stretching the mutant prevention concentration (MPC) beyond its limits. *J Antimicrob Chemother*. (2003) 51:1323–5. doi: 10.1093/jac/dkg255

72. Farnell MB, Donoghue AM, Cole K, Reyes-Herrera I, Blore PJ, Donoghue DJ. *Campylobacter* susceptibility to ciprofloxacin and corresponding fluoroquinolone concentrations within the gastrointestinal tracts of chickens. *J Appl Microbiol*. (2005) 99:1043–50. doi: 10.1111/j.1365-2672.2005.02712.x

73. Beyi AF, Mochel JP, Magnin G, Hawbecker T, Slagel C, Dewell G, et al. Comparisons of plasma and fecal pharmacokinetics of danofloxacin and enrofloxacin in healthy and *Mannheimia haemolytica* infected calves. *Sci Rep*. (2022) 12:5107. doi: 10.1038/s41598-022-08945-z

74. Nielsen EM. Occurrence and strain diversity of thermophilic campylobacters in cattle of different age groups in dairy herds. *Lett Appl Microbiol*. (2002) 35:85–9. doi: 10.1046/j.1472-765X.2002.01143.x

frontiers | Frontiers in Veterinary Science

TYPE Systematic Review
PUBLISHED 20 April 2023
DOI 10.3389/fvets.2023.1155772

OPEN ACCESS

EDITED BY
William Calero-Cáceres,
Technical University of Ambato, Ecuador

REVIEWED BY
Getahun E. Agga,
Agricultural Research Service (USDA), United States
Michael Apley,
Kansas State University, United States

*CORRESPONDENCE
Kayla M. Strong
✉ kayla.strong@ucalgary.ca

SPECIALTY SECTION
This article was submitted to Veterinary Epidemiology and Economics, a section of the journal Frontiers in Veterinary Science

RECEIVED 31 January 2023
ACCEPTED 29 March 2023
PUBLISHED 20 April 2023

CITATION
Strong KM, Marasco KL, Invik J, Ganshorn H, Reid-Smith RJ, Waldner CL, Otto SJG, Kastelic JP and Checkley SL (2023) Factors associated with antimicrobial resistant enterococci in Canadian beef cattle: A scoping review.
Front. Vet. Sci. 10:1155772.
doi: 10.3389/fvets.2023.1155772

Factors associated with antimicrobial resistant enterococci in Canadian beef cattle: A scoping review

Kayla M. Strong[1,2]*, Kaitlin L. Marasco[1], Jesse Invik[1], Heather Ganshorn[3], Richard J. Reid-Smith[2,4,5], Cheryl L. Waldner[6], Simon J. G. Otto[2,7], John P. Kastelic[1] and Sylvia L. Checkley[1,2]

[1]Faculty of Veterinary Medicine, University of Calgary, Calgary, AB, Canada, [2]AMR—One Health Consortium, Calgary, AB, Canada, [3]Libraries and Cultural Resources, University of Calgary, Calgary, AB, Canada, [4]Centre for Food-borne, Environmental and Zoonotic Infectious Diseases, Public Health Agency of Canada, Guelph, ON, Canada, [5]Department of Population Medicine, University of Guelph, Guelph, ON, Canada, [6]Western College of Veterinary Medicine, University of Saskatchewan, Saskatoon, SK, Canada, [7]HEAT-AMR (Human-Environment-Animal Transdisciplinary Antimicrobial Resistance) Research Group, School of Public Health, University of Alberta, Edmonton, AB, Canada

Introduction: Antimicrobial resistance (AMR) is a global health concern, occurring when bacteria evolve to render antimicrobials no longer effective. Antimicrobials have important roles in beef production; however, the potential to introduce AMR to people through beef products is a concern. This scoping review identifies factors associated with changes in the prevalence of antimicrobial-resistant *Enterococcus* spp. applicable to the Canadian farm-to-fork beef continuum.

Methods: Five databases (MEDLINE, BIOSIS, Web of Science, Embase, and CAB Abstracts) were searched for articles published from January 1984 to March 2022, using *a priori* inclusion criteria. Peer-reviewed articles were included if they met all the following criteria: written in English, applicable to the Canadian beef production context, primary research, *in vivo* research, describing an intervention or exposure, and specific to *Enterococcus* spp.

Results: Out of 804 screened articles, 26 were selected for inclusion. The included articles discussed 37 factors potentially associated with AMR in enterococci, with multiple articles discussing at least two of the same factors. Factors discussed included antimicrobial administration (n=16), raised without antimicrobials (n=6), metal supplementation (n=4), probiotics supplementation (n=3), pen environment (n=2), essential oil supplementation (n=1), grass feeding (n=1), therapeutic versus subtherapeutic antimicrobial use (n=1), feeding wet distiller grains with solubles (n=1), nutritional supplementation (n=1) and processing plant type (n=1). Results were included irrespective of their quality of evidence.

Discussion: Comparability issues arising throughout the review process were related to data aggregation, hierarchical structures, study design, and inconsistent data reporting. Findings from articles were often temporally specific in that resistance was associated with AMR outcomes at sampling times closer to exposure compared to studies that sampled at longer intervals after exposure. Resistance was often nuanced to unique gene and phenotypic resistance patterns that varied with species of enterococci. Intrinsic resistance and interpretation of minimum inhibitory concentration varied greatly among enterococcal species, highlighting the importance of caution when comparing articles and generalizing findings.

Systematic Review Registration: [http://hdl.handle.net/1880/113592]

KEYWORDS

antimicrobial resistance, *Enterococcus* spp., beef, cattle, scoping review

1. Introduction

In 2015, the World Health Organization stated there was a "global consensus that antimicrobial resistance poses a profound threat to human health" and released a call to action to address antimicrobial resistance (AMR) (1). Antimicrobial resistance is a quintessential One Health problem interwoven within and across human, animal, and environmental health. Antimicrobial resistance can spread within and between various interconnecting continuums; however, the means and extent of resistance transmission and maintenance are not fully elucidated. The epidemiology of AMR is complex. Antimicrobial resistant enterococci and genetic material coding for AMR can undergo multi-directional transmission among people, animals and the environment, related to numerous factors that influence development of resistance, likelihood of transmission, and/or likelihood of colonization in host and/or reservoir. The environment remains a largely unquantified reservoir of AMR. Antimicrobial-resistant bacteria and genetic material coding for AMR could be transmitted to people in various ways along the beef production continuum, including: direct contact between livestock and humans; environmental contamination by sewage or waste contaminating water, food or other fomites; and contamination during slaughter, processing, food handling, or home preparation (2). In 2016, the Food and Agriculture Organization (FAO) released a call for further research to address knowledge gaps in livestock-driven AMR (3) that was echoed in academic literature (4).

Canadian beef producers use antimicrobials to prevent and treat diseases. For example, tetracyclines and macrolides are commonly used in beef production in Canada (5). Studies examining tetracycline and macrolide resistance trends have reported varying extents of bacterial resistance in enteric bacteria (2, 6, 7). An Alberta enterococci study identified 59% of bovine fecal *Enterococcus hirae* resistant to tetracycline and 33% resistant to macrolides (2) based on Clinical and Laboratory Standards Institute (CLSI) interpretive criteria.

Antimicrobial resistance is a concern for the beef industry due to increasing AMR in common bacteria (e.g., *Mannheimia haemolytica*) coupled with infrequent commercialization of new antimicrobials. Consequently, available antimicrobial options may become limited, especially for metaphylaxis (8, 9). In response to these concerns, government and industry have launched surveillance programs across the production continuum to monitor resistance trends (10).

Enterococcus spp. are commensal bacteria present in the gastrointestinal flora of humans and livestock, comprising up to 1% of intestinal microbiota in adults (11). Enterococci are becoming an important multi-drug-resistant nosocomial pathogen associated with human infections, including endocarditis, bacteremia, and urinary tract infections (12, 13). *Enterococcus* spp. have intrinsic resistance to most cephalosporins and semi-synthetic penicillins and to low concentrations of penicillin and ampicillin (14). Enterococci are also intrinsically resistant (*in vivo*) to clindamycin, trimethoprim-sulfamethoxazole and low concentrations of aminoglycosides (14, 15). Aminoglycosides can be used for treatment when used with a combination of high concentrations of penicillin (14, 15). In addition, various species of enterococci have varying intrinsic resistance. For example, *Enterococcus faecalis* is naturally resistant to quinupristin-dalfopristin whereas *Enterococcus faecium* is not (14). *Enterococcus gallinarum* and *Enterococcus casseliflavus* are intrinsically resistant to low concentrations of vancomycin, although other species of *Enterococcus* are not (16).

Enterococci can also acquire resistance to most antimicrobial classes (including higher concentrations of penicillin and ampicillin) and can transfer mobile genetic elements to other bacteria. More than 62 species of enterococci are associated with infections in numerous hosts, with variations of intrinsic resistance and likely differing niches within the microbiome (17, 18). The population structure of *Enterococcus* spp. within the mammalian gastrointestinal tract is influenced by host species, host age, diet and environmental stress, season, portion of the gastrointestinal tract, and isolates studied (19). Given their location in the mammalian gut, enterococci are exposed to numerous other bacteria. Consequently, enterococci can efficiently accumulate resistance genes from other bacterial species, making them useful for assessing AMR in the gastrointestinal microbiome. Therefore, they are often used as a Gram-positive indicator in AMR surveillance. Of specific concern are human hospital-acquired vancomycin-resistant enterococcal infections; they are associated with higher mortality, extended patient stays, increased risk of re-admission, and higher treatment costs (20).

A scoping review may be used to describe available literature (including the volume and complexion of publications), evaluate the feasibility of a meta-analysis, or identify knowledge gaps in available literature (21). No published scoping reviews were identified that addressed associations between antimicrobial resistant enterococci and factors along the beef production continuum. In this context, factors are defined as modifiable actions or interventions that could be associated with an increase or decrease in antimicrobial-resistant enterococci or related resistance genes. A notable systematic review considered the effects of macrolide use on enteric bacteria and was scrutinized in the development of this scoping review (22).

The objectives of this scoping review were to: identify articles that investigate factors potentially associated with a change in the prevalence of AMR in *Enterococcus* spp. during various production stages applicable to the Canadian beef cattle industry; collate factors during beef cattle production (cow-calf and feedlot operations), processing, and retail markets; and identify the existing range of evidence and knowledge gaps in the literature.

2. Methods

This scoping review followed Arksey and O'Malley's framework (23) and is reported using the PRISMA-ScR (Preferred Reporting Items for Systematic reviews and Meta-Analyses extension for Scoping Reviews) checklist (24). The scoping review used a population, concept and context framework when developing the question (25). The population in question was beef cattle and beef products; the concept examined was antimicrobial-resistant *Enterococcus* spp.; and the context was from cow–calf operations to retail meats.

2.1. Scoping review protocol

A search strategy was developed *a priori* following consultation with a multidisciplinary team that included a health science librarian, biostatisticians, geographers, veterinarians, and epidemiologists. The protocol for this review was registered on PRISM: University of Calgary Digital Repository (26). To identify articles published from January 1984 to the search date of March 2022, a search was done on the following databases: MEDLINE (Ovid platform), BIOSIS Previews (Web of Science platform), Web of Science Core Collection (Science Citation Index and Emerging Sources Citation Index), Embase (Ovid platform), and CAB Abstracts (EBSCO platform). The CAB Abstracts search is provided in Supplementary Figure S1; this search was translated to the syntax and vocabulary of the other databases. Covidence systematic review software (Veritas Health Innovation, Melbourne, Australia) was used to support abstract and full-text screening. The title and abstract screening and full-text screening were subjected to a double-blinded process. Each article was reviewed by a minimum of two reviewers (KS and JI), with a third reviewer (SC) introduced in instances of conflict. Articles were initially screened by title and abstract; those that appeared to meet inclusion criteria had full-text screening and were included or excluded, based on the following inclusion criteria:

(1) Articles must be written in English and published after 1984, coinciding with formal acceptance of the genus *Enterococcus* (17).
(2) Article factors must apply to the Canadian beef production context. Depending on the intervention described, the population of interest must have antimicrobial stewardship practices and animal production policies comparable to Canadian beef production. This would include similarities in legislation related to antimicrobial use and residues, plus similar production and management including cow-calf and feedlot production.
(3) Articles must present peer-reviewed primary research; therefore, reviews, opinion articles, editorials, theses, and conference abstracts were not eligible. Consequently, research findings were evidence-based, peer-reviewed, and not replicated.
(4) Articles must present *in vivo* studies. To ensure that the review was evidence-based and to improve generalizability in beef cattle production, only field trials were evaluated.
(5) Articles must have a comparison of the effects of a factor that measured AMR in *Enterococcus* spp.

The decision guide used by the review team is provided (Supplementary S2). Cohen's Kappa was used to assess the reproducibility of the screening process. Data extracted from articles were entered using an integrated Covidence extraction form. Detailed instructions were developed to guide the extraction process (Supplementary S3). Following extraction, data were exported and stored in a standardized Microsoft Excel spreadsheet.

3. Results

3.1. Screening process

Given the selection parameters, 1,313 studies were imported for screening and 509 duplicates were removed. A total of 804 articles were screened and 26 were selected, with characteristics summarized in Table 1. The article screening process is detailed in Figure 1. Cohen's Kappa was 0.71 for the title and abstract and 0.66 for full-text screening, considered substantial agreement (53).

3.2. Study characteristics

Article attributes are summarized in Supplementary Tables S1, S2. No quality assessment was made regarding the results or interpretation of the articles. Articles published from 2019 to 2022 accounted for 38% (10/26) of included studies. All studies included were conducted in North America, with 22 and four from the United States and Canada, respectively. Geographic distribution is illustrated in Figure 2. International studies examined interventions considered applicable to the Canadian context, having antimicrobial stewardship practices and animal production policies comparable to the Canadian beef production system.

Most studies were done in a feedlot environment ($n = 17$), followed by retail ($n = 6$), abattoir ($n = 1$), and farm ($n = 2$). Enterococci samples came predominately from fecal samples or beef products. Fecal samples were collected either directly from the rectum ($n = 13$), from the pen-floor ($n = 4$), or from an unspecified site ($n = 2$). The remaining samples were collected post-evisceration ($n = 1$) or from retail beef ($n = 6$). Cattle represented in the articles were yearlings ($n = 10$), fall-placed calves ($n = 5$), cows and pre-weaned calves at cow-calf operations ($n = 2$), and finished cattle preslaughter ($n = 2$). Five studies described cattle whose age or weight was not defined; however, these parameters were estimated based on the study context (36, 42–45). The remaining seven studies examined beef samples at retail.

The study design and associated sample collection varied widely across studies. Eight of 17 feedlot studies had cattle acclimatized to the feedlot prior to the study for variable intervals, ranging from 3 days (34, 35) to 3 months (39), whereas study design and trial duration ranged from cross-sectional to cohort studies with longitudinal sampling up to 225 days post-trial initiation (one-day preslaughter) (30). There was also variation in the timing of sample collection compared to the time of intervention. For example, in the 17 feedlot studies, samples were collected during or following the intervention, whereas other studies, at farm, abattoir and retail stages, compared interventions that may have occurred up to several years before sampling. There was also notable variation in the type of feedlot study environments, with five studies reporting on cattle housed in

TABLE 1 Summary of the attributes of 26 articles included in the scoping review of reported factors associated with antimicrobial resistant enterococci in Canadian beef cattle.

First author and year of publication	Study design	Location	Beef production stage	Age of cattle (if applicable)	Exposure or intervention studied	Sample collection site	Compound administered (if applicable)
Agga (2016) (27)	Cross-sectional study	Nebraska, United States	Farm	Cows	Antimicrobial administration	Rectum	Ceftiofur
Amachawadi (2013) (28)	Randomized controlled trial	Kansas, United States	Feedlot	Yearlings	Metal supplementation	Pen floor	Copper
Amachawadi (2015) (29)	Randomized controlled trial	Kansas, United States	Feedlot	Fat cattle preslaughter	Metal and antimicrobial administration	Pen floor	Copper and Tylosin
Beukers (2015) (30)	Randomized controlled trial	Alberta, Canada	Feedlot	Fall placed calves	Antimicrobial administration	Rectum	Tylosin
Chan (2008) (31)	Cross-sectional study	Rhode Island, United States	Retail	Not applicable	"All natural" labelling	Retail	Not applicable
Davedow (2020) (32)	Randomized controlled trial	Alberta, Canada	Feedlot	Yearlings	Antimicrobial administration	Pen floor	Tylosin
Edrington (2014) (33)	Randomized controlled trial	Texas, United States	Feedlot	Fall-placed calves	Antimicrobial administration	Rectum	Virginiamycin
Halleran (2021) (34)	Non-randomized trial	North Carolina, United States	Feedlot	Fall placed calves	Antimicrobial administration	Rectum	Danofloxacin
Halleran (2021) (35)	Non-randomized trial.	North Carolina, United States	Feedlot	Fall placed calves	Antimicrobial administration	Rectum	Florfenicol
Hershberger (2005) (36)	Cross-sectional study	United States, multiple states	Farm	Cows and pre-weaned calves at cow-calf operations	Antimicrobial administration	Rectum	Not specified
Innes (2021) (37)	Cross-sectional study	United States, multiple states	Retail	Not applicable	USDA-Certified Organic labeling Processing plant type	Retail	Not applicable
Jacob (2008) (38)	Randomized controlled trial	Kansas, United States	Feedlot	Yearling	Wet distillers grains with solubles Antimicrobial administration	Rectum	Monensin and Tylosin
Jacob (2010) (39)	Randomized controlled trial	Kansas, United States	Feedlot	Fat cattle preslaughter	Metal supplementation	Rectum	Copper and Zinc
LeJeune (2004) (40)	Cross-sectional study	United States, Multiple States	Retail	Not applicable	"Raised without Antibiotics"	Retail	Not applicable
Muller (2018) (41)	Randomized controlled trial	Kansas, United States	Feedlot	Yearling	Antimicrobial administration	Pen floor	Tylosin
Murray (2020) (42)	Randomized controlled trial	Texas, United States	Feedlot	Yearling	Antimicrobial administration Probiotic supplementation	Unspecified site	Tylosin, *Saccharomyces cerevisiae* and *E. faecium* probiotic
Murray (2021) (43)	Randomized controlled trial	Kansas, United States	Feedlot	Yearling	Metal supplementation Essential oil supplementation	Rectum	Zinc

(Continued)

TABLE 1 (Continued)

First author and year of publication	Study design	Location	Beef production stage	Age of cattle (if applicable)	Exposure or intervention studied	Sample collection site	Compound administered (if applicable)
Murray (2022) (44)	Randomized controlled trial	Texas, United States	Feedlot	Yearling	Antimicrobial administration Probiotic supplementation	Rectum	Tylosin and *Enterococcus faecium* probiotic
Platt (2008) (45)	Randomized controlled trial	Texas, United States	Feedlot	Yearling	Antimicrobial administration	Rectum	Chlortetracycline
Schmidt (2020) (46)	Randomized controlled trial	Nebraska, United States	Feedlot	Fall-placed calves	Antimicrobial administration	Rectum	Tylosin
Schmidt (2021) (47)	Cross-sectional study	United States, Multiple States	Retail	Not applicable	"Raised without Antibiotics"	Retail	Not applicable
Shen (2019) (48)	Randomized controlled trial	Alberta, Canada	Feedlot	Yearling	Antimicrobial administration Probiotic supplementation	Unspecified site	Tylosin, Monensin, *Saccharomyces cerevisiae*
Vikram (2017) (49)	Cross-sectional study	United States, state not indicated	Abattoir	Not applicable	"Raised without Antibiotics"	Post-evisceration	Not applicable
Vikram (2018) (50)	Cross-sectional study	United States, multiple states	Retail	Not applicable	"Raised without Antibiotics"	Retail	Not applicable
Zaheer (2013) (51)	Non-randomized trial	Alberta, Canada	Feedlot	Yearling	Antimicrobial administration	Rectum	Tilmicosin, Tulathromycin, Tylosin
Zhang (2010) (52)	Cross-sectional study	United States, multiple States	Retail	Not applicable	Grass fed	Retail	Not applicable

individual pens at an experimental facility, whereas the remaining 12 studies discussed cattle housed in pairs, small groups with 15 or fewer, or in commercial feedlots with more than 100 cattle per pen.

3.3. Antimicrobial susceptibility

Studies were screened based on the inclusion of *Enterococcus* species-specific findings. Of the 26 included articles, 12 *Enterococcus* species were reported, with *Enterococcus faecium* being the most common, followed by *Enterococcus hirae* and *Enterococcus casseliflavus*. Articles reported one to nine unique *Enterococcus* spp., with a median of four species, whereas 12 articles only reported results to the *Enterococcus* spp. level. The counts of *Enterococcus* species reported are in Figure 3.

Antimicrobial resistance was identified based on phenotypic susceptibility or the presence of AMR genes. AMR in *Enterococcus* spp. isolates were measured phenotypically for 26 studies; and phenotypically and genotypically for 14 studies. Five articles (42, 46, 47, 49, 50) measured genotypic resistance, but outcomes were not reported specific to enterococci but rather the broader sample microbiome using quantitative polymerase chain reaction (PCR) and metagenomic sequencing. An additional three articles reported genotypic resistance specific to enterococci, but were not stratified or statistically evaluated in the comparison of intervention (exposure) or referent groups (30, 32, 51). Similarly, three articles reported phenotypic resistance; however, the resistance findings were not stratified or statistically evaluated in the comparison of intervention (exposure) or referent groups (28, 29, 42) (Supplementary Table S5).

There were diverse methods used to measure antimicrobial susceptibility of enterococci in these studies, including selective media, automated methods (i.e., broth microdilution), and manual methods (i.e., disc diffusion) for phenotypic patterns, whereas PCR and whole genome sequencing were used for genotypic resistance. One study used PCR and whole genome sequencing (49). Of the 26 studies examining phenotypic resistance, 16 cited standardized guidelines for setting interpretive criteria, with multiple methods often described within a single study. If a study stated the use of a specific Sensititre™ (Thermofisher Scientific, United States) antimicrobial susceptibility panel, the associated organization's interpretive guidelines were assumed. Notably, 17 studies used selective, antimicrobial-impregnated media when identifying resistant bacteria. Seventeen studies stated the interpretive criteria or breakpoints used to classify isolates as susceptible, intermediate or resistant in the text, whereas 11 studies stated MICs in the text. The most common guidelines for interpretive breakpoints were referenced from the Clinical and Laboratory Standards Institute (CLSI; $n = 15$), followed by National Antimicrobial Resistance Monitoring System for Enteric Bacteria (NARMS; $n = 4$). In that regard, NARMS describes using CLSI breakpoints when available, and uses their own data to help infer breakpoints when not available (54). In addition, a single study described using a European Committee on Antimicrobial Susceptibility Testing (EUCAST) breakpoint when none was available through CLSI.

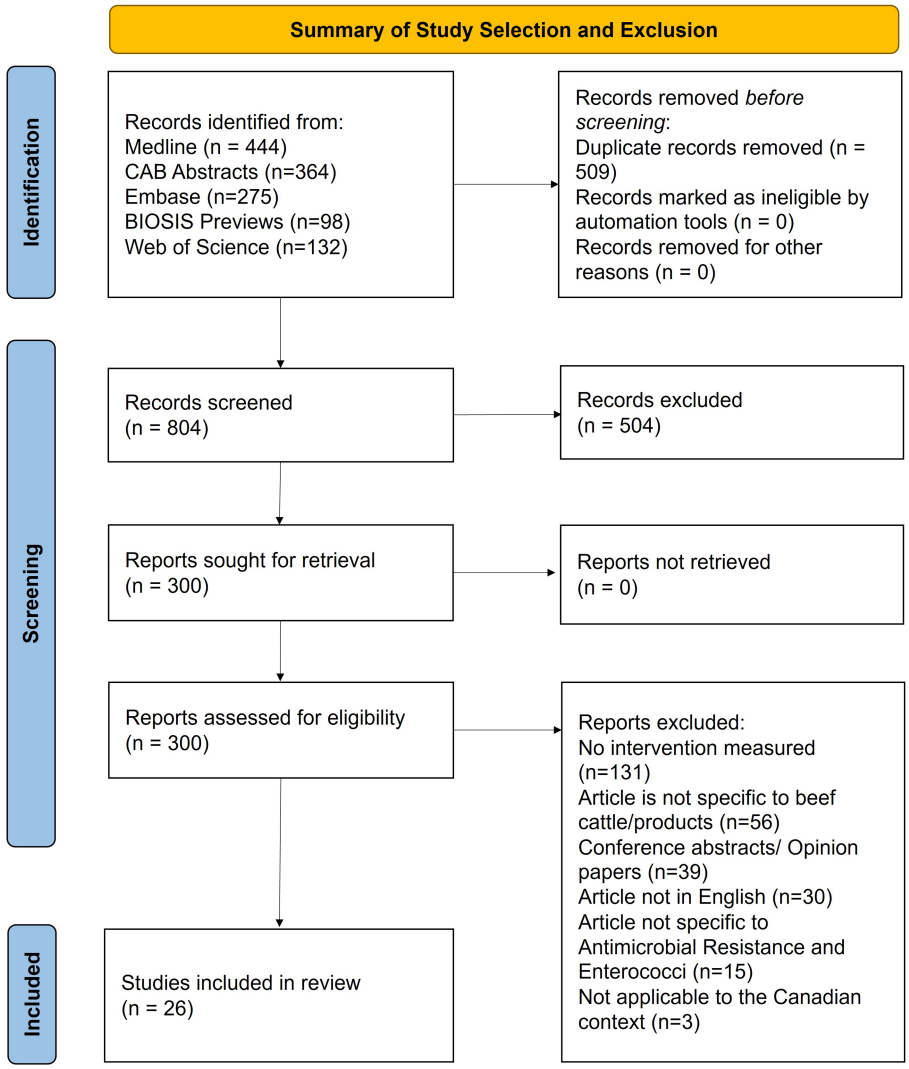


FIGURE 1
PRISMA-ScR flow diagram of the stages of article selection for inclusion in the Scoping review of reported factors associated with antimicrobial resistant enterococci in Canadian beef cattle.

3.4. Statistics

The hierarchical nature of the data (i.e., multiple isolates per sample, multiple samples per animal, and multiple cattle per pen) often required sophisticated modelling to properly account for potential clustering effects. There was much variation in the types of data analyses used, ranging from descriptive statistics (10) to mixed-effects modelling (16). Sample sizes ranged from 12 (31, 34) to 7576 (32) cattle; however, the studies' experimental units included individual cattle, pens of cattle, or other aggregated features. Only three studies referenced a sample size calculation or justification for the sample size used (34, 35, 37).

3.5. Study findings

Studies often had multiple study questions and objectives, with a wide variety of key findings specific to phenotypic and genotypic resistance (Supplementary Table S3). Outcomes represented AMR in enterococci based on specific genes known to convey antimicrobial resistance or phenotypic antimicrobial susceptibility of isolates (55) at one or more time points. Many studies reported varying temporal associations between AMR outcomes and the timing of the intervention (30, 39, 41, 45, 46, 48).

3.5.1. Factors identified within study findings

Overall, 37 factors were reported from the 26 articles. Nine articles reported multiple factors, with five factors overlapping between studies (i.e., "Raised Without Antibiotics" labelling). Factors were compared between exposed and unexposed groups to assess if they were associated with specific AMR outcomes in *Enterococcus* spp. Factors were broadly summarized based on exposure class, exposure (factor), and whether the article reported a statistically significant association with AMR outcomes in enterococci (Supplementary Table S3). Studies reported associations derived from comparisons between factors and multiple outcomes, such as

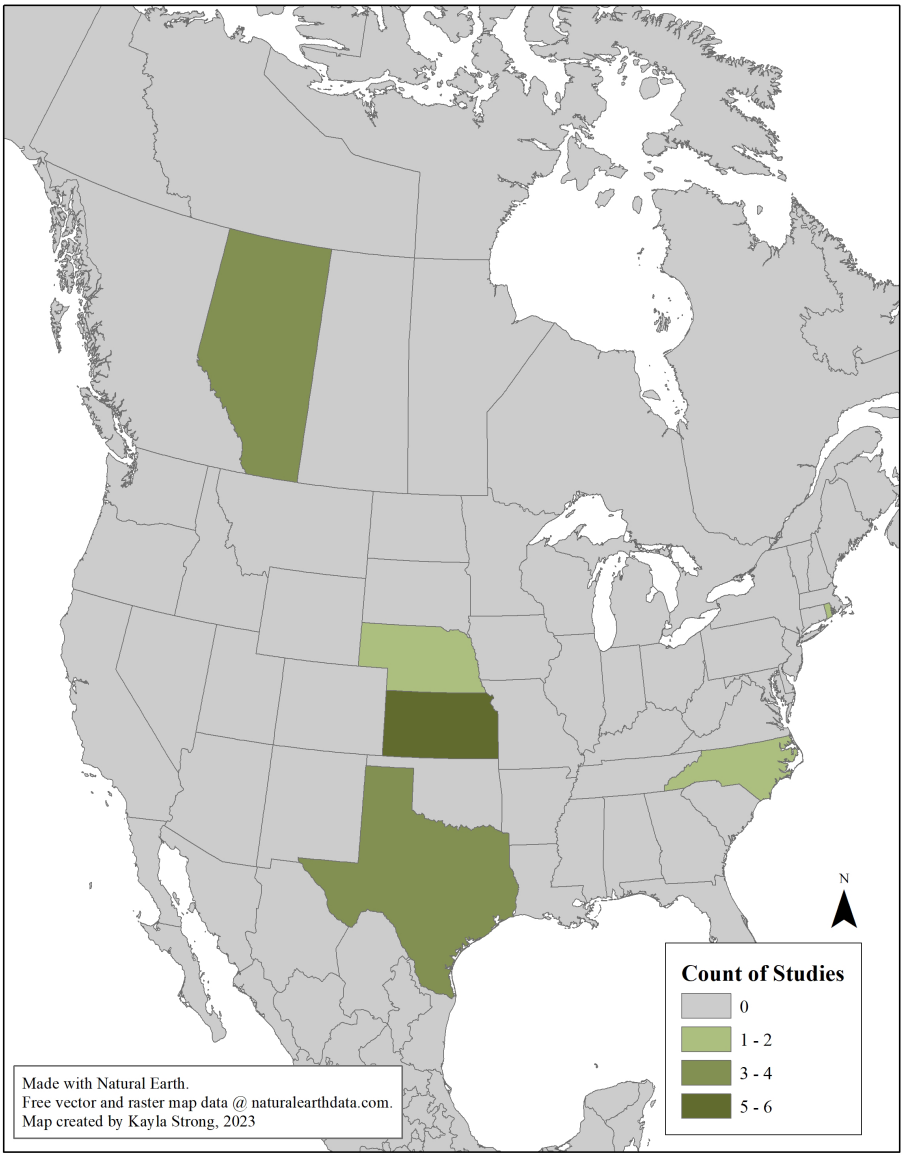


FIGURE 2
Geographic distribution of articles included in the Scoping review of reported factors associated with antimicrobial resistant enterococci in Canadian beef cattle. Seven articles' research locations in the United States were not state-specific and are thus not included in the map.

genotypic and/or phenotypic resistance. These comparisons sometimes resulted in significant associations for one resistance measurement but not another. In addition, some exposures/factors may include multiple exposure groups. For example, some antimicrobial administration studies examined more than one antimicrobial, enabling multiple comparisons to be made to the null when describing that factor.

3.5.1.1. Antimicrobial use

At the genus level, specific to antimicrobial use, studies reported that the use of injectable enrofloxacin (36) or in feed monensin (38) were associated with AMR in enterococci strains. However, other studies reported that injectable formulations of florfenicol (35), danofloxacin (34), or ceftiofur (27) were not associated with AMR in enterococci. One study reported that in-feed virginiamycin use (33) was not associated with phenotypic resistance but with a higher prevalence of identification of the *ermB* gene, associated with resistance to macrolides, lincosamides, and streptogramin B. Reported associations between in-feed macrolide use and resistance were mixed, with inconsistent results across studies and variation between phenotypic and genotypic resistance detection. One study reported an association between macrolide use (both in-feed and injectable) and increased erythromycin resistance in enterococci (51), whereas other studies reported no similar association, specific to in-feed supplementation (32, 41). An association between macrolide feed supplementation and detection

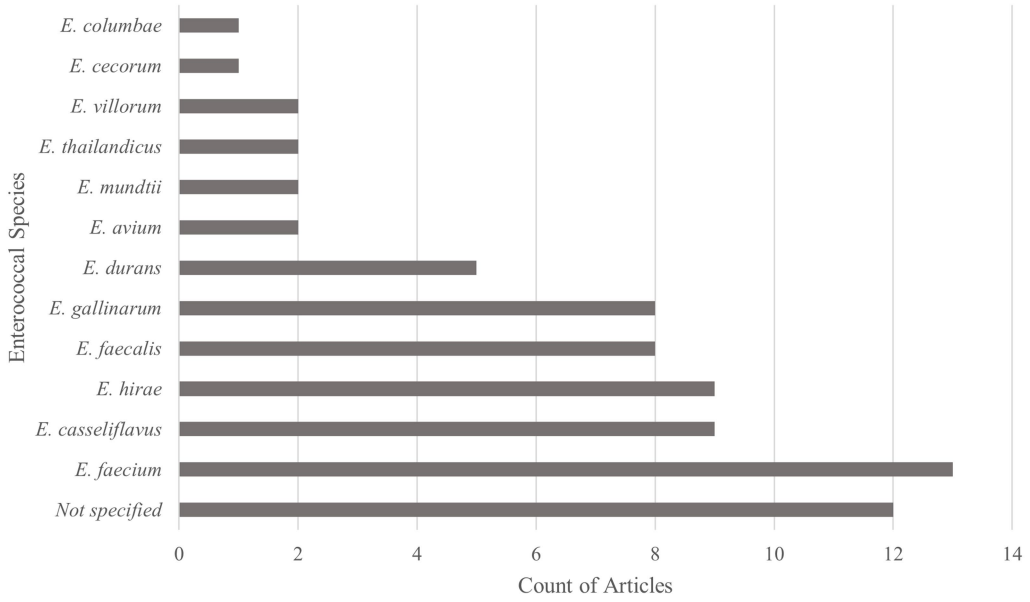


FIGURE 3
Types of enterococcal species reported within Scoping review of reported factors associated with antimicrobial resistant enterococci in Canadian beef cattle (*n*=26). Graph reports the number of articles reporting each enterococcal species. Thirteen articles discussed more than one *Enterococcus* species, with a maximum of nine species discussed within one article.

of resistance genes *tcr(B)*, associated with copper resistance and *erm(B)* were identified in fecal isolates (29).

3.5.1.2. Production factors

Two studies reported that conventionally raised cattle and beef products were associated with increased resistant enterococci in comparison to those labelled as "Raised Without Antibiotics," when comparing phenotypic resistance (47, 49). A separate study concluded that these differences were modest and may be linked to product suppliers, based on a significant interaction with the production system (50). Given the time interval and production steps that occurred between factor occurrence (administration of antimicrobials) and time of measurement (retail beef products), several potential confounders may have influenced studies examining the impacts of "Raised Without Antibiotics." Three studies compared the presence of vancomycin-resistant enterococci (VRE) isolates in conventionally raised beef and beef "Raised Without Antibiotics" or similar labelling, with no evidence of VRE detected in either sample set (31, 40, 47).

The type of processing facility (organic, conventional, or split) was also associated with resistance (37). In a single study that specifically compared grass-fed beef products to conventional beef product, isolates from conventional beef samples were more frequently resistant to daptomycin and linezolid (52). However, other resistance phenotypes assessed were relatively comparable. The study's authors noted the possibility of the sample including enterococci with intrinsic resistance, given the low resistance to daptomycin and linezolid in most *Enterococcus* spp. (52). Perhaps other studies investigating antimicrobial use labelling of retail meats also involved grass-fed cattle, but this was not explicitly stated in the sample collection strategy, and therefore not considered a grass-fed factor.

3.5.1.3. Other supplements

Antimicrobial resistance was not associated with feeding wet distillers grains with solubles (WDGS), except for flavomycin, where WDGS was associated with decreased frequency of resistance in enterococci isolates (38). Probiotics were also examined, with one study reporting a non-significant trend of decreased antimicrobial resistance when probiotics were used compared to not used (44), whereas another reported no association (48). In one article, supplementing an *Enterococcus faecium*, and *S. cerevisiae*-based probiotic increased the probiotic enterococci sequence type (ST296), with a subsequent decrease in sequence type ST240 that tended to include *erm(B)* and *tet(M)* AMR genes (42). Notably, the probiotic *Enterococcus faecium* strain ST296 was isolated from the manure pack sample 112 days post-trial. The probiotic strain survived drying and milling, simulating the process of manure turning to dust and establishing cyclic transmission of a macrolide-susceptible ST296 strain (slightly altered from the original) within a feedlot (42).

Reported associations related to metal supplementation were mixed and inconsistent across phenotypic and genotypic resistance outcomes for various antimicrobials. The resistance gene *ermB* was reported to be associated (29) or not associated (39) with copper supplementation. Similarly, *tcrB* was reported to be associated with (28, 29) or not identified in either the copper-supplemented or control sample set (39). The resistance gene *tet(M)* was not associated with copper supplementation (29, 39). Phenotypic resistance to chloramphenicol, ciprofloxacin, gentamicin, linezolid, penicillin, streptomycin, vancomycin, zinc, and copper were not associated with copper supplementation (39). Comparably, an association between zinc supplementation and tetracycline resistance was reported (43) but had no other resistance associations (39, 43).

Four articles reported that AMR varied across sampling periods of various study designs, with associations between AMR and AMU

during the study but no significant association at the end of the study. These associations were related to the timing of sample collection and timing of antimicrobial treatment, more commonly described in feedlot trials due to study design. Temporary AMR associations were reported following the administration of either chlortetracycline (45), or tylosin (30, 48), but these did not persist and had disappeared by the end of the trials. These factors were all specific to in-feed treatments. Eleven trials included sampling over the entire feeding period or sampling pre-slaughter and were therefore comparable with preslaughter levels, whereas other trials were of shorter intervals, with final sampling dates not representative of the preslaughter period.

4. Discussion

This review examined factors associated with AMR in enterococci isolates at all time points along the beef production continuum, from the cow-calf operation to retail markets. Four broad stages of cow-calf, feedlot, abattoir, and retail were identified. Various sub-stages presented opportunities for further research on potential AMR factors. For example, within cow-calf production, there were unique risks associated with neonatal, pasture-grazed, and pre-weaned calves and cows.

Articles within this review addressed a broad range of research objectives and spanned One Health sectors by including articles across the human, animal, and environmental spectrums, with the analysis done by a cross-disciplinary team. A One Health approach supported a robust interpretation of the available information. There were notable variations in study design, antimicrobial susceptibility testing, data analyses and differences in species of enterococci assessed; thus, caution is required when attempting comparisons or summaries of literature. This scoping review described findings but did not try to compare them.

Findings were often temporally specific in that AMR outcomes were often associated with samples collected soon after antimicrobial exposure. This temporal nature of association was not addressed in the study design of many articles, making comparisons among studies difficult. Sampling plans, such as sampling frequency, also varied across the treatment timeline. Three studies reported temporal associations with antimicrobial use which returned to null by the end of the feeding period (30, 45, 48), whereas other studies did not have a study design appropriate to identify this phenomenon. Studies also varied regarding a period of acclimatization to the feedlot for calves before starting a trial; arguably extended acclimatization renders cattle not a "real" feedlot population and therefore less generalizable to feedlot practice. There are concerns that the microbiome may have differed after cattle commingled compared to cattle not given that opportunity, influencing generalizability to other populations. The size of pens which cattle were commingled may also influence the microbiome and reduce comparisons of differing study designs.

AMR detection and reporting varied by specific antimicrobial and *Enterococcus* spp. (33, 45), highlighting the importance of using caution when comparing study findings across all Entercocci. Various species of enterococci may differ in common acquired resistance patterns and intrinsic resistance. For example, *Enterococcus faecalis* is intrinsically resistant to quinupristin-dalfopristin, whereas *Enterococcus faecium* is not (56). Phenotypic erythromycin and tetracycline resistance, and resistance genes specific to tetracyclines (*tet(M)*) and macrolides (*erm(B)*) were the most common resistance trends identified in enterococci. These were consistent with a prior enterococci-specific review and a surveillance study of AMR in isolates from various stages of beef production (2, 22). Zaheer et al. (2) reported that various enterococci species were highly associated with their environment. Specifically, *Enterococcus hirae* was predominant within feedlot cattle settings yet accounted for < 15% of enterococci in beef processing systems, abattoirs, and retail spaces (2). Instead, *Enterococcus faecalis* was the most predominant enterococcus species in abattoirs and retail spaces, accounting for 74% of samples (2). The predominance of *Enterococcus faecalis* in abattoirs and retail spaces has been seen in additional studies (57–59). Human clinical isolates are primarily *Enterococcus faecalis* (2), the predominant concern in human medicine (12, 60). The relevance of *Enterococcus hirae* for humans is not fully understood as it is rarely recognized in humans, although it may not always be identified due to the limitations of some commercial diagnostic identification methods (61). The shift from *Enterococcus hirae* predominance in cattle to *Enterococcus faecalis* in the abattoir and retail beef did not provide evidence of transmission across the continuum. Furthermore, this observation highlighted the importance of speciating enterococci when evaluating factors that might be associated with AMR. Individual enterococci species were reported in 14 of 26 studies assessed within this review. Twelve studies just reported *Enterococcus* spp., which is also a concern considering intrinsic resistance differs among species.

Several studies investigated associations between a high level of copper supplemented in the feed and the presence of *tcrB*, a transmissible gene conferring resistance to copper, in enterococci isolated from the feces of those cattle compared to enterococci that were isolated from feces of cattle supplemented at a lower concentration covering dietary needs for cattle. The *tcrB* gene has been previously identified co-located on mobile genetic elements that also carry *erm(B)*, a gene that encodes resistance to macrolides, lincosamides, and streptogramin B, and/or tet(M), a gene that encodes resistance to tetracyclines (62, 63).

4.1. Multicausal associations

Articles within this review discussed the long-term and multi-factorial nature of AMR (27, 42, 44). The concept of a multicausal association was further illustrated when considering the number of studies that examined exposures that potentially occurred months or years before sampling, for example, beef products raised conventionally versus those "Raised Without Antibiotics" or similar labelling (31, 37, 40, 47, 50), grass-fed (52), or studies attempting to assess effects of antimicrobial supplementation that had occurred years earlier (27). In the example of "Raised Without Antibiotics" versus conventional beef production, it is difficult to conclude if the reported associations (or lack of) resulted from antimicrobial exposure or were related to other various production, transportation, abattoir, processing, or retail exposures. Differing constellations of factors and confounders in long-term studies may not be measured or adjusted for in statistical analyses.

Some feedlot studies reported a similar increase in the proportion of resistant isolates in both the control and intervention group earlier in the feeding period and a similar decrease in the proportion of resistant isolates over time. In addition to changes in diet and

microbiome mentioned above, perhaps there was an additional environmental transmission of bacteria and/or their resistance genes between the groups in the feedlot over time. For example, Beukers et al. (30) followed the proportion of tylosin resistance fecal enterococci isolates in cattle receiving tylosin phosphate versus those in control cattle. Despite a difference in the frequency of resistance across isolates, there was a similar distribution within both treatment groups, with parallel timing of increases and decreases of the proportion of resistance (30).

4.2. Multilevel data and issues of clustering

Articles included within this scoping review varied widely in sample collection methodologies (i.e., individual versus composite samples) and the experimental unit studied. In many studies, the exposure unit was not the same as the unit of measurement. For example, individual cattle received antimicrobial treatment, but resistance was assessed in *Enterococcus* spp. isolated from pooled fecal samples. Many but not all studies accounted for this multilevel data structure in their statistical analysis using mixed-effects models. Articles that reported adjusted data accounting for its hierarchical structure when discussing significance rarely maintained the hierarchical structure of data within result summaries or supplemental material. Many studies did not reflect the results of these analyses with any level of detail. When data does not present the sampling structure, future use of raw data may introduce clustering biases and misrepresent the data. Going forward, publishers should encourage data to be presented at all appropriate levels when presenting summaries of results and within their supplemental material. In addition, authors should provide details of the stochastic methods of analysis and subsequent interpretation of their findings to promote reproducibility.

4.3. Standardization

Increased standardization and reproducibility of existing research studies would be extremely valuable for strengthening current knowledge in AMR. The earliest published articles included in the review were published in 2004 (40), and standardization of reporting has subsequently evolved. This was evidenced through updated reporting standards and guidelines that have been expanded to account for trial protocol accessibility in randomized trials (64), and developed to address the needs of observational epidemiological studies (65). Despite these advances, there remains wide variation in data presented in articles published in the past 5 years, indicating standardization has not been achieved. This might include further harmonization across national standards, more robust reporting guidelines by journals, or incentivization to provide anonymized hierarchical data and model parameters.

A recent systematic review with a narrower focus on macrolide supplementation in the feedlot setting concluded that long durations of tylosin supplementation are associated with increased proportions of macrolide-resistant gastrointestinal enterococci in feedlot cattle (22). The review encouraged researchers to follow reporting guidelines and publish comparison data for a meta-analysis (22), consistent with the challenges faced in this scoping review.

4.4. Knowledge gaps

This review examined factors occurring within four core stages in beef production: cow-calf operations, feedlot, abattoir, and retail. Within each stage, a series of substages or categorizations were attempted (i.e., neonatal, pasture-grazed, and pre-weaned calves). Of the 26 articles included, only two (27, 36) examined exposures at the cow-calf and "farm" space, making it difficult to differentiate risks across sub-stages.

The feedlot was the second identified stage, where cattle typically spend 90 to >300 days. Most studies identified within the scoping review occurred within the feedlot environment. However, there were knowledge gaps along the temporal timeline, with few studies examining cattle for the total duration at the feedlot. Reproducibility and replication of studies in a comparable environment with similar sampling timelines were limited, presenting an additional knowledge gap. Many feedlot studies occurred in an experimental pen setting, with individual animals or small groups from single sources, and may have included an acclimatization period. In contrast, commercial feedlot settings in North America are often much larger and introduce cattle from numerous sources. Therefore, findings from experimental pen settings may not be generalizable to the commercial environment given multiple potential confounders that may occur in commercial feedlots. This introduces a knowledge gap when interpreting these experimental pen studies.

After the feedlot phase, cattle are transported to the abattoir for slaughter and processing. Similar to the prior two stages, a series of sub-stages occur. These include transport to slaughter, lairage, slaughter, processing, and secondary processing as required. Within the articles identified, only one study applied to these stages, examining abattoir factors (37).

The final stage was the retail space, which includes packaging, storage, transport, potential repackaging, and purchasing conditions of the meat. Although multiple studies examined resistance at the retail stage, the exposure in question was the use of antimicrobials in raising cattle. However, there were no studies specifically examining retail interventions/exposures. Further research and discussion of potential AMR-related factors related to cow-calf operations, transportation, abattoirs, beef processing, and retail spaces are required.

Parallel to knowledge gaps in the scope of research, there are also potential gaps in the depth of information. A recent review discussed the benefits of whole genome sequencing in detecting AMR genes in enterococci and concluded that this approach is well-suited for identifying phenotypically sensitive bacteria that may carry resistance genes (66). Identification of genetic determinants allows for potential outbreak management and understanding of the potential for phylogeographic spread, enhancing understanding of AMR epidemiology (67). Genotypic data regarding potential factors associated with AMR are currently limited and represent a substantial data gap in the literature.

4.5. Limitations

Several limitations may have affected the type of articles retrieved and included in this scoping review. First, articles were not excluded based on the quality of the evidence. A minimal number of

publications met the search criteria, and we wanted to characterize all available information, make interpretations, and suggest future actions. The quality of evidence was assessed internally but was not reflected as a part of this scoping review. Secondly, environmental articles were not included. Environmental transmission is an essential component of AMR within beef production but was outside the context of this scoping review. Thirdly, grey literature (e.g., conference proceedings, dissertations, government publications) was not captured within the scoping review. This potentially excluded smaller studies and emerging, unpublished research. Additionally, only articles written in English were included, potentially excluding international findings applicable to the Canadian context. The requirement of there being a comparison group for inclusion of a factor excluded certain study designs, e.g., descriptive studies and case reports.

The extraction of factors associated with AMR in enterococcal isolates from articles included in the scoping review was unique to this review and identified challenges in data extraction for secondary purposes. Factors were drawn from reported associations and patterns; however, summarized statements were unique to the context of the study and often not comparable to other studies examining the same factor. This was due to differences in sampling timelines, antimicrobial susceptibility testing protocols, type of data presented, and confounding variables considered. In addition, methods of bacterial analysis, antimicrobial susceptibility testing, and even minimum inhibitory concentrations and breakpoint cut-offs have changed over time. Therefore, caution must be used when interpreting findings and drawing conclusions beyond the scope of the original article. The differences in articles examining similar factors limit opportunities for meta-analysis and other quantitative analyses.

Results from the data extraction were not presented specific to each enterococcal species, and instead discussed as a collective genus. The decision to report at the genus level was due to variations in detail provided by the articles, with 14 articles providing species of Enterococci and 12 not. Of those who did report the enterococci species, there were varying speciation methodologies and standards used. The decision not to report enterococci species was a limitation in this article given the intrinsic resistance trends that are unique to many Enterococci species, and differing environments in which species are detected.

The scoping review faced similar challenges as prior antimicrobial-specific reviews in the area, with limited articles for inclusion, variable study designs, limited data available for extraction, inadequate adjustments for potential confounders, and reporting of non-significant results by omission, potentially furthering publication bias (22, 68, 69). A general limitation of scoping reviews is the possibility that the search strategy did not identify all published articles within the study scope; however, this risk was minimized by having a multidisciplinary team involved in syntax development and study design.

5. Conclusion

This scoping review identified factors that may be associated with increases or decreases in the prevalence of AMR in *Enterococcus* spp. isolated at various points along the beef production continuum, including cow-calf and feedlot operations, slaughter, and retail markets. A series of factors associated with antimicrobial administration, metal supplementation, probiotics supplementation, and meat processing were characterized. Resistance was associated with certain heavy metals and antimicrobial supplementation but was highly specific to the timing of sampling related to exposure, and specific phenotypic and/or genotypic resistance assessed. Inconsistencies in the amount of detail, availability of reported results, and interpretation of hierarchical data limited the interpretability and comparison of factors on a broader One-Health scope. Data gaps were identified in antimicrobial treatment and other management factors occurring during breeding, neonatal environment, and pasture grazing stages at cow-calf operations; transportation between production stages; abattoir lairage, slaughter, processing, and potential secondary processing; and packaging, storage and purchasing conditions in retail environments. Variations in sampling methods, sampling framework, intervention/exposure timeline and duration, data presentation, and resistance information collected were additional limitations. Future research should focus on filling identified research gaps that have limited or no published articles, along with standardization of laboratory, analytical and reporting methodologies. In addition, manuscripts should prioritize access to anonymized raw data with associated metadata for secondary analyses for future transdisciplinary projects and applications.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary material, further inquiries can be directed to the corresponding author.

Author contributions

KS, RR-S, CW, SO, and SC: conceptualization. KS, RR-S, SO, SC, and HG: methodology. KS, KM, JI, and SC: investigation. KS, KM, JI, and HG: data curation. KS: writing—original draft. KS, RR-S, CW, SO, KM, JI, JK, SC, and HG: writing—review and editing. RR-S and SC: supervision and funding acquisition. All authors contributed to the article and approved the submitted version.

Funding

This research is part of the AMR—One Health Consortium, funded by the Major Innovation Fund program of the Ministry of Jobs, Economy and Innovation, Government of Alberta. This project is also funded by the Natural Sciences and Engineering Research Council of Canada and the Genomics Research Development Initiative Shared Priority Project on AMR (GRDI-AMR 1).

Acknowledgments

The authors thank Dr. Carl Ribble for his early contributions in project visualization, and the GRDI-AMR 1 Work Package 5 team for their support in project design and methodology.

Conflict of interest

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.

Publisher's note

All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations, or those of the publisher, the editors and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.

Supplementary material

The Supplementary material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fvets.2023.1155772/full#supplementary-material

References

1. World Health Organization. *Global Action Plan on Antimicrobial Resistance [internet]*. Geneva, Switzerland: World Health Organization (2015).

2. Zaheer R, Cook SR, Barbieri R, Goji N, Cameron A, Petkau A, et al. Surveillance of enterococcus spp. reveals distinct species and antimicrobial resistance diversity across a one-health continuum. *Sci Rep*. (2020) 10:3937. doi: 10.1038/s41598-020-61002-5

3. Wall B, Mateus A, Marshall L, Pfeiffer D, Lubroth J, Ormel H, et al. *Drivers, Dynamics and Epidemiology of Antimicrobial Resistance in Animal Production*. Rome: Food and Agriculture Organization of the United Nations (2016).

4. McCubbin KD, Anholt RM, de Jong E, Ida JA, Nóbrega DB, Kastelic JP, et al. Knowledge gaps in the understanding of antimicrobial resistance in Canada. *Front Public Health*. (2021) 9:726484. doi: 10.3389/fpubh.2021.726484

5. Brault SA, Hannon SJ, Gow SP, Warr BN, Withell J, Song J, et al. Antimicrobial use on 36 beef feedlots in western Canada: 2008–2012. *Front Vet Sci*. (2019) 6:329. doi: 10.3389/fvets.2019.00329

6. Benedict KM, Gow SP, McAllister TA, Booker CW, Hannon SJ, Checkley SL, et al. Antimicrobial resistance in Escherichia coli recovered from feedlot cattle and associations with antimicrobial use. *PLoS One*. (2015) 10:e0143995. doi: 10.1371/journal.pone.0143995

7. Waldner CL, Gow S, Parker S, Campbell JR. Antimicrobial resistance in fecal Escherichia coli and campylobacter spp. from beef cows in western Canada and associations with herd attributes and antimicrobial use. *Can J Vet Res*. (2019) 83:80–9.

8. Cameron A, McAllister TA. Antimicrobial usage and resistance in beef production. *J Animal Sci Biotechnol*. (2016) 7:68. doi: 10.1186/s40104-016-0127-3

9. Economic Research Service. The U.S. and EU animal pharmaceutical industries in the age of antibiotic resistance. U.S. Department of Agriculture; (2019). Report no.: ERR-264.

10. BCRC. Antimicrobial Resistance [Internet]. Beef Cattle Research Council. (2020) [cited 2020 Oct 20]. Available at: http://www.beefresearch.ca/research-topic.cfm/antimicrobial-resistance-11

11. Dubin K, Pamer EG. Enterococci and their interactions with the intestinal microbiome. *Microbiol Spectr*. (2017) 5:5.6.01. doi: 10.1128/microbiolspec.BAD-0014-2016

12. Arias CA, Murray BE. Emergence and management of drug-resistant Enterococcal infections. *Expert Rev Anti-Infect Ther*. (2008) 6:637–55. doi: 10.1586/14787210.6.5.637

13. Linden PK. Optimizing therapy for vancomycin-resistant enterococci (VRE). *Semin Respir Crit Care Med*. (2007) 28:632–45. doi: 10.1055/s-2007-996410

14. Kristich CJ, Rice LB, Arias CA. Enterococcal infection—treatment and antibiotic resistance In: MS Gilmore, DB Clewell, Y Ike and N Shankar, editors. *Enterococci: From Commensals to Leading Causes of Drug Resistant Infection [internet]*. Boston, MA: Massachusetts Eye and Ear Infirmary (2014)

15. CLSI. *Performance Standards for Antimicrobial Disk and Dilution Susceptibility Tests for Bacteria Isolated From Animals. 6th* ed. Berwyn, PA, USA: Clinical and Laboratory Standards Institute (2023) (CLSI supplement VET01S).

16. Eliopoulos GM, Gold HS. Vancomycin-resistant enterococci: mechanisms and clinical observations. *Clin Infect Dis*. (2001) 33:210–9. doi: 10.1086/321815

17. Lebreton F, Willems RJL, Gilmore MS. Enterococcus diversity, origins in nature, and gut colonization In: MS Gilmore, DB Clewell, Y Ike and N Shankar, editors. *Enterococci: From Commensals to Leading Causes of Drug Resistant Infection [internet]*. Boston, MA: Massachusetts Eye and Ear Infirmary (2014)

18. Parte AC, Sardà Carbasse J, Meier-Kolthoff JP, Reimer LC, Göker M. List of prokaryotic names with standing in nomenclature (LPSN) moves to the DSMZ. *Int J Syst Evol Microbiol*. (2020) 70:5607–12. doi: 10.1099/ijsem.0.004332

19. Silva N, Igrejas G, Gonçalves A, Poeta P. Commensal gut bacteria: distribution of *Enterococcus* species and prevalence of *Escherichia coli* phylogenetic groups in animals and humans in Portugal. *Ann Microbiol*. (2012) 62:449–59. doi: 10.1007/s13213-011-0308-4

20. Chiang HY, Perencevich EN, Nair R, Nelson RE, Samore M, Khader K, et al. Incidence and outcomes associated with infections caused by vancomycin-resistant enterococci in the United States: systematic literature review and meta-analysis. *Inf Control Hospital Epidemiol*. (2017) 38:203–15. doi: 10.1017/ice.2016.254

21. Sargeant JM, O'Connor AM. Scoping reviews, systematic reviews, and meta-analysis: applications in veterinary medicine. *Front Vet Sci*. (2020) 7:11. doi: 10.3389/fvets.2020.00011

22. Cazer CL, Eldermire ERB, Lhermie G, Murray SA, Scott HM, Grohn YT. The effect of tylosin on antimicrobial resistance in beef cattle enteric bacteria: a systematic review and meta-analysis. *Prev Vet Med*. (2020) 176:104934. doi: 10.1016/j.prevetmed.2020.104934

23. Arksey H, O'Malley L. Scoping studies: towards a methodological framework. *Int J Soc Res Methodol*. (2005) 8:19–32. doi: 10.1080/1364557032000119616

24. Tricco AC, Lillie E, Zarin W, O'Brien KK, Colquhoun H, Levac D, et al. PRISMA extension for scoping reviews (PRISMA-ScR): checklist and explanation. *Ann Intern Med*. (2018) 169:467–73. doi: 10.7326/M18-0850

25. Aromataris E, Munn Z, (eds.) JBI Manual for Evidence Synthesis [internet]. JBI; (2020). Available at: https://synthesismanual.jbi.global.

26. Strong K, Marasco K, Invik J, Ganshorn H, Reid-Smith R, Waldner C, et al. Factors associated with antimicrobial resistant enterococci in north American beef cattle: A scoping review protocol. (2021); Available at: https://prism.ucalgary.ca/handle/1880/113592. Accessed August 10, 2021.

27. Agga GE, Schmidt JW, Arthur TM. Antimicrobial-resistant fecal bacteria from ceftiofur-treated and nonantimicrobial-treated comingled beef cows at a cow-calf operation. *Microb Drug Resist*. (2016) 22:598–608. doi: 10.1089/mdr.2015.0259

28. Amachawadi RG, Scott HM, Alvarado CA, Mainini TR, Vinasco J, Drouillard JS, et al. Occurrence of the transferable copper resistance gene tcrB among fecal enterococci of U.S. feedlot cattle fed copper-supplemented diets. *Appl Environ Microbiol*. (2013) 79:4369–75. doi: 10.1128/AEM.00503-13

29. Amachawadi RG, Scott HM, Aperce C, Vinasco J, Drouillard JS, Nagaraja TG. Effects of in-feed copper and tylosin supplementations on copper and antimicrobial resistance in faecal enterococci of feedlot cattle. *J Appl Microbiol*. (2015) 118:1287–97. doi: 10.1111/jam.12790

30. Beukers AG, Zaheer R, Cook SR, Stanford K, Chaves AV, Ward MP, et al. Effect of in-feed administration and withdrawal of tylosin phosphate on antibiotic resistance in enterococci isolated from feedlot steers. *Front Microbiol*. (2015) 6:483. doi: 10.3389/fmicb.2015.00483

31. Chan PA, Wakeman SE, Angelone A, Mermel LA. Investigation of multi-drug resistant microbes in retail meats. *Journal of Food Agriculture & Environment*. (2008) 6:71–5.

32. Davedow T, Narvaez-Bravo C, Zaheer R, Sanderson H, Rodas-Gonzalez A, Klima C, et al. Investigation of a reduction in tylosin on the prevalence of liver abscesses and antimicrobial resistance in enterococci in feedlot cattle. *Front vet sci*. (2020) 7:90. doi: 10.3389/fvets.2020.00090

33. Edrington TS, Bischoff KM, Loneragan GH, Nisbet DJ. Evaluation of feeding distiller's grains, containing virginiamycin, on antimicrobial susceptibilities in fecal isolates of enterococcus and Escherichia coli and prevalence of resistance genes in cattle. *J Anim Sci*. (2014) 92:1144–9. doi: 10.2527/jas.2013-6954

34. Halleran JL, Callahan BJ, Jacob ME, Sylvester HJ, Prange T, Papich MG, et al. Effects of danofloxacin dosing regimen on gastrointestinal pharmacokinetics and fecal microbiome in steers. *Sci Rep*. (2021) 11:11249. doi: 10.1038/s41598-021-90647-z

35. Halleran JL, Minch R, Slyvester HJ, Jacob ME, Prange T, Baynes R, et al. Comparison of the intestinal pharmacokinetics of two different florfenicol dosing regimens and its impact on the prevalence and phenotypic resistance of E. coli and enterococcus over time. *Microorganisms*. (2021) 9:1835. doi: 10.3390/microorganisms9091835

36. Hershberger E, Oprea SF, Donabedian SM, Perri M, Bozigar P, Bartlett P, et al. Epidemiology of antimicrobial resistance in Enterococci of animal origin. *J Antimicrob Chemother*. (2005) 55:127–30. doi: 10.1093/jac/dkh508

37. Innes GK, Nachman KE, Abraham AG, Casey JA, Patton AN, Price LB, et al. Contamination of retail meat samples with multidrug-resistant organisms in relation to organic and conventional production and processing: a cross-sectional analysis of data from the United States National Antimicrobial Resistance Monitoring System, 2012–2017. *Environ Health Perspect*. (2021) 129:057004. doi: 10.1289/EHP7327

38. Jacob ME, Fox JT, Narayanan SK, Drouillard JS, Renter DG, Nagaraja TG. Effects of feeding wet corn distillers grains with solubles with or without monensin and tylosin on the prevalence and antimicrobial susceptibilities of fecal foodborne pathogenic and commensal bacteria in feedlot cattle. *J Anim Sci*. (2008) 86:1182–90. doi: 10.2527/jas.2007-0091

39. Jacob ME, Fox JT, Nagaraja TG, Drouillard JS, Amachawadi RG, Narayanan SK. Effects of feeding elevated concentrations of copper and zinc on the antimicrobial susceptibilities of fecal bacteria in feedlot cattle. *Foodborne Pathog Dis*. (2010) 7:643–8. doi: 10.1089/fpd.2009.0401

40. LeJeune JT, Christie NP. Microbiological quality of ground beef from conventionally-reared cattle and "raised without antibiotics" label claims. *J Food Prot*. (2004) 67:1433–7. doi: 10.4315/0362-028X-67.7.1433

41. Muller HC, Van Bibber-Krueger CL, Ogunrinu OJ, Amachawadi RG, Scott HM, Drouillard JS. Effects of intermittent feeding of tylosin phosphate during the finishing period on feedlot performance, carcass characteristics, antimicrobial resistance, and incidence and severity of liver abscesses in steers. *J Anim Sci*. (2018) 96:2877–85. doi: 10.1093/jas/sky166

42. Murray SA, Holbert AC, Norman KN, Lawhon SD, Sawyer JE, Scott HM. Macrolide-susceptible probiotic *Enterococcus faecium* ST296 exhibits faecal-environmental-oral microbial community cycling among beef cattle in feedlots. *Lett Appl Microbiol*. (2020) 70:274–81. doi: 10.1111/lam.13269

43. Murray SA, Amachawadi RG, Norman KN, Lawhon SD, Nagaraja TG, Drouillard JS, et al. Effects of zinc and menthol-based diets on co-selection of antibiotic resistance among *E. coli* and *Enterococcus* spp. in beef cattle. *Animals*. (2021) 11:259. doi: 10.3390/ani11020259

44. Murray SA, Holbert AC, Norman KN, Lawhon SD, Sawyer JE, Scott HM. Effects of tylosin, a direct-fed microbial and feedlot pen environment on phenotypic resistance among enterococci isolated from beef cattle feces. *Antibiotics (Basel)*. (2022) 11:106. doi: 10.3390/antibiotics11010106

45. Platt TM, Loneragan GH, Scott HM, Norby B, Thomson DU, Brown MS, et al. Antimicrobial susceptibility of enteric bacteria recovered from feedlot cattle administered chlortetracycline in feed. *Am J Vet Res*. (2008) 69:988–96. doi: 10.2460/ajvr.69.8.988

46. Schmidt JW, Vikram A, Miller E, Jones SA, Arthur TM. In-feed tylosin phosphate administration to feedlot cattle minimally affects antimicrobial resistance. *J Food Prot*. (2020) c48:350–64. doi: 10.4315/0362-028X.JFP-19-342

47. Schmidt JW, Vikram A, Doster E, Thomas K, Weinroth MD, Parker J, et al. Antimicrobial resistance in U.S. retail ground beef with and without label claims regarding antibiotic use. *J Food Prot*. (2021) 84:827–42. doi: 10.4315/JFP-20-376

48. Shen Y, Davedow T, Tao R, Saleem AM, Yoon I, Narvaez C, et al. Ruminally protected and unprotected Saccharomyces cerevisiae fermentation products as alternatives to antibiotics in finishing beef steers. *J Anim Sci*. (2019) 97:4323–33. doi: 10.1093/jas/skz270

49. Vikram A, Rovira P, Agga GE, Arthur TM, Bosilevac JM, Wheeler TL, et al. Impact of "raised without antibiotics" beef cattle production practices on occurrences of antimicrobial resistance. *Appl Environ Microbiol*. (2017) 83:e01682–17. doi: 10.1128/AEM.01682-17

50. Vikram A, Miller E, Arthur TM, Bosilevac JM, Wheeler TL, Schmidt JW. Similar levels of antimicrobial resistance in U.S. food service ground beef products with and without a "raised without antibiotics" claim. *J Food Prot*. (2018) 81:2007–18. doi: 10.4315/0362-028X.JFP-18-299

51. Zaheer R, Cook SR, Klima CL, Stanford K, Alexander T, Topp E, et al. Effect of subtherapeutic vs. therapeutic administration of macrolides on antimicrobial resistance in Mannheimia haemolytica and enterococci isolated from beef cattle. *Front Microbiol*. (2013) 4:133. doi: 10.3389/fmicb.2013.00133

52. Zhang J, Wall SK, Xu L, Ebner PD. Contamination rates and antimicrobial resistance in bacteria isolated from "grass-fed" labeled beef products. *Foodborne Pathog Dis*. (2010) 7:1331–6. doi: 10.1089/fpd.2010.0562

53. McHugh ML. Interrater reliability: the kappa statistic. *Biochem Med (Zagreb)*. (2012) 22:276–82. doi: 10.11613/BM.2012.031

54. National Antimicrobial Resistance Monitoring System. The National Antimicrobial Resistance Monitoring System Manual of laboratory methods [internet]. (2021). Report No.: 4th Edition. Available at: https://www.fda.gov/media/101423/download

55. Miller WR, Munita JM, Arias CA. Mechanisms of antibiotic resistance in enterococci. *Expert Rev Anti-Infect Ther*. (2014) 12:1221–36. doi: 10.1586/14787210.2014.956092

56. Hollenbeck BL, Rice LB. Intrinsic and acquired resistance mechanisms in enterococcus. *Virulence*. (2012) 3:421–569. doi: 10.4161/viru.21282

57. Holman DB, Klima CL, Gzyl KE, Zaheer R, Service C, Jones TH, et al. Antimicrobial resistance in enterococcus Spp. isolated from a beef processing plant and retail ground beef. *Microbiol Spectr*. (2021) 9:e01980–21. doi: 10.1128/Spectrum.01980-21

58. Aslam M, Diarra MS, Service C, Rempel H. Characterization of antimicrobial resistance in enterococcus spp. recovered from a commercial beef processing plant. *Foodborne Pathog Dis*. (2010) 7:235–41. doi: 10.1089/fpd.2009.0380

59. Tyson GH, Nyirabahizi E, Crarey E, Kabera C, Lam C, Rice-Trujillo C, et al. Prevalence and antimicrobial resistance of enterococci isolated from retail meats in the United States, 2002 to 2014. *Appl Environ Microbiol*. (2018) 84:e01902–17. doi: 10.1128/AEM.01902-17

60. Hammerum AM. Enterococci of animal origin and their significance for public health. *Clin Microbiol Infect*. (2012) 18:619–25. doi: 10.1111/j.1469-0691.2012.03829.x

61. Tan CK, Lai CC, Wang JY, Lin SH, Liao CH, Huang YT, et al. Bacteremia caused by non-faecalis and non-faecium enterococcus species at a medical center in Taiwan, 2000 to 2008. *J Infect*. (2010) 61:34–43. doi: 10.1016/j.jinf.2010.04.007

62. Amachawadi RG, Shelton NW, Shi X, Vinasco J, Dritz SS, Tokach MD, et al. Selection of fecal enterococci exhibiting tcrB-mediated copper resistance in pigs fed diets supplemented with copper. *Appl Environ Microbiol*. (2011) 77:5597–603. doi: 10.1128/AEM.00364-11

63. Hasman H, Kempf I, Chidaine B, Cariolet R, Ersbøll AK, Houe H, et al. Copper resistance in enterococcus faecium, mediated by the tcrB gene, is selected by supplementation of pig feed with copper sulfate. *Appl Environ Microbiol*. (2006) 72:5784–9. doi: 10.1128/AEM.02979-05

64. Moher D, Hopewell S, Schulz KF, Montori V, Gøtzsche PC, Devereaux PJ, et al. CONSORT 2010 explanation and elaboration: updated guidelines for reporting parallel group randomised trials. *BMJ*. (2010) 340:c869. doi: 10.1136/bmj.c869

65. von Elm E, Altman DG, Egger M, Pocock SJ, Gøtzsche PC, Vandenbroucke JP. The strengthening the reporting of observational studies in epidemiology (STROBE) statement: guidelines for reporting observational studies. *J Clin Epidemiol*. (2008) 61:344–9. doi: 10.1016/j.jclinepi.2007.11.008

66. Rogers LA, Strong K, Cork SC, McAllister TA, Liljebjelke K, Zaheer R, et al. The role of whole genome sequencing in the surveillance of antimicrobial resistant *Enterococcus* spp.: a scoping review. *Front Public Health*. (2021) 9:599285. doi: 10.3389/fpubh.2021.599285

67. Ellington MJ, Ekelund O, Aarestrup FM, Canton R, Doumith M, Giske C, et al. The role of whole genome sequencing in antimicrobial susceptibility testing of bacteria: report from the EUCAST subcommittee. *Clin Microbiol Infect*. (2017) 23:2–22. doi: 10.1016/j.cmi.2016.11.012

68. Tang KL, Caffrey NP, Nóbrega DB, Cork SC, Ronksley PE, Barkema HW, et al. Restricting the use of antibiotics in food-producing animals and its associations with antibiotic resistance in food-producing animals and human beings: a systematic review and meta-analysis. *Lancet Planetary Health*. (2017) 1:e316–27. doi: 10.1016/S2542-5196(17)30141-9

69. Jans C, Sarno E, Collineau L, Meile L, Stark KDC, Stephan R. Consumer exposure to antimicrobial resistant bacteria from food at Swiss retail level. *Front Microbiol*. (2018) 9:362. doi: 10.3389/fmicb.2018.00362

frontiers | Frontiers in Veterinary Science

TYPE Original Research
PUBLISHED 25 April 2023
DOI 10.3389/fvets.2023.1155811

OPEN ACCESS

EDITED BY
João Pedro Rueda Furlan,
University of São Paulo, Brazil

REVIEWED BY
Clair L. Firth,
University of Veterinary Medicine Vienna, Austria
Lucy Brunton,
Royal Veterinary College (RVC), United Kingdom

*CORRESPONDENCE
Lis Alban
✉ lia@lf.dk

RECEIVED 31 January 2023
ACCEPTED 05 April 2023
PUBLISHED 25 April 2023

CITATION
Moura P, Sandberg M, Høg BB, Niza-Ribeiro J, Nielsen EO and Alban L (2023) Characterisation of antimicrobial usage in Danish pigs in 2020. *Front. Vet. Sci.* 10:1155811. doi: 10.3389/fvets.2023.1155811

Characterisation of antimicrobial usage in Danish pigs in 2020

Pedro Moura[1], Marianne Sandberg[1], Birgitte Borck Høg[1], João Niza-Ribeiro[2,3], Elisabeth Okholm Nielsen[4] and Lis Alban[5,6]*

[1]National Food Institute, Technical University of Denmark, Lyngby, Denmark, [2]Departamento de Estudo de Populações, ICBAS, Instituto de Ciências Biomédicas Abel Salazar, Universidade do Porto, Porto, Portugal, [3]Epidemiology Research Unit (EPIUnit), Instituto de Saúde Pública da Universidade do Porto, Porto, Portugal, [4]SEGES Innovation, Copenhagen, Denmark, [5]Department for Food Safety, Veterinary Issues and Risk Analysis, Danish Agriculture and Food Council, Copenhagen, Denmark, [6]Department of Veterinary and Animal Sciences, University of Copenhagen, Frederiksberg, Denmark

Introduction: Denmark is one of the world's largest exporters of pigs and pig meat, so the sector plays an important role in the national antimicrobial use (AMU). The Danish government has run antimicrobial stewardship programs in collaboration with the pig industry for more than 25years. These have resulted in substantial overall reductions in total AMU and limiting the use of fluoroquinolones, the 3rd and 4th generation cephalosporines and the polymyxin colistin. To understand where further reductions in AMU could take place, it is necessary to investigate which antimicrobials are being used, how, and for which reasons.

Materials and methods: We characterized the AMU in the Danish pig sector in 2020, providing new analytical insights based on data retrieved from the VetStat database. The AMU data were segmented into classes, routes of administration, treatment indications and age groups, and interpreted as an outcome of the interventions taken. We evaluated the current AMU regarding choice of antimicrobial class. Moreover, we discussed how to further improve the antimicrobial stewardship in Danish pig production to achieve additional reductions without jeopardizing animal welfare. Where relevant, two pig veterinary specialists were consulted.

Results: In 2020, 43.3mg antimicrobials per population correction unit (PCU) were ascribed to the Danish pig sector. There was practically no use of fluoroquinolones, 3rd and 4th generation cephalosporins and polymyxins. Weaners related to 45% of the total AMU in pigs when measured in tonnes and 81% when measured in defined animal daily doses, of these 76% were ascribed to gastrointestinal indications and overall, 83% were administered perorally.

Conclusion: To enable further reductions in AMU, it should be investigated how and when to replace group treatments (e.g., all animals in section or a pen) with individual treatments. Moreover, prevention of disease and promotion of animal health should be prioritized, e.g., through focus on feed, vaccination, biosecurity, and disease eradication.

KEYWORDS

antimicrobials, surveillance, consumption, usage, stewardship, Denmark, pigs, one-health

1. Introduction

In modern medicine, antimicrobials (AM) constitute fundamental instruments for the control of bacterial infectious diseases. However, when AM are used, a natural evolutionary selection is triggered, selecting the most well-adapted bacteria that can obtain, express, and propagate genes more fitting to survival than other bacteria (1). Therefore, the use of AM should be prudent, especially of those that have been defined as critically important by the European Medicines Agency (EMA) (2), so the last line of defence against infections is maintained.

The World Health Organization (WHO) has recognized the fight against AM resistance (AMR) as one of the most important challenges that humanity will have to face in the present decade (3). Given that AMR genes can circulate in any direction within and between a global and complex system composed of the environment, humans and animals, a coordinated One Health (OH) approach is crucial to comprehend the problem (4). Prudent use of AM will ensure that humans and animals in need of AM treatment can be treated not just now but also in the future (5). However, animal welfare may be challenged, if animals with severe infections are not treated with AM. To take both these concerns into account, the Danish pig industry has developed the approach called "As little as possible, but as much as necessary" (6), where improving animal health and, consequently, reducing the need for AMU are central to antimicrobial stewardship.

Antimicrobial stewardship can be defined as "A coherent set of actions which promotes using antimicrobials responsibly" (7), with the primary goal being "to optimize clinical outcomes while minimizing unintended consequences of antimicrobial use" (8). Monitoring both AMU and the development of AMR allows the interpretation of patterns and trends of AMU, which can be related to the emergence of AMR, enabling risk evaluation and management, and therefore constituting the basis of AM stewardship programs (9). AMU data can, within a given time frame, also be used to evaluate the efficacy of control measures implemented, and when the same indicators are applied establish international comparisons (10).

AM stewardship programs across Europe have received international recognition. Examples of good practices can be made out of the Danish pig sector (11), along with the Dutch model where a combination of mandatory and voluntary actions (12) have resulted in a shift from 3rd and 2nd to 1st choice AM compounds in the dairy sector (13). Likewise, the multisectoral voluntary approach implemented in the United Kingdom has resulted in a 50% reduction in overall AMU in the livestock industry from 2014 and 2021, including a 79% reduction in the use of highest-priority critically important antibiotics during the same period (14).

To elucidate whether the AMU and AMR monitoring systems in place and actions taken to combat AMR are effective, it is necessary to evaluate them at regular intervals. Several tools have been developed to help in this (15). In an international network called Convergence in evaluation frameworks for integrated surveillance of AMU and AMR (CoEvalAMR), guidelines have been developed for evaluation along with assessment of different evaluation tools. One of these tools is the Integrated Surveillance System Evaluation (ISSE), which is a conceptual framework for evaluation of the performance and the value of OH integration in surveillance systems for AMU and AMR. According to ISSE, evaluations can be done at different levels such as production of information and expertise, generation of actionable knowledge, influence on decision-making and contributions to desirable outcomes. All this will enable an evaluation of the impact of the decisions made (16).

Denmark is a "pig country." In 2020, there were 2,921 active and professional farms with pigs registered; the sector produced 32.6 million pigs, with 17.5 million of these being slaughtered in the country, and 14.8 million were exported as weaners at 30kg of weight (17) as seen in Figure 1, in addition 0,3 million finishers and sows were exported for slaughter. Monitoring of AMU is at the age groups (1) sows and piglets and (2) weaners, and (3) finishers (18). In the Danish pig sector, AMR trends are monitored by indicator *Escherichia coli* isolates obtained from arbitrarily selected caecal samples collected at slaughter, and from fresh, chilled meat collected at retail points, tested in accordance with EU requirements (19). Denmark has been implementing AM stewardship measures for over 25 years as shown in the following:

A national ban on the use of AM as growth promoters came into force in finishers in 1998 and in weaners in 1999, whereas this came into force in the European Union (EU) in 2006 (20), Figure 1. Veterinary advisory service contracts are required for the large pig herds, i.e., with more than 300 sows (21), and there is a limitation of veterinarians' profits from AM sales (11). Moreover, direct marketing of prescription-only drugs and vaccines to layman is prohibited (22). Since 2001, AMU is reported into the VetStat database, which has been the basis for implementing sector interventions and measuring their impact. Pharmacies are obliged to report the amount of AM that the veterinarians prescribe and specify the target age group and treatment indication. Feed mills similarly report AM-medicated feed sales at farm level, while veterinarians directly report the amounts of AM they prescribe and use in clinical practice (23).

The Danish Integrated Antimicrobial Resistance Monitoring and Research Program (DANMAP) provides a complete and transparent picture of AMU and the occurrence of AMR in bacteria from food animals, food and humans in Denmark. For that reason, DANMAP serves as the basis for implementing evidence-based policies (24), e.g., since 2002, the prescription of fluoroquinolones is antibiogram-dependent (11), and in 2010 a voluntary industry ban on the use of 3rd and 4th generation cephalosporins was introduced in Danish pig production (11).

The Yellow Card initiative, which was established in 2010, sets limits to the acceptable AMU at the individual herd level. Benchmarking figures are defined for the different animal age group, based on the average AMU over the last 9 months, calculated as defined animal daily dosages (DADD) per 100 animals per day. These figures for the individual herd are then compared with national permit limits (20). Originally, these permit limits were defined as twice the national average within the age group. These have been lowered over time. The current thresholds in DADDs are 3.2 for sows and piglets, 4.4 for finishers and 17.2 for weaners (25). Some critically important AMs are weighted by a factor above 1 to reflect the AM's perceived negative impact on AMR development, and this increases the registered DADD value: i.e. 3rd and 4th generation cephalosporins, colistin and fluoroquinolones have a weighting factor of 10, whereas tetracyclines have a weighting factor of 1.5, while unrestricted AMs, such as penicillins have a weight factor of 1 (20).

To understand the current AMU in the Danish pig sector, it is important to provide historical context to the figures regarding when the different risk mitigating measures were implemented and how

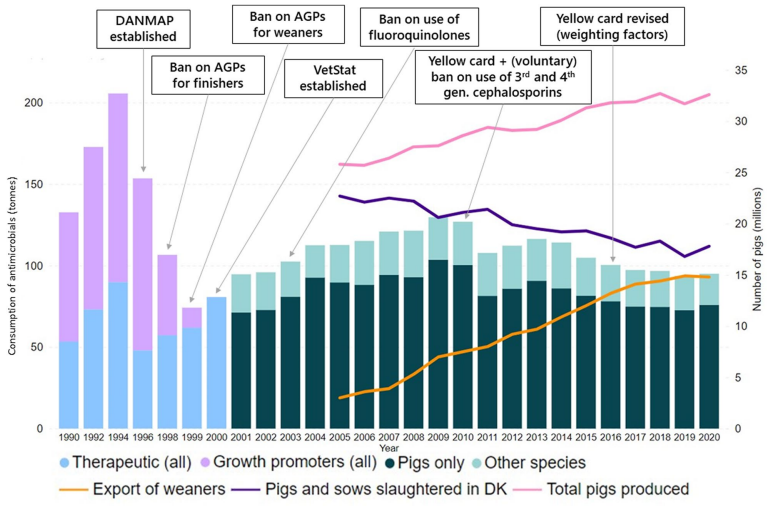


FIGURE 1
Total amount of AMU in Danish livestock from 1990 to 2000 and in the pig sector from 2001, total number of pigs produced in Denmark since 2005, divided into weaners exported and pigs and sows slaughtered domestically, and risk mitigating initiatives. AGP, Antimicrobial growth promoters; DK, Denmark; DANMAP, Danish Integrated Antimicrobial Resistance Monitoring and Research Program.

the sector developed. As can be seen in Figure 1, the overall AMU in tonnes was reduced by 52% from 1994 when consumption reached its maximum to 2020, despite concurrent growth of the pig sector. AMU per species only became available after the establishment of VetStat.

To combine an optimal clinical effect with the lowest possible adverse impact from the development of AMR, it is crucial to select the appropriate AM. The European Medicines Agency's EMA's Antimicrobial Advice *Ad Hoc* Expert Group (AMEG) classification is based on considering the probability and consequences associated with the use of a specific AM regarding the development of AMR, as well as its importance in human medicine, while considering the existence of alternative substances. In this classification: Category A "Avoid" consists of AM that are not licensed for use in animals; Category B substances include 3rd and 4th generation cephalosporins, quinolones and polymyxins, which should be of "Restricted" use in veterinary medicine, as these are critically important substances in human medicine; Category C "Caution" includes AMs for which there are reliable alternatives in human medicine, but few veterinary alternatives; Category D "Prudence" covers AM for use as a first line of treatment, in a prudent way whenever possible (Supplementary Table S1) (2).

In Denmark, prescription guidelines have been released by the Danish Veterinary and Food Administration (DVFA), classifying certain AM substances as first choice (Group 1), as alternatives (Group 2) or of restricted use (Group 3) (26), Supplementary Table S1. As part of that work, risk assessments have been undertaken for selected AMs in pigs, i.e., macrolides (27) and pleuromutilin (28). These guidelines are progressively updated according to new knowledge about AMR development. Preventing the occurrence of disease by investing in use of vaccination is also an important health promoting initiative, which ultimately comes down to each farmer's decision (29). As can be seen, in Supplementary Figure S1, vaccination sales for some of the most common pathogens have gone up in recent years.

The overarching question is: What is the current state in Denmark with respect to AMU after more than 25 years of interventions to combat AMR development?

The detailed objectives of this paper are to:

- Characterize Danish AMU in the three age groups: weaners; finishers; sows and piglets in 2020.
- Based upon the figures produced, discuss the current AMU in Danish pig production with respect to risk of development of AMR, where AMU is seen as a driver for AMR. Discuss whether it would be feasible to further improve the antimicrobial stewardship through reduction of AMU by moving usage from oral to parenteral treatment or alter use patterns, without jeopardizing animal welfare.

2. Materials and methods

2.1. Data

The data regarding AMU in the Danish pig sector in 2020 originated from Vetstat and consisted of the data used in 2020 DANMAP report. The same data, which also encompass the national antimicrobial sales figures, are reported annually to the European Surveillance of Veterinary Antimicrobial Consumption (ESVAC) report. This report contains the total sales of antimicrobial agents for veterinary use in livestock production from 31 European countries (30). ESVAC figures are published using a statistic called mg per population correction unit (PCU), hence the use in milligrams (mg) of active substance is normalized by a standardized estimate of the national animal biomass (30).

To calculate this statistic specifically for the Danish pig sector, the figure describing national AMU sales was taken from the 2020 DANMAP Report: 7.59×10^{10} mg of AM active substance sold (20).

For PCU, a figure of 1.754×10^9 kg, calculated according to EMA's directives and originating from the European database of sales of veterinary antimicrobial agents was used (31).

AMU measured in defined animal daily dosages (DADD) per licensed medicinal product had previously been calculated by the DANMAP team and these figures were made available for this analysis. DADD is the average maintenance dose per day for the main indication of a drug in the appropriate animal species. It is calculated using the following formula:

$$DADD = \frac{mg\ of\ active\ substance}{DANMAP\ dosage\ per\ kg\ of\ body\ weight \times standard\ weight\ of\ animal\ age\ group} \quad \text{(Formula 1)}$$

In this formula, standardized weight categories for animal age groups are used, implying weaners: 15 kg, finishers: 50 kg and sows and piglets: 200 kg. In the last age group, the weight of the piglets is embedded in the weight of the sows (32). DADD usage constants are not defined per product, but for each AM agent, administration route, and animal species as mg active compound per kg live animal. These values are related to the standardized use as defined in DANMAP and can vary from the actual prescribed daily dose or from the recommended dosage in the summary of the product characteristics (SPC) or from the values used to calculate VetStat ADD's (20).

To set the overall AMU into perspective, the proportion, in thousands, of the population under treatment per day was also calculated per each of the animal age groups using the DADD per 1,000 animals per day (DAPD) indicator. As an example of the application of this indicator, 20 DAPDs stands for 2% of the population being treated with AM, on average, on any given day in 2020. It was calculated by dividing the total estimated number of kg doses (DADDs) used per year by the estimated live biomass in the age group (in tonnes, cumulated over 365 days) using the following formula:

$$DAPD = \frac{\sum number\ of\ kg\ doses\ (DADDs)}{estimated\ live\ biomass} \quad \text{(Formula 2)}$$

where the estimated live biomass figures given per animal age group, in million tonnes, represent the number of standard animals with an estimated average weight on any given day in Denmark in 2020. For 2020, these were: sows and piglets: 9 million tonnes, weaners: 33 million tonnes, and finishers: 107 million tonnes. These figures were taken from the DANMAP database and were based on the animal census from Statistics Denmark and from the export records curated by the Danish Agriculture & Food Council.

2.2. Evaluation of AMU

In line with the definition set up by Aenishaenslin et al. (16), the current state of AMU was seen as an outcome of the actions to combat AMR development taken so far in Denmark, implying the last 25 years. We chose this approach because we see AMU as a driver for AMR. We did not intend to assess the impact of the individual elements of the complete Danish OH stewardship program (DANMAP). To do this, detailed investigations should be undertaken, where inspiration for these can be found in Aenishaenslin et al. (2021) (16). The AMU in Danish pig production was evaluated with respect to the risk of AMR assessing the following:

2.2.1. Total AMU in the sector

The AMU for each of the three pig age groups segmented into classes, routes of administration, and treatment indications was calculated in tonnes as well as in DADD, as absolute values and as proportions of the total AMU in pigs, together with the proportion of pigs estimated as being treated on any given day in 2020. National and sectorial AMU figures, based on sales data, were also calculated in mg/PCU and interpreted in the context of the ESVAC figures for the year 2020 and compared with data from other selected countries.

2.2.2. Risk related to national choice of am class in comparison with EMA recommendations

The use of the different antimicrobial AM classes, segmented according to EMA's AMEG classification was summarized and the results interpreted according to the current risk management recommendations issued by the Danish Veterinary and Food Administration (DVFA).

2.2.3. Feasibility of improving the Danish pig sector's antimicrobial stewardship

As suggested by (33) the approach to further reduce AMR caused by AMU in Danish pig production could focus on the selection of the administration route, choice of antibiotics, as well as management improvements to lower the incidence of diseases requiring antibiotic treatment.

A visual depiction of the AMU in the sector was made, dividing the AMU by indication for treatment and administration route for each of the three age groups. This enabled us to discuss the feasibility of moving some of the AMU from oral to parenteral treatment to make further reductions and hereby improving the antimicrobial stewardship further.

Estimating the prevalence of disease in the pig sector and connecting it to the consumption of AM with precision is very difficult, hence, past works by (34) and (35) were used as to discuss this. Moreover, to get further insights and updates, we consulted two external pig veterinarians each with more than 25 years of clinical experience and associated with different major Danish veterinary advisory company.

The data analysis was performed, and the graphical outputs were produced using Microsoft Power BI® Version: 2.102.845.0.

3. Results and discussion

3.1. Total AMU in the sector

Segmenting AMU into the three different pig age groups clarified that the three age groups have different relevance for the total AMU (Table 1). Weaners registered the highest use, both in tonnes (34.4) and in DADD (201.5 million). Sows and piglets registered the second highest consumption in tonnes (22.7) but third when calculated as DADD (9.2 million). Finishers were associated with the second highest consumption when AMU was measured in DADD (37.9 million) and the third when measured in tonnes (18.8). This difference can be explained by the DADD formula, where a treatment is

TABLE 1 Distribution of antimicrobial (AM) treatments, in tonnes and DADD units as well as the proportion of the population under treatment per day in the Danish pig sector in 2020, per pig age group category.

	Pig age group			
	Sows and piglets	Finishers	Weaners	Total
Total use				
Measured in tonnes	22.7	18.8	34.4	75.9
Proportion of total AMU (tonnes)	29.9%	24.8%	45.3%	100.0%
Measured in million DADD	9.2	37.9	201.5	248.6
Proportion of total AMU (DADD)	3.7%	15.2%	81.1%	100.0%
Measured in DADD per 1,000 animals per day (DAPD)	19 (1.9%)	18 (1.8%)	92 (9.2%)	

attributed to 50 kg individuals in the case of finishers and to 200 kg individuals in the case of sows, as described in Formula 1. By examining the consumption in DAPD units, given that this indicator is a proportion per 1,000 individuals, on average and on any given day in 2020, 1.9% of the sows were being treated with AM, as were 1.8% of the finishers and 9.2% of the weaners.

According to the 2020 ESVAC report, a 43.2% reduction was observed in the 25 countries which provided AM sales data form 2011 to 2020 (30). Even though the figures reported in ESVAC correspond to the yearly overall sales in food producing animals, given that pig production plays a major role in several European countries, it is safe to assume that the pig sector is responsible for some of these reductions (36). In the same report, Denmark's livestock industry as a whole registered a total AMU of 37.2 mg/PCU, while the median of the 31 reporting countries was 51.9 mg/PCU (30). Our calculations show that the Danish pig sector registered an AMU of 43.3 mg/PCU. Hence, a higher value than the one registered when evaluating the consumption by the entire Danish livestock industry. The higher use in pigs is in line with a global trend, where AMU measured in mg/PCU is higher in pigs than the in other predominant livestock species which are cattle and chicken (37).

Direct international AMU comparisons based on sales data need to be interpreted carefully, as they can lead to misinterpretations (38), especially because statistics to measure AMU and denominators to estimate the animal biomass are not harmonized and different methods of data collection are applied (39). National AMU figures should also be interpreted in the context of the country's production objectives, as these will shape the sector, by influencing the size and specialisation of the farms (40). As an example, the Danish pig sector has evolved to export weaners, which are raised in other countries, as seen in Figure 1. This means that almost half of the pigs are exported after going through the critical post weaning stage in Denmark (17). As seen in Table 1, weaners are the most treated age group, so the specialisation of the sector and consequent large proportion of very young individuals, naturally creates pressure in the overall consumption of the entire Danish sector, when measured in mg/PCU. If the exported weaners would have reached slaughter weight in Denmark, the overall AMU of the country in mg/PCU would likely have been lower than observed.

In an intensive pig production context, Denmark is usually regarded as an international example of good AMU practice (11). In a previous work by (41), the number of treatments per animal per 100 days (TI100) was estimated in a sample of heavy fattening farms, where finishers are slaughtered at 160 kg or more and destined for Parma ham production. Acknowledging the limitations of a direct comparison, the authors still highlighted a five-times higher use in Italy compared to Denmark (41). Good practices of the Danish pig sector regarding AM prescription were also underlined by Carmo et al., in a study comparing Danish and Swiss prescription patterns (42).

In conclusion, the current AMU per pig in Denmark may be considered low for finishers and for sows, given that only a small percentage of individuals is being treated, on average, on any given day during the year. Due to the specialisation of the sector, the focus for optimisation should be on the weaners, as they are responsible for the largest part of the national consumption.

3.2. Risk related to national choice of am class in comparison with EMA recommendations

In 2020, there was no recorded use of category A "Avoid" compounds (25). This result was expected, given that the use of these compounds is illegal in farm animals (2). In addition, no residues of these substances were found by the Danish pig meat residue monitoring program (43). Moreover, only 90 g of category B "Restrict" AM were ascribed to the entire Danish pig sector, a figure too low to be actionable in DADD units. According to the last ESVAC report, Denmark's livestock industry has reported the use of less than 0.01 mg/PCU each for fluoroquinolones, 3rd and 4th generation cephalosporins and polymyxins, while the median value registered by ESVAC for each of these groups was 1.1 mg/PCU, 0.2 mg/PCU and 0.8 mg/PCU, respectively (30). However, it should be emphasized that given their low defined dosage per animal kg, the use of critically important AM to human medicine tends to appear lower, when expressed in mass-based units of measure, such as the mg/PCU, when compared to dose-based units, such as the DADD (44).

Overall, there is a higher consumption of AM category C "Caution" than that of category D" Prudence" compounds in Danish pig production, as can be seen in Figure 2. The high use of category C "Caution" AMs covers macrolides (81 million DADDs) and pleuromutilins (34 million DADDs). In contrast to the AMEG classification, these AMs are 1st choice AM according to the DVFA. EMA is also more restrictive in its classification of aminoglycosides (neomycin and streptomycin) and lincosamides (lincomycin) than the DVFA (2, 26).

Regarding category D "Prudence" AMs, tetracyclines are placed in the most acceptable category by EMA but are considered a 2nd choice by DVFA and therefore associated with a weighting multiplier of 1.5 (instead of 1) in the Danish Yellow Card initiative. The decision to attribute this weighting factor is connected with the perceived role of the pig production in the emergence of livestock-associated methicillin-resistant *Staphylococcus aureus* (11, 45). This weighting likely discourages pig producers from using tetracyclines as they will more easily reach the Yellow Card limits than they would by using a non-weighted AM.

Evaluating the effect of AM exposure in the development of AMR genes is complex and each individual gene has its own dynamics in terms of emergence and dispersion (46), also studies of a representative size are required to acquire enough strength to make conclusions. Still, AMU reductions are expected to have a positive effect in reducing AMR in finisher pig gut microbiome, providing that the affected AM class is not replaced by another one (47). Risk mitigating initiatives such as the abolishment of growth promoters had a direct effect on the AMR levels detected in both pigs and broilers (48); the ban on using tylosine as a growth promoter resulted in a plummeting of the macrolide resistance in *Campylobacter* in pigs (49). Similarly, the voluntary industry ban on cephalosporin use in pig production had a significant impact in resistant *E. coli* detected at slaughter (50).

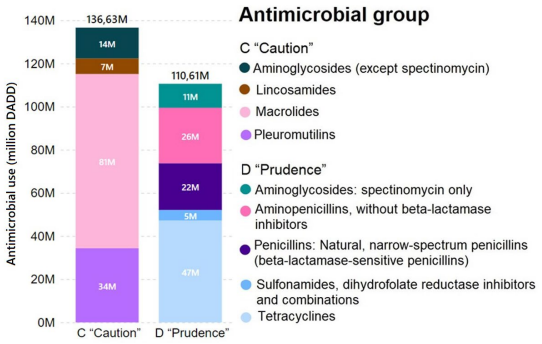


FIGURE 2
Total AM treatments in the Danish pig sector in 2020, measured as million DADD and divided according to AMEG classifications and AM class. The four AM classes of amphenicols, some penicillins (anti-staphylococcal penicillins, ß-lactamase-resistant penicillins), 3rd and 4th generation cephalosporins and polymyxins were excluded from the graph, as each class constituted less than 1% of the treatments.

In the EU, Denmark is among those countries that report the lowest occurrence of chloramphenicol and ciprofloxacin resistance, while the occurrence of ampicillin, azithromycin, sulphonamide, trimethoprim and tetracycline resistance is comparable to the average reported by all EU Member States. Moreover, over the last 6 years, the percentage of fully sensitive *E. coli* isolates collected from caecal samples has remained approximately constant, around 46% (20).

Andersen et al., 2023 (33) estimated the quantitative effect of AMU fluctuations in Danish pig farms on the abundance of AMR genes, demonstrating that an increase or decrease in AMU is expected to cause, respectively, and increase or decrease in the abundance of AMR genes, with the stronger effects being observed over longer periods of time. The causal association between the occurrence of antimicrobial resistant bacteria in both the animal and human populations is not always clear, as shown by (51). Also, only potential associations between AMU and the emergence of AMR in humans and food-producing animals have been demonstrated (52, 53). This is partially because not many studies of the same size, as (33) have been done. In summary, for Denmark, the fact that there is effectively no use of 3rd and 4th generation cephalosporins, colistin or fluoroquinolones likely lowers the risk related to AMR development related to these substances. Moreover, the Danish legislation has led to a decrease in the use of tetracyclines since 2016, but a rise in the use of aminoglycosides, macrolides and extended-spectrum penicillin (54) in line with the recommendations by the DVFA.

3.3. Feasibility of improving the Danish pig sector's antimicrobial stewardship

As can be seen in Table 2 and in Figure 3, the contributions of the three animal age groups to the total AMU of the pig sector differ

TABLE 2 AM treatments shown as % of DADD units used by the Danish pig sector in 2020, divided according to age category, treatment indication and administration route.

	Relative distribution (in %) of DADD according to animal age group			
	Sows and piglets	Finishers	Weaners	Total
Treatment indication				
Gastrointestinal	9%	62%	76%	71%
Locomotor + CNS + skin*	47%	31%	15%	19%
Reproduction, urogenital system	17%	0%	0%	1%
Respiratory disorders	17%	7%	9%	9%
Udder	10%	0%	0%	0%
Total	100%	100%	100%	100%
Administration route				
Parenteral	91%	34%	17%	23%
Peroral	9%	66%	83%	77%
Total	100%	100%	100%	100%

*: Treatment of joints, limbs, hooves, central nervous system and skin.

10.3389/fvets.2023.1155811

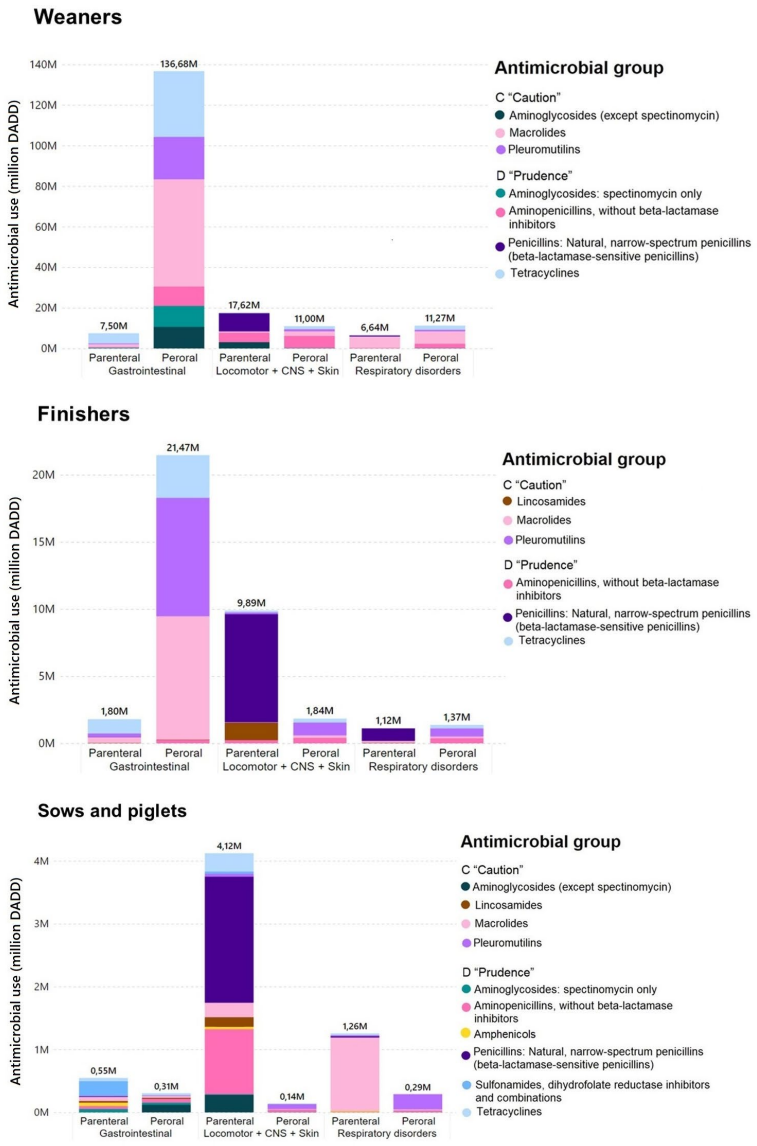


FIGURE 3
Distribution of AMU, in DADD units, in the Danish pig sector in 2020, per treatment indication, divided by AM classes, where each graph represents an age group. AM classes that constituted less than 5% of the treatments in all selected indications were excluded. Only the three most frequent treatment indications are shown. To facilitate the visualization of the antimicrobial classes consumed, 100% stacked column charts are provided in Supplementary Figure S2.

widely, as do the treatment indications as defined by VetStat, the AM classes used and the administration route. Overall, around 99% of the pig sector AMU can be connected to three indications, among the 11 treatment indications encompassed in VetStat: "Gastrointestinal"; "Locomotor + central nervous system (CNS) + skin," grouped together due to the low overall figures per each of the solo indications (55) and "Respiratory disorders." Therefore, our analysis focused on these three indications. A particular emphasis was given to peroral treatments, given their major proportion and perhaps less selective usage. In Denmark, metaphylactic treatment is allowed, as in other countries (56), however, only for herds with a veterinary advisory service contract and regular use of diagnostics to confirm the diagnosis and identify resistance patterns (57). Also, in past sector interventions, major AMU reductions were connected to declines in group treatments with oral medication (58).

According to the two external pig veterinarians consulted, the results presented are an accurate depiction of the Danish pig sector's routinely prescribed AM to treat the most frequent pathologies, overall speaking. Within the sows and piglets age group, most of the registered "gastrointestinal" and "respiratory" treatment indications can likely be attributed to use in piglets. Moreover, the treatment indications "locomotor + CNS + skin" and "reproduction, urogenital system and mammary gland" are mostly connected with treatment of sows.

To identify whether it would be possible to further reduce AMU in Danish pig production, the data were divided according to age group, indication, AM class and administration route. In the following,

the distribution of AMU into these subgroups is discussed in relation to the types of infection causing the symptoms observed. Moreover, the focus is on the administration route, which is believed to greatly impact the effectiveness of AM treatment, while having in mind that individual treatment will most likely lead to lower AMU than group treatment (47). However, it may not be feasible to treat a higher number of pigs individually, given the logistical challenges connected to do so (56).

Table 2 shows that gastrointestinal diseases in weaners and finishers account for most of the AMU., and that the AM treatment of these infections are primarily macrolides, pleuromutilins and tetracyclines, all administered perorally. This is probably because the infections are often caused by *Lawsonia intracellularis*, *Escherichia coli* or *Brachyspira pilosicoli* (34). Macrolides and pleuromutilins can be used to treat *Lawsonia intracellularis*, and tetracyclines can be effective against both *Lawsonia intracellularis* and *E. coli* (35). For gastrointestinal disease, peroral administration of AM is commonly the preferable administration route, as the AM will work immediately in the organ of interest. As an example, it has been assessed that to threat diarrhea related *Lawsonia intracellularis* with oxytetracycline, batch treatment with oral medication is more effective than individual parental treatment (59). According to the pig veterinary specialists consulted, parenteral use of AM for gastrointestinal indications is mostly prescribed for animals that are too weak to eat or drink. Individual treatment of gastrointestinal disease is not feasible in large pig herds, where hundreds of weaners may need treatment over a short period of time. Still, the focus should be in placing ill pigs that may not be able to drink in quarantine pens and ensure individual treatments, e.g., by using injectables.

The AMEG expert advisory group also stated that to minimize AMR, individual treatments given parenteral or oral, in this order, should be preferred to oral group medication *via* drinking water and feed, given that the individual treatments are thought to have a lower general effect on AMR selection (2).

As shown by Andersen et al., (2020) parenteral AMU appear to have a high effect on resistance genes for the specific AM classes used, whereas peroral AMU tended to have a lower effect on resistance genes but for a broader range of AM classes (47). Compared to parenteral AMU, the broader impact of peroral AMU can be due to their widespread irregular during the weaner and finisher rearing periods (60, 61). Given that in Denmark, most peroral AM treatments are commonly administered in the drinking water, and weaners are routinely sorted in pens by their weight, peroral use of AM could be considerably reduced if it is targeted to as few pens or sections as necessary. This is already in practice, where a double pipe drinking water system is installed.

To treat respiratory infections in the Danish pig sector, the most used AM classes are perorally administered macrolides in weaners; penicillins are given parenterally and pleuromutilins perorally to finishers, and macrolides are given parenterally to piglets. The most common respiratory pathogens in Danish pigs are, in order of frequency according to the veterinary pig specialists consulted: *Mycoplasma hyopneumoniae*, *Mycoplasma hyorhinis*, *Glaesserella parasuis* followed by *Pasteurella multocida*, *Streptococcus suis* and *Actinobacillus pleuropneumoniae*. The last three are usually susceptible to penicillin (35), whereas *M. hyopneumoniae* is most often treated using either parenteral or peroral macrolides or peroral tetracyclines (62). Moreover, *Bordetella bronchoseptica* and *M. hyorhinis* infections are treated with either parenteral or peroral macrolides, usually soon after weaning. Because of the low weight of piglets and the way DADDs are calculated (Formula 1), with sows and piglets being in the same group, relatively few DADDs can be used to treat several animals, considering the DANMAP dosage per kg of body weight. Peroral administration of AM for respiratory indications is mostly used for metaphylactic treatments.

For locomotor, CNS and skin indications, the most prescribed AM classes were parenterally administered penicillins and aminopenicillins, in all three age groups. Infections related to these indications are mostly due to *Streptococcus suis* and *Erysipelothrix rhusiopathiae*, which tend to be susceptible to ß-lactams, and *Mycoplasma hyosynoviae* that can be successfully treated with macrolides or tetracyclines (35, 63). Peroral administration of AM for locomotor, CNS and skin indications is most likely used for metaphylaxis.

In a cross-sectional study conducted in four European countries, 227 farrow-to-finish pig farms were comparing regarding AMU by age category, antimicrobial class and administration route (64). According to this study, Sweden stood out with a comparatively low AMU in weaners, and an overall predominance of parenteral treatments (87%). These figures, along with the Swedish livestock sector registering an AMU of 11.1 mg/PCU (30) in 2020, and a pig sector internationally praised for its actions on herd treatments (65), where individual treatments accounted for 80% of the AMU (66) suggest that Sweden can be mentioned as an example of a country that has managed to reduce AMU to a larger extent than many other countries. The Swedish legislation specifies that group treatment can only be prescribed on a case-by-case basis, after the implementation of a written and compulsory disease control program based upon a systematic analysis of the disease issue (67). Likewise, the national guidelines state that group treatment of post-weaning diarrhea should only be considered if more than 25% of the pigs in a litter are affected (50). However, Swedish pig herds are smaller than Danish pig herds, in general, which makes it easier to treat animals individually than in Denmark. The mean Swedish sow and fattening farms have 185 sows and 945 pigs, respectively (68); in contrast, the median Danish sow and fattening farms have around 500 to 599 sows and 2,000 to 2,999 pigs, respectively (17). Since 2014, in Denmark, group treatment prescriptions require laboratory diagnosis and the elaboration of an action plan aiming to reduce the need for group treatments. Furthermore, the prescription's effect on the herd health must be followed up in the farm's trimestral veterinary advisory report together with an evaluation conducted by the farm veterinarian justifying the need to continue the treatments, 3 months after the first assessment (69).

In conclusion, the extended use of the peroral AM administration route in Danish pigs is mostly related to treatment of gastrointestinal disease in weaners. This makes sense from the veterinary and economic points of view, due to feasibility when treating numerous animals simultaneously. Peroral AMU is higher than parenteral AMU for gastrointestinal indications for weaners and finishers. For all the other indications, most of the use is parenteral, implying individual treatment that should result in less AMU than in the case of peroral treatment.

The last 10 years' reductions and shifts achieved in the use of AMs of critical importance in European pig production suggest

that further reductions in AMU in the pig sector are possible (36). The question is which initiatives will achieve this. According to the two external pig field veterinarians consulted, to further decrease AMU in the Danish pig sector, the focus should be on disease prevention and animal health promotion. This could be achieved by providing better quality feed, increasing group vaccination, and improving external and internal biosecurity. This is in line with international pig specialists, who ranked these measures among the most promising to promote responsible AMU, taking into consideration the measures' combined effectiveness, feasibility and return on investment (70). Numerous studies have shown that targeted use of vaccines in animal populations can lead to a significant decrease in the consumption of AMs (71). Moreover, the two veterinary pig specialists recommended that the need of vaccines should be considered individually for each farm, especially vaccines against the bacteria *E. coli*, *Mycoplasma* spp., *Lawsonia intracellularis* and *Actinobacillus pleuropneumoniae* and the viruses PCV2, influenza and PRRS. Improvement of the overall animal health combined with prevention of the outset of primary or secondary infections can act as effective measure. This is despite the study by Kruse et al. (72) showing that vaccine use was not related to lower AMU, because vaccines may have been used to handle existing disease problems, and hence, reverse causality was observed (72). Moreover, new and possibly more effective vaccines have been developed since the study by Kruse et al. (72) was published. Vaccination cannot be considered a stand-alone measure but should be part of a multi-action plan also involving external and internal biosecurity. Danish farmers often state the cost of vaccination as a major limitation; despite this, vaccines sales have been increasing in Denmark, as can be seen in Supplementary Figure S1.

Denmark's specific pathogen free (SPF) system aims to avoid the introduction of specific pathogens into pig herds, and has led to eradication of swine dysentery, an infection for which high AMU is ascribed (11). Other national eradication programs are being considered in Denmark, with that against PRRS virus being recently initiated (73). An initiative that could result in significant reduction of AMU at the national level.

To further promote responsible AMU, the Danish Agriculture & Food Council has released a manual on good antibiotic practices, with simple and easy to follow guidelines on the prevention and diagnosis of diarrhea in weaners and finishers and the handling of antibiotic treatments (6). The manual is promoted among farmers and updated when necessary or as new relevant knowledge arises.

The Yellow Card permit limits were originally defined as twice the average use among the country's pig farms. Further AMU reduction targets should be accompanied by careful animal health and welfare assessments (57) to ensure that animals are being treated "As little as possible, but as often as necessary." Finally, it was found that the VetStat monitoring system is working effectively, as it is set up to present AMU in detail, which includes age group, indication, AM and administration route. This allows the identification of high use segments, which potentially could lead to implementation of targeted interventions. Still, one issue to consider when operating a Yellow Card-like system is that permitted limits could be interpreted as acceptable limits, which is not the intention of the system.

Sanders et al. (2020) analysed the multiple strategies followed concerning the essential system design elements and management processes of AM stewardship initiatives, based on farm-level AMU, demonstrating that there is no widely accepted approach to implement such initiatives (44). The decisions made in Denmark should be considered in the context of the country and may not be universally applicable. As an example, in Italy the ClassyFarm system benchmarks farms by comparing their usage either at age group or herd level to median of all farms and classifies them according to quartiles. However, a similar approach to the Danish yellow card has been followed by a private system in the Czech Republic (Q VET pigs) and two quality assurance system in Switzerland (SuisSano and Safety+) also define a multiplication factor for the use of high priority critically important AM (74).

4. Conclusion

After more than 25 years of AMU stewardship-related interventions in the Danish pig sector, the sector's AMU can be considered responsible in an intensive livestock production context. There is no use of AM in the category A "Avoid" and a minuscule use of category B" Restrict." Most peroral use is related to weaners suffering from gastrointestinal infections. To further reduce the pig sector's AMU, a further shift from section to pen or individual treatments should be considered. To ensure prudent use of AM, enhanced focus should be on the prevention of disease and the promotion of animal health through the rearing of more robust pigs, use of better feed, more vaccines and increased biosecurity.

Data availability statement

The data analyzed in this study is subject to the following licenses/restrictions: The data set may be consulted under the terms defined by the Danish Program for surveillance of antimicrobial consumption and resistance in bacteria from food animals, food and humans. Requests to access these datasets should be directed to MS, marsan@food.dtu.dk.

Author contributions

PM, MS, and LA took equal leadership in all steps of the study, from the conception to the design of the work, and drafting the first versions of the manuscript. JN-R provided a critical external view to the study. BH supervised the data analysis and figure production. EN contributed with clinical and legal expertise. All authors contributed to the article and approved the submitted version.

Funding

This work was partly undertaken as part of CoEvalAMR Phase 2, which is funded by the JPIAMR and led by the Faculty of Veterinary Medicine at the University of Montreal, receiving funding from the Canadian Institutes for Health Research.

Acknowledgments

Frede Keller from DanVet and Claus Heisel from LVK are acknowledged for input and comments to the analyses undertaken.

Conflict of interest

LA and EN work for two organisations which provide advice to farmers and meat producing companies.

The remaining 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.

Publisher's note

Supplementary material

The Supplementary material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fvets.2023.1155811/full#supplementary-material

References

1. Kahn LH. Antimicrobial resistance: a one health perspective. *Trans R Soc Trop Med Hyg [Internet]*. (2017) 111:255–60. doi: 10.1093/trstmh/trx050

2. European Medicines Agency. Categorisation of antibiotics in the European Union. Ema/Cvmp/Chmp/682198/2017 [Internet]. (2019);31(December):73. Available at: https://www.ema.europa.eu/en/documents/report/categorisation-antibiotics-european-union-answer-request-european-commission-updating-scientific_en.pdf

3. World Health Organization. Urgent health challenges for the next decade 2030: World Health Organization [internet]. NeuroPharmac Journal. (2020). p. 113–117. Available at: https://www.who.int/news-room/photo-story/photo-story-detail/urgent-health-challenges-for-the-next-decade

4. Palma E, Tilocca B, Roncada P. Antimicrobial resistance in veterinary medicine: an overview. *Int J Mol Sci [Internet]*. (2020) 21:1–21. doi: 10.3390/ijms21061914

5. Aarestrup FM. The livestock reservoir for antimicrobial resistance: a personal view on changing patterns of risks, effects of interventions and the way forward. *Philos Trans R Soc B Biol Sci [Internet]*. (2015) 370:20140085. doi: 10.1098/rstb.2014.0085

6. Danish Agriculture & Food Council. Manual til god Antibiotikapraksis [Internet]. (2019). Available at: https://svineproduktion.dk/viden/i-stalden/management/manualer/antibiotika

7. Dyar OJ, Huttner B, Schouten J, Pulcini C. On behalf of, ESGAP (ESCMID study group for antimicrobial stewardship). *Clinical Microbiology and Infection*. (2017) 23:793–798. doi: 10.1016/j.cmi.2017.08.026

8. Dellit TH, Owens RC, McGowan JE, Gerding DN, Weinstein RA, Burke JP, et al. Infectious Diseases Society of America and the Society for Healthcare Epidemiology of America guidelines for developing an institutional program to enhance antimicrobial stewardship [internet] In: . *Clinical Infectious Diseases*. United Kingdom: Oxford Academic (2007). *Vol. 44*, 159–77.

9. Ferreira JP, Staerk K. Antimicrobial resistance and antimicrobial use animal monitoring policies in Europe: where are we ? *J Public Health Policy [Internet]*. (2017) 38:185–202. doi: 10.1057/s41271-017-0067-y

10. European Medicines Agency. Guidance on provision of data on antimicrobial use by animal species from national data collection systems guidance on provision of data on antimicrobial use by animal species from national data collection systems Table of contents. EMA/489035/2016 [Internet]. (2018); Available at: http://www.ema.europa.eu/docs/en_GB/document_library/Scientific_guideline/2017/03/WC500224492.pdf

11. Food and Agriculture Organization of the United Nations and Ministry of Environment and Food of Denmark. Tackling antimicrobial use and resistance in pig production lessons learned in Denmark [internet]. (2019). Available at: https://www.fao.org/documents/card/es/c/CA2899EN

12. Speksnijder DC, Mevius DJ, Bruschke CJM, Wagenaar JA. Reduction of veterinary antimicrobial use in the Netherlands. The dutch success model. *Zoonoses Public Health*. (2015) 62:79–87. doi: 10.1111/zph.12167

13. Moura P, Sanders P, Heederik D, Van Geijlswijk IM, Niza-Ribeiro J. Use of a new antimicrobial consumption monitoring system (vet-AMNet): application to Dutch dairy sector over a 9-year period. *Front Vet Sci*. (2022) 9:984771. doi: 10.3389/fvets.2022.984771

14. FAO and VMD. *Tackling Antimicrobial Use and Resistance in Food-Producing Animals: Lessons Learned in the United Kingdom of Great Britain and Northern Ireland [internet]*. FAO, Veterinary Medicines Directorate. (2022).

15. Sandberg M, Hesp A, Aenishaenslin C, Bordier M, Bennani H, Bergwerff U, et al. Assessment of evaluation tools for integrated surveillance of antimicrobial use and resistance based on selected case studies. *Front Vet Sci [Internet]*. (2021) 8:620998. doi: 10.3389/fvets.2021.620998

16. Aenishaenslin C, Häsler B, Ravel A, Parmley EJ, Mediouni S, Bennani H, et al. Evaluating the integration of one health in surveillance Systems for Antimicrobial use and Resistance: a conceptual framework. *Front Vet Sci [Internet]*. (2021) 8:611931. doi: 10.3389/fvets.2021.611931

17. Danish Agriculture & Food Council. Pigmeat statistics 2020 [internet]. (2021). Available at: https://agricultureandfood.dk/prices-and-statistics/annual-statistics

18. Lopes Antunes AC, Jensen VF. Close to a decade of decrease in antimicrobial usage in Danish pig production–evaluating the effect of the yellow card scheme. *Front Vet Sci [Internet]*. (2020) 7:1–11. doi: 10.3389/fvets.2020.00109

19. European Parliament and Council of the European Union. Commission implementing decision 2013/652/EU of 12 November 2013 on the monitoring and reporting of antimicrobial resistance in zoonotic and commensal bacteria. Off J Eur Union [Internet] (2013);(2011):26–39. Available at: https://publications.europa.eu/en/publication-detail/-/publication/83e1934f-4d39-11e3-ae03-01aa75ed71a1/language-en

20. *Statens serum Insitut. National Veterinary Institue. National Food Institue. Danmap 2020*. (2021);1–174. Available at: https://www.danmap.org/reports/2020

21. Danish Veterinary and Food Administration. Health advice pigs [internet]. (2021). Available at: https://www.foedevarestyrelsen.dk/Leksikon/Sider/Sundhedsrådgivning-svin.aspx

22. Danish Ministry of Health. Danish medicines act [internet]. (2005). Available at: https://laegemiddelstyrelsen.dk/en/about/targets-and-tasks/legislation/the-danish-medicines-act/#:~:text=The Danish Medicines Act regulates,and advertising of medicinal products

23. Stege H, Bager F, Jacobsen E, Thougaard A. VETSTAT -the Danish system for surveillance of the veterinary use of drugs for production animals. *Prev Vet Med [Internet]*. (2003) 57:105–15. doi: 10.1016/S0167-5877(02)00233-7

24. Wielinga PR, Jensen VF, Aarestrup FM, Schlundt J. Evidence-based policy for controlling antimicrobial resistance in the food chain in Denmark. *Food Control [Internet]*. (2014) 40:185–92. doi: 10.1016/j.foodcont.2013.11.047

25. Danish Veterinary and Food Administration. Nye grænseværdier for antibiotikaforbruget til grise [Internet]. Available at: https://svineproduktion.dk/aktuelt/nyheder/2018/06/290618_nye_graensevaerdier_gult_kort

26. Danish Veterinary and Food Administration. *Guideline for prescribing antimicrobial for pigs guideline for Prescibing antimicrobial for pigs [internet]*. (2018). Available at: https://www.foedevarestyrelsen.dk/english/Animal/AnimalHealth/Veterinary_medicine/Documents/Antimicrobial guideline for pigs Denmark 2018.pdf

27. Alban L, Nielsen EO, Dahl J. A human health risk assessment for macrolide-resistant campylobacter associated with the use of macrolides in Danish pig production. *Prev Vet Med [Internet]*. (2008) 83:115, 74–29. doi: 10.1016/j.prevetmed.2007.06.006

28. Alban L, Ellis-Iversen J, Andreasen M, Dahl J, Sönksen UW. Assessment of the risk to public health due to use of antimicrobials in pigs-an example of pleuromutilins in Denmark. *Front Vet Sci [Internet]*. (2017) 4:74. doi: 10.3389/fvets.2017.00074

29. Sok J, Fischer EAJ. Farmers' heterogeneous motives, voluntary vaccination and disease spread: an agent-based model. *Eur Rev Agric Econ [Internet]*. (2020) 47:1201–22. doi: 10.1093/erae/jbz041

30. European Medicines Agency. Sales of veterinary antimicrobial agents in 31 European countries in 2019 and 2020 [internet]. *Europ Surveillance Veterinary Antimicrobial Consumption*. (2020). 2021:1–130. doi: 10.2809/167341

31. European Medicines Agency -EMA. The European database of sales of veterinary antimicrobial agents [internet]. Available at: https://esvacbi.ema.europa.eu/analytics/saw.dll?PortalPages

32. Dupont N, Fertner M, Kristensen CS, Toft N, Stege H. Reporting the national antimicrobial consumption in Danish pigs: influence of assigned daily dosage values and population measurement. *Acta Vet Scand [Internet]*. (2016) 58:27–9. doi: 10.1186/s13028-016-0208-5

33. Andersen VD, Møller FD, Jensen MS, Aarestrup FM, Vigre H. The quantitative effect of antimicrobial usage in Danish pig farms on the abundance of antimicrobial resistance genes in slaughter pigs. *Prev Vet Med [Internet]*. (2023) 214:105899. doi: 10.1016/j.prevetmed.2023.105899

34. Aarestrup FM, Oliver Duran C, Burch DGS. Antimicrobial resistance in swine production. *Anim Health Res Rev [Internet]*. (2008) 9:135–48. doi: 10.1017/S1466252308001503

35. Jensen VF, Emborg HD, Aarestrup FM. Indications and patterns of therapeutic use of antimicrobial agents in the Danish pig production from 2002 to 2008. *J Vet Pharmacol Ther [Internet]*. (2012) 35:33–46. doi: 10.1111/j.1365-2885.2011.01291.x

36. Dewulf J, Joosten P, Chantziaras I, Bernaerdt E, Vanderhaeghen W, Postma M, et al. Antibiotic use in European pig production: less is more. *Antibiotics*. (2022) 11:1493. doi: 10.3390/antibiotics11111493

37. Van Boeckel TP, Brower C, Gilbert M, Grenfell BT, Levin SA, Robinson TP, et al. Global trends in antimicrobial use in food animals. *Proc Natl Acad Sci U S A [Internet]*. (2015) 112:5649–54. doi: 10.1073/pnas.1503141112

38. Bondt N, Jensen VF, Puister-Jansen LF, van Geijlswijk IM. Comparing antimicrobial exposure based on sales data. *Prev Vet Med [Internet]*. (2013) 108:10–20. doi: 10.1016/j.prevetmed.2012.07.009

39. Collineau L, Belloc C, Stärk KDC, Hémonic A, Postma M, Dewulf J, et al. Guidance on the selection of appropriate indicators for quantification of antimicrobial usage in humans and animals. *Zoonoses Public Health [Internet]*. (2017) 64:165–84. doi: 10.1111/zph.12298

40. Lekagul A, Tangcharoensathien V, Yeung S. Patterns of antibiotic use in global pig production: a systematic review. *Vet Anim Sci [Internet]*. (2019) 7:100058. doi: 10.1016/j.vas.2019.100058

41. Scali F, Santucci G, Maisano AM, Giudici F, Guadagno F, Tonni M, et al. The use of antimicrobials in italian heavy pig fattening farms. *Antibiotics [Internet]*. (2020) 9:1–12. doi: 10.3390/antibiotics9120892

42. Carmo LP, Nielsen LR, Alban L, Müntener CR, Schüpbach-Regula G, Magouras I. Comparison of antimicrobial consumption patterns in the Swiss and Danish cattle and swine production (2007-2013). *Front Vet Sci [Internet]*. (2017) 4:26. doi: 10.3389/fvets.2017.00026

43. EFSA. Report for 2020 on the results from the monitoring of veterinary medicinal product residues and other substances in live animals and animal products. *EFSA Support Publ [Internet]*. (2022) 19:91. doi: 10.2903/sp.efsa.2022.en-7143

44. Sanders P, Vanderhaeghen W, Fertner M, Fuchs K, Obritzhauser W, Agunos A, et al. Monitoring of farm-level antimicrobial use to guide stewardship: overview of existing systems and analysis of key components and processes. *Front Vet Sci*. (2020) 7:540. doi: 10.3389/fvets.2020.00540

45. Danish Ministry of the Environment and Food. *MRSA Risiko og håndtering Rapport ved MRSA-ekspertgruppen*. Danish Veterinary and Food Administration. (2017).

46. Birkegård AC, Halasa T, Græsbøll K, Clasen J, Folkesson A, Toft N. Association between selected antimicrobial resistance genes and antimicrobial exposure in Danish pig farms. *Sci Rep [Internet]*. (2017) 7:9683–8. doi: 10.1038/s41598-017-10092-9

47. Andersen VD, Aarestrup FM, Munk P, Jensen MS, de Knegt LV, Bortolaia V, et al. Predicting effects of changed antimicrobial usage on the abundance of antimicrobial resistance genes in finisher' gut microbiomes. *Prev Vet Med [Internet]*. (2020) 174:104853. doi: 10.1016/j.prevetmed.2019.104853

48. Aarestrup SAM, Emborg HD, Pedersen K, Hendriksen RS, Bager F. Effect of abolishment of the use of antimicrobial agents for growth promotion on occurrence of antimicrobial resistance in fecal enterococci from food animals in Denmark. *Antimicrob Agents Chemother [Internet]*. (2001) 45:2054–9. doi: 10.1128/AAC.45.7.2054-2059.2001

49. *Statens Serum Institut Danish Veterinary and Food Administration Danish Medicines Agency National Veterinary Institute, Technical University of Denmark National Food Institute, Technical University of Denmark*. The Danish Integrated Antimicrobial Resistance Monitoring and Research Programme. (2006).

50. AgersoØ Y, Aarestrup FM. Voluntary ban on cephalosporin use in Danish pig production has effectively reduced extended-spectrum cephalosporinase-producing Escherichia coli in slaughter pigs. *J Antimicrob Chemother [Internet]*. (2013) 68:569–72. doi: 10.1093/jac/dks427

51. Mather AE, Reid SWJ, Maskell DJ, Parkhill J, Fookes MC, Harris SR, et al. Distinguishable epidemics of multidrug-resistant salmonella typhimurium DT104 in different hosts. *Science [Internet]*. (2013) cited 2023 Mar 6) 341:1514–7. doi: 10.1126/science.1240578

52. European Centre for Disease Prevention and Control (ECDC) EFSA (EFSA) and EMA (EMA). Third joint inter-agency report on integrated analysis of consumption of antimicrobial agents and occurrence of antimicrobial resistance in bacteria from humans and food-producing animals in the EU/EEA. *EFSA J [Internet]*. (2021) 19:1–164. doi: 10.2903/j.efsa.2021.6712

53. Muloi D, Ward MJ, Pedersen AB, Fèvre EM, Woolhouse MEJ, Van Bunnik BAD. Are food animals responsible for transfer of antimicrobial-resistant Escherichia coli or their resistance determinants to human populations? A systematic review. *Foodborne Pathog Dis [Internet]*. (2018) 15:467–74. doi: 10.1089/fpd.2017.2411

54. Statens Serum Institut National & Food Institute, Technical University of Denmark. *DANMAP 2021 -use of antimicrobial agents and occurrence of antimicrobial resistance in bacteria from food animals, food and humans in Denmark*. (2022).

55. Statens Serum Institut National & Food Institute, Technical University of Denmark. *DANMAP 2019 Web Annex*. Lyngby, Denmark: National Food Institute. (2020).

56. Jerab J, Jansen W, Blackwell J, Van HJ, Palzer A, Lister S, et al. Real-world data on antibiotic group treatment in European livestock: drivers, conditions, and alternatives. *Antibiotics*. (2022) 11:1–19. doi: 10.3390/antibiotics11081046

57. Alban L, Dahl J, Andreasen M, Petersen JV, Sandberg M. Possible impact of the "yellow card" antimicrobial scheme on meat inspection lesions in Danish finisher pigs. *Prev Vet Med [Internet]*. (2013) 108:334–41. doi: 10.1016/j.prevetmed.2012.11.010

58. Dupont N, Diness LH, Fertner M, Kristensen CS, Stege H. Antimicrobial reduction measures applied in Danish pig herds following the introduction of the "yellow card" antimicrobial scheme. *Prev Vet Med [Internet]*. (2017) 138:9–16. doi: 10.1016/j.prevetmed.2016.12.019

59. Larsen I, Nielsen SS, Olsen JE, Nielsen JP. The efficacy of oxytetracycline treatment at batch, pen and individual level on Lawsonia intracellularis infection in nursery pigs in a randomised clinical trial. *Prev Vet Med*. (2016) 124:25–33. doi: 10.1016/j.prevetmed.2015.12.018

60. Wiuff C, Lykkesfeldt J, Svendsen O, Aarestrup FM. The effects of oral and intramuscular administration and dose escalation of enrofloxacin on the selection of quinolone resistance among salmonella and coliforms in pigs. *Res Vet Sci*. (2003) 75:185–93. doi: 10.1016/S0034-5288(03)00112-7

61. Zhang L, Huang Y, Zhou Y, Buckley T, Wang HH. Antibiotic administration routes significantly influence the levels of antibiotic resistance in gut microbiota. *Antimicrob Agents Chemother [Internet]*. (2013) 57:3659–66. doi: 10.1128/AAC.00670-13

62. Maes D, Segales J, Meyns T, Sibila M, Pieters M, Haesebrouck F. Control of mycoplasma hyopneumoniae infections in pigs. *Vet Microbiol [Internet]*. (2008) 126:297–309. doi: 10.1016/j.vetmic.2007.09.008

63. Pieters MG, Maes D. Mycoplasmosis. *Diseases of Swine. 8th* Edn. eds. J. J. Zimmerman, L. A. Karriker, A. Ramirez, K. J. Schwartz, G. W. Stevenson and J. Zhang. (John Wiley & Sons, Inc), (2019):863–883.

64. Sjölund M, Postma M, Collineau L, Lösken S, Backhans A, Belloc C, et al. Quantitative and qualitative antimicrobial usage patterns in farrow-to-finish pig herds in Belgium, France. *Germany and Sweden Prev Vet Med*. (2016) 130:41–50. doi: 10.1016/j.prevetmed.2016.06.003

65. Waluszewski A, Cinti A, Perna A. Antibiotics in pig meat production: restrictions as the odd case and overuse as normality? Experiences from Sweden and Italy. *Humanit Soc Sci Commun* 2021 [Internet];8:1–12. doi: 10.1057/s41599-021-00852-4

66. Public Health Agency of Sweden and National Veterinary Institute. *2020 Swedres | Svarm. Sales of antibiotics and occurrence of antibiotic resistance in Sweden*. Public Health Agency of Sweden and National Veterinary Institute. (2021).

67. Swedish Veterinary Association. Guidelines for the use of antibiotics in production animals. (2013);54. Available at: https://www.svf.se/media/vd5ney4l/svfs-riktlinje-antibiotika-till-produktionsdjur-eng-2017.pdf

68. (SVA) Swedish National Veterinary Institute. Pig production and health status of pigs in Sweden [internet]. (cited 2022 Jun 26). Available at: https://www.sva.se/en/animals/pigs/

69. Ministry of Environment and Food. *Bekendtgørelse om sundhedsrådgivningsaftaler for svinebesætninger [Internet]*. (2016) p. BEK nr. 33. Available from: https://www.retsinformation.dk/eli/lta/2014/534

70. Postma M, Stärk KDC, Sjölund M, Backhans A, Beilage EG, Lösken S, et al. Alternatives to the use of antimicrobial agents in pig production: a multi-country expert-ranking of perceived effectiveness, feasibility and return on investment. *Prev Vet Med [Internet]*. (2015) 118:457–66. doi: 10.1016/j.prevetmed.2015.01.010

71. EMA Committee for Medicinal Products for Veterinary Use (CVMP) and EFSA Panel on Biological Hazards (BIOHAZ)Murphy D, Ricci A, Auce Z, Beechinor JG, Bergendahl H, et al. EMA and EFSA joint scientific opinion on measures to reduce the need to use antimicrobial agents in animal husbandry in the European Union, and the resulting impacts on food safety (RONAFA). *EFSA J*. (2017) 15:4666. doi: 10.2903/j.efsa.2017.4666,

72. Kruse AB, de Knegt LV, Nielsen LR, Alban L. No clear effect of initiating vaccination against common endemic infections on the amounts of prescribed antimicrobials for Danish weaner and finishing pigs during 2007-2013. *Front Vet Sci*. (2017) 3:1–12. doi: 10.3389/fvets.2016.00120

73. Danish Agriculture & Food Council. National strategy for reducing PRRS in pigs [internet]. Available at: https://svineproduktion.dk/Aktuelt/Temaer/PRRS

74. The AACTING-network. Description of existing monitoring systems for collection, analysis, benchmarking and reporting of farm-level veterinary antimicrobial usage [internet]. (2021). Available at: www.aacting.org

frontiers | Frontiers in Veterinary Science

TYPE Brief Research Report
PUBLISHED 03 May 2023
DOI 10.3389/fvets.2023.1154550

OPEN ACCESS

EDITED BY
Kohei Makita,
Rakuno Gakuen University, Japan

REVIEWED BY
Amjad Islam Aqib,
Cholistan University of Veterinary and Animal Sciences, Pakistan
Aneliya Milanova,
Trakia University, Bulgaria

*CORRESPONDENCE
Ghazal Nemati
✉ ghazal.nemati@agroscope.admin.ch

†These authors share first authorship

RECEIVED 30 January 2023
ACCEPTED 04 April 2023
PUBLISHED 03 May 2023

CITATION
Nemati G, Romanó A, Wahl F, Berger T, Rojo LV and Graber HU (2023) Bovine *Staphylococcus aureus*: a European study of contagiousness and antimicrobial resistance.
Front. Vet. Sci. 10:1154550.
doi: 10.3389/fvets.2023.1154550

Bovine *Staphylococcus aureus*: a European study of contagiousness and antimicrobial resistance

Ghazal Nemati[1,2*†], Alicia Romanó[2†], Fabian Wahl[3], Thomas Berger[1], Laura Vazquez Rojo[2] and Hans Ulrich Graber[2]

[1]Food Microbial Systems, Risk Assessment and Mitigation Group, Agroscope, Bern, Switzerland, [2]Food Microbial Systems, Microbiological Safety of Foods of Animal Origin Group, Agroscope, Bern, Switzerland, [3]Food Microbial Systems, Agroscope, Bern, Switzerland

In dairy herds managements, mastitis is the leading cause of economic losses. One of the most important pathogens responsible for intra-mammary infections is *Staphylococcus aureus*. The genetic properties of *S. aureus* have a strong influence on its pathogenicity and contagiousness. In this study, we aimed to obtain a comprehensive overview of the key bovine *S. aureus* clinical properties, such as contagiousness and antimicrobial resistance, present in European strains. For this, 211 bovine *S. aureus* strains from ten European countries that were used in a previous study were used in this study. Contagiousness was assessed using qPCR for the detection of the marker gene *adlb*. Antimicrobial resistance was evaluated using a broth microdilution assay and mPCR for the detection of genes involved in penicillin resistance (*blaI*, *blaR1*, and *blaZ*). It was found that *adlb* was present in CC8/CLB strains; however, in Germany, it was found in CC97/CLI and in an unknown CC/CLR strains. CC705/CLC strains from all countries were found to be susceptible to all tested antibiotics. Major resistance to penicillin/ampicillin, chloramphenicol, clindamycin and tetracycline was detected. Resistance to oxacillin, trimethoprim/sulfamethoxazole and cephalosporins was rarely observed. In addition, contagiousness and antibiotic resistance seem to correlate with different CCs and genotypic clusters. Hence, it is recommended that multilocus sequence typing or genotyping be utilized as a clinical instrument to identify the most appropriate antibiotic to use in mastitis treatment. Actualization of the breakpoints of veterinary strains is necessary to address the existing antibiotic resistance of the bacteria involved in veterinary mastitis.

KEYWORDS

***Staphylococcus aureus*, *adlb*, antimicrobial resistance, minimum inhibitory concentration, multidrug resistant**

1. Introduction

In veterinary medicine, mastitis is the leading cause of economic losses in dairy herds management. It contributes to reductions in milk quality and production, there are costs associated with its treatment, and animal culling can be a consequence of treatment failures (1, 2). In Switzerland, the total cost of mastitis is ~$131 million annually, according to Heiniger et al. (3). One of the most important pathogens responsible for intramammary infections (IMIs) is *Staphylococcus aureus* (4). *S. aureus* may infect only some individual animals or may be contagious and infect the entire herd; infections usually resulting in subclinical chronic mastitis (5, 6). As shown previously (5, 7–12), the genetic properties of

S. aureus have a strong influence on its pathogenicity and contagiousness, making subtyping necessary to improve treatment success and dairy herd management. Using ribosomal spacer PCR (RS-PCR), it has been shown that the rate of infected cows in a herd is highly dependent on the bacterial genotype (GT) (7–10), and, *S. aureus* genotype B (GTB) and its variants may infect up to 100% of cows in the same herd (7–9, 11) due to its high contagiousness (13, 14). In contrast, other genotypes and their variants (e.g., GTC, GTF, GTS) are restricted to one or a few cows in a herd (7–10, 15, 16). In the electrophoresis of the RS-PCR product, variants differ in 1 electrophoretic band and as consequence, are named by superscripted roman numerals (e.g., GTR^{I}). For further simplification, genotypes and their variants are combined into genotypic clusters (CL). For example, GTB and its variants form a cluster named CLB. Multilocus sequence typing (MLST) (17) results have shown that CLB is almost exclusively associated with clonal complex 8 (CC8), whereas CLC corresponds largely to CC705, and CLR to CC97 and CC133 (9, 18). In Europe, CLB, CLC, CLF, CLI, and CLR account for 76.6% of all *S. aureus* isolates obtained from clinical milk samples (19).

RS-PCR is particularly suitable for clinical applications as it is a low-cost, high-throughput method that provides analytical resolution at least as good as *spa* typing in bovine strains (9, 18). However, it is more appropriate to use MLST for subtyping at the biological level because a *S. aureus* clone can be used (17) and, consequently, evolutionary identity established (20, 21). To sanitize Swiss dairy herds infected with the contagious *S. aureus* CLB, Sartori et al., developed a real-time quantitative PCR (qPCR) assay to identify this pathogen in milk samples and achieved diagnostic sensitivity and specificity at the cow level of 99 and 100%, respectively (22). This new assay has been used to detect, with high specificity, the gene *adlb* which encodes the bovine adhesion-like protein located in the GTB-specific staphylococcal cassette chromosome SCCgtb (16, 22). It is a marker for contagiousness and high prevalence of intra-mammary infection (IMI) in dairy herds (11, 16).

Antibiotic (AB) treatment is still one of the most important measures for controlling bovine mastitis (23). However, the frequently unsatisfactory cure rates remain a serious concern, particularly for IMI caused by *S. aureus* (6, 24–27). One major reason for this drawback is the improper use of ABs (28, 29). Additionally, AB treatments applied at the herd level are usually not reported, even though various mastitis control plans strongly recommend performing these analyses and collecting the resultant data (30). Since 2019, it has been required for Swiss's farms to declare the AB treatments used at the herd level (31). In terms of the ABs used to treatment bovine IMIs caused by *S. aureus,* various classes of AB are used: typically, ß-lactams (penicillins and cephalosporins), aminoglycosides, lincosamides, and macrolides (32, 33). Penicillin G is the most commonly used AB for treating IMI in cows caused by *S. aureus* and other Gram-positive mastitis pathogens. In *S. aureus*, the *bla* operon mediates AB resistance against penicillin G and other β-lactamase-sensitive penicillins. The *bla* operon can be located on plasmids (as transposon) or on the chromosome (34, 35) and contains three genes: (1) *blaZ,* which encodes the βlactamase that hydrolyzes the β-lactam ring of AB, rending them inactive; (2) *blaI*, which encodes the repressor; (3) *blaR1* which encodes the sensor and antirepressor (35, 36). Ivanovic et al., recently showed that the *bla* operon plays a key role in phenotypic resistance to penicillin. Furthermore, for *S. aureus,* they highlighted the importance of using the minimum inhibitory concentration (MIC) value as the gold standard when assessing resistance to penicillin and probably other ABs (33).

As contagiousness and antimicrobial resistance (AMR) are critical pathogenic factors of the *S. aureus* strains responsible for bovine mastitis, a comprehensive study was performed to assess the distribution of these key clinical properties in strains from across Europe. Contagiousness was assessed using qPCR to detect the *adlb* gene, which is a staphylococcal marker for contagiousness and for high prevalence of intra-mammary infection in dairy herds. Furthermore, AMR was evaluated using an MIC assay and melting curve PCR (mPCR) to detect genes involved in penicillin resistance (*blaI*, *blaR1*, and *blaZ*).

2. Materials and methods

2.1. Strain collection

A total of 211 bovine strains of *S. aureus* were used in this study that had been collected from 10 European countries; Austria, Belgium, France, Germany, Ireland, Italy, Macedonia, Norway, Slovenia, and Switzerland. These strains were originally collected during two previous studies by Boss et al. (18) and Cosandey et al. (19). As described by Cosandey et al., the strains were aseptically collected from milk samples from individual quarters (19). The strains had been stored in skim milk at −20°C. They were plated onto Columbia agar plates containing 5% sheep blood (Biomérieux Suisse s.a., Geneva, Switzerland) and incubated at 37°C for 24 h (18, 19). The genotypes (GT) and the clonal complexes (CCs) information was obtained from previous studies. The distribution of the different CCs and the GT across the 10 European countries is shown in Table 1 (19).

2.2. DNA extraction

DNA was extracted from single *S. aureus* colonies. One colony was picked and resuspended in 100 μL of 10 mM Tris-HCl and 10 mM EDTA (pH = 8.5), incubated at 95°C for 10 min, and immediately placed on ice. The lysates were diluted 1:100 in qPCR H_2O (SINTETICA S.A, Mendrisio, Switzerland) for use as templates. The samples were stored at −20°C and were analyzed within 2 weeks of extraction (18).

2.3. Quantitative PCR (qPCR) with *adlb* and internal control gene

Real-time qPCR was performed with *adlb* and the internal control gene (N gene of canine distemper virus [CDVN]) according to the protocol of Sartori et al. (22). The characteristics of the utilized primers are listed in Supplementary Table S1. DNA amplification was performed using a Magnetic Induction Cycler

TABLE 1 Distribution of *Staphylococcus aureus* genotypes and clonal complexes across 10 European countries.

	Clonal complex (CC)	Genotype (GT)
Austria	CC8 (9)	GTB (5), GTAM (2), GTI^{IV} (1), GTBE (1)
	CC97 (10)	GTR (2), GTR^{I} (1), GTR^{VI} (3), GTBC (1), GTBL (2), GTE (1)
	CC705 (10)	GTC (7), GTC^{I} (1), GTR^{VI} (1), GTZ (1)
	CC20 (1)	GTF (1)
	CC9 (5)	GTF^{III} (4), GTR^{VI} (1)
	Other CC (13)	
	CC5 (1)	GTE (1)
	CC25 (1)	GTAK (1)
	CC30 (1)	GTB (1)
	CC71 (3)	GTR (2), GTR^{X} (1)
	CC101 (3)	GTAH (2), GTR^{VII} (1)
	CC133 (2)	GTR^{I} (1), GTR^{VI} (1)
	CC479 (1)	GTC (1)
	Unknown (1)	GTAH (1)
Belgium	CC8 (1)	GTB^{II} (1)
	CC97 (4)	GTI^{I} (4)
	CC705 (7)	GTC (4), GTC^{I} (2), GTC^{II} (1)
	CC20 (1)	GTF (1)
	Other CC (5)	
	CC70 (1)	GTC^{I} (1)
	CC71 (1)	GTI (1)
	CC133 (2)	GTR (1), GTZ (1)
	CC479 (1)	GTBG (1)
France	CC8 (2)	GTB (2)
	CC705 (3)	GTC (1), GTC^{I} (2)
	CC20 (4)	GTF (4)
	Other CC (2)	
	CC15 (1)	GTJ^{I} (1)
	CC133 (1)	GTR^{I} (1)
Germany	CC8 (11)	GTB (8), GTB^{I} (3)
	CC97 (5)	GTI^{I} (3), GTR^{VI} (2)
	CC705 (3)	GTC (1), GTC^{II} (2)
	CC9 (2)	GTF^{III} (2)
	Other CC (18)	
	CC1 (3)	GTAN (1), GTBA (1), GTBJ (1)
	CC7 (2)	GTL (1), GTM (1)
	CC15 (1)	GTJ (1)
	CC50 (1)	GTAU (1)
	CC71 (1)	GTI (1)
	CC133 (2)	GTR^{I} (1), GTR^{II} (1)
	CC398 (3)	GTS (3)
	CC479 (4)	GTP (1), GTZ (3)
	Unknown (1)	GTR^{I} (1)
Ireland	CC97 (2)	GTR (1), GTR^{VI} (1)
	CC705 (2)	GTC^{I} (1), GTO^{I} (1)
	Other CC (7)	
	CC5 (1)	GTE (1)
	CC71 (6)	GTAN (1), GTR (2), GTR^{VI} (3)
Italy	CC8 (9)	GTB (9)
	CC97 (3)	$GTBE^{I}$ (1), GTF (1), GTI^{I} (1)
	CC20 (1)	GTF (1)
	CC9 (1)	GTF^{III} (1)
	Other CC (6)	
	CC22 (1)	GTP (1)
	CC30 (1)	$GTBE^{I}$ (1)
	CC71 (1)	GTI^{I} (1)
	CC126 (2)	GTS^{I} (2)
	CC398 (1)	GTS (1)
Macedonia	CC97 (1)	GTR^{VI} (1)
	Other CC (2)	
	CC7 (1)	GTM (1)
	Unknown (1)	GTR^{VI} (1)
Norway	CC97 (2)	GTR (2)
	Other CC (4)	
	CC133 (2)	GTZ (2)
	CC479 (1)	GTZ (1)
	Unknown (1)	GTC (1)
Slovenia	CC97 (6)	GTR (1), GTR^{II} (2), GTAA (1), GTO (1), GTZ (1)
	CC20(1)	GTAT (1)
	CC9 (1)	GTBB (1)
	Other CC (5)	
	CC22 (1)	GTI^{II} (1)
	CC49 (2)	GTAA (1), GTR^{I} (1)
	CC101 (1)	GTAA (1)
	CC71 (1)	GTR (1)
Switzerland	CC8 (18)	GTB (18)
	CC97 (1)	GTR (1)
	CC705 (19)	GTC (16), GTC^{I} (1), GTA (1), GTH (1)
	Other CC (4)	
	CC5 (1)	GTE (1)

(Continued)

TABLE 1 (Continued)

	Clonal complex (CC)	Genotype (GT)
	CC59 (1)	GTD (1)
	CC70 (1)	GTC (1)
	Unknown (1)	GTC (1)

qPCR real-time thermal cycler (Bio Molecular Systems, Australia) and the following cycling conditions: initial denaturation at 95°C for 3 min followed by 45 running cycles of denaturation at 95°C for 3 s and annealing/elongation at 60°C for 20 s. Two reference strains that were positive for both targets were included as positive controls.

2.4. PCR analysis of the *bla* operon genes

The mPCR was performed according to the protocol of Ivanovic et al. (33). Each of the 211 strains was analyzed for the presence of *blaI*, *blaR1*, and *blaZ;* each gene was detected separately. As per Ivanovic et al., amplicons with a single melting peak identical to the positive control for *blaI*, *blaR1*, or *blaZ* were considered positive. The characteristics of the utilized primers are listed in Supplementary Table S2.

2.5. Assessment of antimicrobial sensitivity

The sensitivity of each strain to 30 antimicrobial agents was tested by minimum inhibitory concentration (MIC) using a PM32 panel (Beckman Coulter, Inc., Brea, CA, USA) following the manufacturer's instructions. The tested ABs concentrations (μg/mL) were as follows: amoxicillin/K clavulanate (0.5/0.25–8/4), ampicillin (0.5–8), azithromycin (1–2), cefepime (4–8), cefotaxime (1–2), cefuroxime (4–8), chloramphenicol (8), ciprofloxacin (0.5–1), clindamycin (0.25–0.5, 2), daptomycin (0.5–4), ertapenem (0.5–1), erythromycin (1–2), fosfomycin (32), fusic acid (2), gentamycin (1–4), imipenem (2–8), levofloxacin (1–2), linezolid (0.5–4), meropenem (2–8), moxifloxacin (0.5–1), nitrofurantoin (64), oxacillin (0.25–2), penicillin (0.03–0.25, 2), rifampin (0.5–2), synercid (1–4), teicoplanin (1–8), tetracycline (1–2), tobramycin (1–4), trimethoprim/sulfamethoxazole (1/19–4/76), and vancomycin (0.25–8). Additionally, cefoxitin (4 μg/mL) screening was performed to determine the presence of methicillin resistant *Staphylococcus aureus* (MRSA) strains. When possible, the current clinical breakpoint of the EUCAST was used (37), otherwise the range specified by the CLSI was applied (38). All the ABs tested and their breakpoints are listed in Supplementary Table S3.

2.6. Statistical analysis

Data are expressed as absolute numbers or percentage. To assess the associations among different AB, the corresponding *phi* coefficients were computed and plotted using R 4.0.5 (39) together with the corrplot package v. 0.84. *Phi* values range from −1 to 1 (40). Negative *phi* values indicate a negative, inverse association among both variables, whereas positive *phi* values indicate a positive association. The Kappa test was performed using R 4.0.5 (39) to evaluate the agreement between the MIC and the *bla* mPCR results. Kappa values range from 0 to 1, with values of 0 and 1 indicating no and perfect agreement, respectively (41). To assess penicillin resistance, a loglinear model was computed to analyze the relationships among the factors penicillin, CC, country, and their interactions. The analysis was performed using Systat 13 (Systat Software Inc., Richmond, CA).

TABLE 2 Detailed distribution of *adlb* across different genotypes and clonal complexes, listed by country.

	Genotype	Clonal complexes
Austria	GTB (6)	CC8 (5)
		Other CC (1)
Belgium	ND	ND
France	GTB (2)	CC8 (2)
Germany	GTB (10)	CC8 (10)
	GTI (1)	CC97 (1)
	Other GT (1)	Other CC (1)
Ireland	ND	ND
Italy	GTB (8)	CC8 (8)
Macedonia	ND	ND
Norway	ND	ND
Slovenia	ND	ND
Switzerland	GTB (18)	CC8 (18)

ND, Not detected.

3. Results

3.1. Presence of *adlb* in European *S. aureus* strains

The 211 *S. aureus* strains collected from 10 European countries were assessed using qPCR for the presence of *adlb* and its association with GTs and CCs. Among the 211 strains, 46 were positive for *adlb*. The distribution of *adlb* among the different GTs and CCs and among the 10 European countries is shown in Table 2.

An analysis of the GTs found to contain *adlb*, showed that 44 of 47 (94%) CLB strains were positive for *adlb* and that only two strains were positive for *adlb* in the remaining 164 strains (1.2%). Furthermore, the gene was also observed in a German GTI^{I} and a GTR^{I} strain. GTB was not detected in Ireland, Macedonia, Slovenia, or Norway. In Italy, Germany, and Belgium, three GTB strains were found that did not contain *adlb*.

3.2. AMR overview in European *S. aureus* strains

An analysis of the MIC data showed that 65% of the strains ($n = 137$) were inhibited by all the tested ABs. Table 3 shows the strains that demonstrated AMR, sorted by CC. Only the ABs to which resistance was exhibited are included.

Among all the ABs, the greatest number of AMR strains were found to be resistant to penicillin/ampicillin, chloramphenicol, clindamycin and tetracycline. There was no AMR observed against most of the tested antibiotics, including vancomycin, trimethoprim/sulfamethoxazole, rifampin, synercid, meropenem, linezolid, imipenem, daptomycin, and ertapenem. Interestingly, no MRSA strains were found.

A total of nine strains (4.3%) were multidrug resistant (MDR). The MDR strains were detected in only four countries: Belgium ($n = 4$, 1.8%), Austria ($n = 1$, 0.5%), Italy ($n = 3$, 1.4%) and Germany ($n = 1$, 0.5%). It is worth noting that the four Belgian strains showed the same pattern of resistance to β-lactams (ampicillin and penicillin), chloramphenicol, and clindamycin. The most resistant strain originated in Italy and showed resistance to β-lactams (ampicillin and penicillin), chloramphenicol, quinolones (ciprofloxacin, levofloxacin, and moxifloxacin), tetracycline, and trimethoprim/sulfamethoxazole.

Supplementary Figure S1 shows the AMR associations found among different ABs (ampicillin, chloramphenicol, clindamycin, penicillin and tetracycline). A strong association was found between the β-lactam ABs (ampicillin and penicillin, $phi = 1.0$; $P < 0.001$). Additionally, a strong association ($phi = 0.79$; $P < 0.001$) was found between clindamycin and chloramphenicol.

To analyze the observed penicillin resistance in more detail, a statistical model was computed to analyze the relationships among the following factors: resistance to penicillin, the most abundant CCs (CC8, CC97, and CC705), countries, and their interactions. For penicillin ($n = 54$), significant interactions ($P < 0.001$ in each case) were observed between penicillin resistance and CCs and between penicillin resistance and countries. Significant values ($P < 0.001$ in each case) were also obtained for the interaction between the CCs and countries, and for individual factors except the CCs ($P = 0.055$). Regarding the CCs, 50% and 14% of the CC97 and CC8 strains, respectively, showed resistance to penicillin. In contrast, CC705 was always sensitive to penicillin. Resistance to penicillin was particularly prominent in Austria, Belgium, Germany, and Ireland, and was absent in Slovenia and Switzerland. An identical loglinear model was also calculated for the genotypic clusters; the most observed CCs were replaced by the three most common CLs (CLB, CLC, and CLR). Significant interactions were found between penicillin resistance and CLs ($P = 0.014$) and between the penicillin resistance and countries ($P < 0.001$). CLC strains were always sensitive to penicillin, whereas 13% of CLB strains and 37% of CLR strains were resistant to penicillin. The distribution of penicillin resistance among the countries was identical to the found in the CCs model. Similar analyses for ABs other than penicillin were not performed due to a lack of sufficient data. In fact, for chloramphenicol and tetracycline, the next most common resistance targets after penicillin, only 20 (9.5%) and 12 (5.7%) of strains demonstrated resistance to these ABs, respectively.

CC705 was not only susceptible to penicillin but also to all other ABs except for one strain that was resistant to azithromycin and erythromycin (both macrolides) and another one that was resistant to chloramphenicol (Table 3). CC97 showed resistance to penicillin, chloramphenicol, and clindamycin. Increased AMR rates, in particular to penicillin/ampicillin and chloramphenicol, were also detected in CC9, CC20, and CC133 (Supplementary Table S4).

3.3. Association between MIC and *bla* operon genes

All 54 strains that exhibited phenotypic resistance to penicillin (26% of all strains) showed the simultaneous presence of all *bla* operon genes. In contrast, in 34 strains that were positive for all *bla* genes, the corresponding MIC value was always $< 0.12\,\mu g/mL$. Interestingly, this discrepancy was observed exclusively in CC8/CLB strains with the exception of one strain CC20/GTAT. For 123 trains, the MIC assay and mPCR for *bla* operon genes gave negative results.

4. Discussion

4.1. Prevalence of *adlb* in European *S. aureus* strains

Previous studies demonstrated that *S. aureus* CC8/CLB is highly contagious (13, 14) and can be detected very specifically by the qPCR assay for *adlb* (22) as also used in the present study. Indeed, with an inclusivity of 97% and exclusivity of 98%, the specificity of this test is very high (22), a fact that was recently confirmed by Gazzola et al. (42). Because of the tight association between CC8/CLB (contagious) and *adlb*, the gene turned out to be a marker for contagiousness and for high prevalence of IMI in dairy herds as shown by Sartori et al. in Swiss and by Maisano et al. in Italian dairy herds (11, 16). Based on the present results we further suggest that high staphylococcal IMI prevalence is also present in Austrian, French, and German dairy herds as *adlb* was regularly observed in the corresponding strains. Indeed, a recent examination of an Austrian and German dairy herd with high IMI prevalence caused by *S. aureus* revealed again the presence of the *adlb* gene. Whether *adlb* is the only staphylococcal marker for contagiousness and high IMI prevalence remains to be elucidated. In fact, the study by Maisano et al. demonstrated that in a small percentage of herds *adlb* was not linked to high staphylococcal IMI prevalence (16).

Interestingly, we detected the *adlb* gene in a German GTI^I and a GTR^I strain, genotypes that are not part of CLB/CC8. From ongoing studies, we know that the *adlb* gene is located on the staphylococcal cassette chromosome (SCC). As reviewed by Malachowa et al., SCCs may be transmitted among *S. aureus* strains by horizontal gene transfer; hence, the presence of *adlb* gene in GTI^I and GTR^I strains may be the result of this mechanism, with an *S. aureus* CC8/CLB most likely being the SCC donor (43).

TABLE 3 Detailed description of the isolates (n = 211), their genotypes, and their phenotypic (and mPCR) resistance to the tested antibiotics.

CCs	Country	Genotype cluster (CL)	Phenotypic results (MIC)																			mPCR
			GEN[a]	TOB[a]	CIP[b]	LEV[b]	MOX[b]	TEI[c]	CLI[d]	AZI[e]	ERY[e]	AMP[f]	OXA[f]	PEN[f]	TET[g]	CHL[h]	FOS[h]	FUA[h]	MOX[h]	NIT[h]	T/S[h]	*bla*
CC8 (50)	Austria	CLB (5)																				Pos (4)
		CLI (1)												1								Pos (1)
		CLOG (3)										2		2	1			1				Pos (2)
	Belgium	CLB (1)										1		1								Pos (1)
	France	CLB (2)										2		2								Pos (2)
	Germany	CLB (11)												1								Pos (8)
	Italy	CLB (9)							1			2		2	1	2						Pos (6)
	Switzerland	CLB (18)																				Pos (17)
CC97 (34)	Austria	CLR (6)										3		3								Pos (3)
		CLOG (4)										3		3								Pos (3)
	Belgium	CLI (4)							4			4		4		4						Pos (4)
	Germany	CLI (3)										2		2								Pos (2)
		CLR (2)										1		1								Pos (1)
	Ireland	CLR (2)										2		2								Pos (2)
	Italy	CLF (1)																				Neg
		CLI (1)										1		1								Pos (1)
		CLOG (1)							1			1		1	1							Pos (1)
	Macedonia	CLR (1)																				Neg
	Norway	CLR (2)																				Neg
	Slovenia	CLR (3)													1							Neg
		CLOG (3)						1								1						Neg
	Switzeland	CLR (1)																				Neg
CC705 (44)	Austria	CLC (8)																				Neg
		CLR (1)										1		1								Pos (1)
		CLOG (1)																				Neg
	Belgium	CLC (7)																				Neg

(Continued)

10.3389/fvets.2023.1154550

TABLE 3 (Continued)

CCs	Country	Genotype cluster (CL)	Phenotypic results (MIC)																			mPCR
			GEN[a]	TOB[a]	CIP[b]	LEV[b]	MOX[b]	TEI[c]	CLI[d]	AZI[e]	ERY[e]	AMP[f]	OXA[f]	PEN[f]	TET[g]	CHL[h]	FOS[h]	FUA[h]	MOX[h]	NIT[h]	T/S[h]	*bla*
	France	CLC (3)																				Neg
	Germany	CLC (3)																				Neg
	Ireland	CLC (1)																				Neg
		CLOG (1)																				Neg
	Switzerland	CLC (17)								1	1					1						Neg
		CLOG (2)																				Neg
CC20 (8)	Austria	CLF (1)							1			1		1								Pos (1)
	Belgium	CLF (1)																				Neg
	France	CLF (4)															1					Neg
	Italy	CLF (1)												1								Pos (1)
	Slovenia	CLOG (1)																				Pos (1)
CC9 (9)	Austria	CLF (4)										2		2		2						Pos (2)
		CLR (1)										1		1								Pos (1)
	Germany	CLF (2)										1		1		1						Pos (1)
	Italy	CLF (1)																				Neg
	Slovenia	CLOG (1)													1							Neg
Other CC (66)	Austria	CLB (1)																				Pos (1)
		CLC (1)																				Neg
		CLR (6)							1	1	1	1		1	1	1						Pos (1)
		CLOG (5)	1	1								1				2						Pos (1)
	Belgium	CLC (1)																				Neg
		CLI (1)										1		1								Pos (1)
		CLR (1)														1						Neg
		CLOG (2)																				Neg
	France	CLR (1)																				Neg
		CLOG (1)										1		1								Pos (1)
	Germany	CLI (1)										1		1								Pos (1)

(Continued)

10.3389/fvets.2023.1154550

TABLE 3 (Continued)

CCs	Country	Genotype cluster (CL)	Phenotypic results (MIC)																			mPCR
			GEN[a]	TOB[a]	CIP[b]	LEV[b]	MOX[b]	TEI[c]	CLI[d]	AZI[e]	ERY[e]	AMP[f]	OXA[f]	PEN[f]	TET[g]	CHL[h]	FOS[h]	FUA[h]	MOX[h]	NIT[h]	T/S[h]	*bla*
		CLR (3)																				Neg
		CLOG (14)			1	1	1					5	1	5	2	1			1		1	Pos (5)
	Ireland	CLR (5)										5		5		1				3		Pos (5)
		CLOG (2)										1		1	1							Pos (1)
	Italy	CLI (1)							1													Neg
		CLOG (5)			1	1	1		1			4		4	2	1			1		1	Pos (4)
	Macedonia	CLR (1)																				Neg
		CLOG (1)										1		1								Pos (1)
	Norway	CLC (1)																				Neg
		CLOG (3)														1						Neg
	Slovenia	CLI (1)																				Neg
		CLR (2)																				Neg
		CLOG (2)													1							Neg
	Switzerland	CLC (2)																				Neg
		CLOG (2)														1						Neg
Total No.			1	1	2	2	2	1	10	2	2	51	1	53	12	20	1	1	2	3	2	88

The abbreviation used in the table for the antibiotics are listed below, and the antibiotics are categorized according to class:

[a]Aminoglycosides: GEN, gentamycin; TOB, tobramycin.

[b]Fluoroquinolones: CIP, ciprofloxacin; LEV, levofloxacin; MOX, moxifloxacin.

[c]Glycopeptides: TEI, teicoplanin.

[d]Lincosamides: CLI, clindamycin.

[e]Macrolides: AZI, azithromycin; ERY, erythromycin.

[f]Penicillins: AMP, ampicillin; OXA, oxacillin; PEN, penicillin.

[g]Tetracyclines: TET, tetracycline.

[h]CH, chloramphenicol; FOS, fosfomycin; FUA, fusic acid; NIT, nitrofurantoin; T/S, trimethoprim/sulfamethoxazole.

The different color gradient is based on the number of positive samples. One is the lowest number of positive samples (light yellow) and the highest number of positive samples is in red.

10.3389/fvets.2023.1154550

4.2. Prevalence of AMR in 10 European countries

In recent years, a general increase in AMR has been reported, and this increase is thought to mainly be due to AB misuse and abuse in agriculture (44, 45). In the worst-case scenario, this AMR could be transmitted to humans, which would aggravate the existing AMR situation faced in human medicine (29). Nevertheless, ABs continue to be a key factor in the treatment of bovine mastitis caused by *S. aureus* (11, 23, 46). Hence, it is vital to use the AB to which an isolate is fully susceptible to guarantee the successful of the therapy. According to our research, despite the large amounts of ABs that have been used to treat bovine IMIs in the past, the AMR status of *S. aureus* isolates from European mastitis cases is promising (47). In fact, all strains were susceptible to most of the 31 ABs tested. AMR was only observed for penicillin (25.6%) ampicillin (24.2%), chloramphenicol (9.5%), clindamycin (4.7%), and tetracyclines (5.7%). Penicillin, chloramphenicol, and tetracycline are ABs that have been widely used in cattle medicine over the past 50 years (48–50). These findings demonstrate and confirm previous observations that the regular use of ABs against *S. aureus* increases the possibility of the emergence of AMR (51, 52). This is in line with our observations that AMR was absent for all ABs whose application, at least in Switzerland, has not been approved for treatment of cattle (50); this is true for all the ABs on the World Health Organization (WHO) reserve list (53, 54), such as daptomycin, linezolid, and fifth-generation cephalosporins. This also holds true for most of the ABs on the WHO watch list (54) including quinolones, carbapenems, fusidic acid (one strain resistant), rifampin, teicoplanin, tobramycin (one strain resistant), and vancomycin; the exceptions were the very limited macrolide (0.9%) and tetracycline (5.7%) resistance. Interestingly, all strains were susceptible to oxacillin and all (except two strains) were susceptible to gentamicin and to trimethoprim/sulfamethoxazole. Obviously, these ABs are still efficient despite their extensive use in cattle medicine. In Switzerland, trimethoprim/sulfamethoxazole is exclusively used as a systemic treatment and is not applied intramammarily (55), so IMI-associated *S. aureus* strains are not in direct contact with this AB, which explain their susceptibility. This contrasts with oxacillin and gentamicin, which have been widely used for the treatment of IMIs in the past 40 years. The minimal AMR prevalence for these AB in bovine *S. aureus* demonstrates that the occurrence of AMRs is not only a matter of frequent use (penicillin and tetracycline). But that it considerably depends on the AB class (aminoglycosides) and even on the properties of the individual compound (oxacillin and penicillin). Considering MRSA, the present study and the one by El Garch co-authors (47) show that MRSA are of no to little concern in the field of bovine mastitis. These observations are in clear contrast to the situation in Swiss human isolates, where the prevalence of MRSA is 6.6% (56). These findings largely suggest that bovine mastitis isolates are not the source of MRSA at the human level.

With a prevalence of 25.6%, penicillin resistance was the most frequently observed type of AMR in our study. This finding aligns with the results of another European study (25.5%) (47) and of an international study (19.4%) that included strains from South America (Argentina, Brazil, and Colombia), South Africa, and the USA (57). Penicillin was introduced for the treatment of bovine mastitis as early as 1945 (58) and is still considered the AB of choice to treat Gram-positive mastitis pathogens (29), which demonstrates its importance in modern medicine.

It is worth noting that resistance to penicillin in bovine *S. aureus* strains can be misreported, as recently shown by Ivanovic et al. (33). Using whole genome sequencing and bioinformatics, the authors showed that the MIC assay, which was also used in the present study, provided the correct results, while analyses conducted using disk diffusion and PCR methods were remarkably flawed (33). Depending on the protocol applied, either too many false negative or false positive results were generated, and false positive results were also generated when the mPCR method was used to assed the *bla* operon genes (*blaI*, *blaR1*, *blaZ*). In the case of mPCR, it turned out that the discrepant results were always associated with *S. aureus* CC8/CLB strains. Further genomic analyses of these strains showed that the promoter of the *bla* operon present in the plasmid of the *S. aureus* CC8/CLB strains was inactivated by a 31-bp deletion (33); consequently, the *bla* operon genes that mediate penicillin resistance, were no longer expressed but could be detected by mPCR. The same association, which was explicit for the CC8/CLB strain, between negative MIC values and positive mPCR results was confirmed in the present study. Compared to the previous study (33), however, considerably more strains were evaluated here.

The present study further revealed two more very relevant findings. First, for the three major CCs (CC8, CC97, and CC705) and CLs (CLB, CLC, and CLR), penicillin resistance was highly dependent on the CC and CL. In fact, the CC705 and CLC strains were always susceptible to penicillin whereas penicillin resistance in the CC97 and CLR strains was high, at 50 and 37%, respectively. Penicillin resistance in the CC8 and CLB strains was intermediate, at 14 and 13%, respectively. Importantly, the CC705 and CLC strains were not only susceptible to penicillin but, with two exceptions, also to all other ABs, a property that was not observed for strains in the other CCs and CLs. Second, the prevalence of penicillin resistance is country dependent. Indeed, resistance to penicillin was particularly observed in strains from Austria, Belgium, Germany, and Ireland; however, it was completely absent in strains from Slovenia and Switzerland. It is likely that resistance to other ABs (i.e., chloramphenicol and tetracycline) is also country dependent, although this could not be assessed in the present study because the rate of resistance of other ABs were low and the data set was too small for statistical analyses. Unfortunately, the reason for the difference in penicillin resistance among countries remains unknown and requires further clinical and epidemiological investigations. Nevertheless, our findings demonstrate at least for penicillin, that the prevalence of AMR is country dependent and that caution is required when interpreting results. However, from a statistical and interpretative perspective there are no concerns about analyzing data from multiple-countries as a single entity. In our case, this means that, except for penicillin resistance, the observed prevalence of AMR reflects that at the European level.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary material, further inquiries can be directed to the corresponding author.

Author contributions

HG conceived and planned the experiments. GN and LR performed the experimental analyses. AR and HG performed the statistical analyses. All authors discussed the results, and critically revised and approved the final submitted manuscript.

Funding

This work was financed by the Agroscope (Bern, Switzerland) research program. Agroscope is the center of excellence for agricultural research of the Swiss administration. Open access funding by Agroscope.

Acknowledgments

The authors thank Aurélie Cosandey and Renate Boss for their valuable support and for sharing samples collected during previous studies.

Conflict of interest

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.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fvets.2023.1154550/full#supplementary-material

References

1. Halasa T, Nielen M, De Roos AP, Van Hoorne R, de Jong G, Lam TJ, et al. Production loss due to new subclinical mastitis in dutch dairy cows estimated with a test-day model. *J Dairy Sci.* (2009) 92:599–606. doi: 10.3168/jds.2008-1564

2. Ruegg PL. A 100-year review: mastitis detection, management, and prevention. *J Dairy Sci.* (2017) 100:10381–97. doi: 10.3168/jds.2017-13023

3. Heiniger D, van den Borne BH, Lechner I, Tschopp A, Strabel D, Steiner A, et al. [Cost-benefit analysis of an intervention to improve udder health in swiss dairy farms]. *Schweiz Arch Tierheilkd.* (2014) 156:473–81. doi: 10.1024/0036-7281/a000634

4. Peton V, Le Loir Y. *Staphylococcus aureus* in veterinary medicine. *Infect Genet Evol.* (2014) 21:602–15. doi: 10.1016/j.meegid.2013.08.011

5. Leuenberger A, Sartori C, Boss R, Resch G, Oechslin F, Steiner A, et al. Genotypes of *Staphylococcus aureus*: on-farm epidemiology and the consequences for prevention of intramammary infections. *J Dairy Sci.* (2019) 102:3295–309. doi: 10.3168/jds.2018-15181

6. Sears PM, McCarthy KK. Management and treatment of *Staphylococcal mastitis*. *Vet Clin North Am Food Anim Pract.* (2003) 19:171–85. doi: 10.1016/S0749-0720(02)00079-8

7. Fournier C, Kuhnert P, Frey J, Miserez R, Kirchhofer M, Kaufmann T, et al. Bovine *Staphylococcus aureus*: association of virulence genes, genotypes and clinical outcome. *Res Vet Sci.* (2008) 85:439–48. doi: 10.1016/j.rvsc.2008.01.010

8. Graber HU, Naskova J, Studer E, Kaufmann T, Kirchhofer M, Brechbuhl M, et al. mastitis-related subtypes of bovine *Staphylococcus aureus* are characterized by different clinical properties. *J Dairy Sci.* (2009) 92:1442–51. doi: 10.3168/jds.2008-1430

9. Cremonesi P, Pozzi F, Raschetti M, Bignoli G, Capra E, Graber HU, et al. Genomic characteristics of *Staphylococcus aureus* strains associated with high within-herd prevalence of intramammary infections in dairy cows. *J Dairy Sci.* (2015) 98:6828–38. doi: 10.3168/jds.2014-9074

10. Capra E, Cremonesi P, Pietrelli A, Puccio S, Luini M, Stella A, et al. Genomic and transcriptomic comparison between *Staphylococcus aureus* strains associated with high and low within herd prevalence of intra-mammary infection. *BMC Microbiol.* (2017) 17:21. doi: 10.1186/s12866-017-0931-8

11. Sartori C, Boss R, Bodmer M, Leuenberger A, Ivanovic I, Graber HU. Sanitation of *Staphylococcus aureus* genotype B-positive dairy herds: a field study. *J Dairy Sci.* (2018) 101:6897–914. doi: 10.3168/jds.2017-13937

12. Sartori C, Perreten V, Ivanovic I, Hardi-Landerer MC, Graber HU. Short communication: lack of intramammary niche recolonization during a sanitation program for the contagious mastitis pathogen *Staphylococcus aureus* genotype B. *J Dairy Sci.* (2018) 101:8296–300. doi: 10.3168/jds.2017-14313

13. Voelk V, Graber HU, van den Borne BH, Sartori C, Steiner A, Bodmer M, et al. A longitudinal study investigating the prevalence of *Staphylococcus aureus* genotype B in seasonally communal dairy herds. *J Dairy Sci.* (2014) 97:4184–92. doi: 10.3168/jds.2013-7291

14. van den Borne BHP, Graber HU, Voelk V, Sartori C, Steiner A, Haerdi-Landerer MC, et al. A longitudinal study on transmission of *Staphylococcus aureus* genotype B in swiss communal dairy herds. *Prev Vet Med.* (2017) 136:65–8. doi: 10.1016/j.prevetmed.2016.11.008

15. Luini M, Cremonesi P, Magro G, Bianchini V, Minozzi G, Castiglioni B, et al. Methicillin-resistant *Staphylococcus aureus* (Mrsa) is associated with low within-herd prevalence of intra-mammary infections in dairy cows: genotyping of isolates. *Vet Microbiol.* (2015) 178:270–4. doi: 10.1016/j.vetmic.2015.05.010

16. Maisano AM, Luini M, Gazzola A, Sala L, Vezzoli F, Bertocchi L, et al. Staphylococcus aureus adlb gene is associated with high prevalence of intramammary infection in dairy herds of northern Italy: A cross-sectional study. *J Dairy Sci.* (2023). doi: 10.3168/jds.2022-22496. [Epub ahead of print].

17. Feil EJ, Cooper JE, Grundmann H, Robinson DA, Enright MC, Berendt T, et al. How clonal is *Staphylococcus aureus*? *J Bacteriol.* (2003) 185:3307–16. doi: 10.1128/JB.185.11.3307-3316.2003

18. Boss R, Cosandey A, Luini M, Artursson K, Bardiau M, Breitenwieser F, et al. Bovine *Staphylococcus aureus*: subtyping, evolution, and zoonotic transfer. *J Dairy Sci.* (2016) 99:515–28. doi: 10.3168/jds.2015-9589

19. Cosandey A, Boss R, Luini M, Artursson K, Bardiau M, Breitenwieser F, et al. *Staphylococcus aureus* genotype B and other genotypes isolated from cow milk in European countries. *J Dairy Sci.* (2016) 99:529–40. doi: 10.3168/jds.2015-9587

20. Schwendimann L, Merda D, Berger T, Denayer S, Feraudet-Tarisse C, Kläui AJ, et al. Staphylococcal enterotoxin gene cluster: prediction of enterotoxin (Seg and Sei) production and of the source of food poisoning on the basis of Vsaβ typing. *Appl Environ Microbiol.* (2021) 87:e0266220. doi: 10.1128/AEM.02662-20

21. Kläui AJ, Boss R, Graber HU. Characterization and comparative analysis of the *Staphylococcus aureus* genomic island Vsa: an *in silico* approach. *J Bacteriol.* (2019) 201:e00777. doi: 10.1128/JB.00777-18

22. Sartori C, Boss R, Ivanovic I, Graber HU. Development of a new real-time quantitative Pcr assay for the detection of *Staphylococcus aureus* genotype B in cow milk, targeting the new gene Adlb. *J Dairy Sci.* (2017) 100:7834–45. doi: 10.3168/jds.2017-12820

23. Algharib SA, Dawood A, Xie S. Nanoparticles for treatment of bovine *Staphylococcus aureus*. mastitis. *Drug Deliv.* (2020) 27:292–308. doi: 10.1080/10717544.2020.1724209

24. Sol J, Sampimon OC, Snoep JJ, Schukken YH. Factors associated with bacteriological cure during lactation after therapy for subclinical mastitis caused by *Staphylococcus aureus*. *J Dairy Sci.* (1997) 80:5. doi: 10.3168/jds.S0022-0302(97)76243-X

25. Sol J, Sampimon OC, Barkema HW, Schukken YH. Physiology and management factors associated with cure after therapy of clinical mastitis caused by *Staphylococcus aureus*. *J Dairy Sci.* (2000) 83:6. doi: 10.3168/jds.S0022-0302(00)74875-2

26. Gruet P, Maincent P, Berthelot X, Kaltsatos V. Bovine mastitis and intramammary drug delivery: review and perspectives. *Adv Drug Deliv Rev.* (2001) 50:14. doi: 10.1016/s0169-409x(01)00160-0

27. Barkema HW, Schukken YH, Zadoks RN. Invited review: the role of cow, pathogen, and treatment regimen in the therapeutic success of bovine *Staphylococcus aureus* mastitis. *J Dairy Sci.* (2006) 89:18. doi: 10.3168/jds.S0022-0302(06)72256-1

28. Pyorala S. *In vitro* studies as supporting evidence on the efficacy of antimicrobials for mastitis treatment. *J Appl Microbiol.* (2009) 107:2116–7. doi: 10.1111/j.1365-2672.2009.04538.x

29. Preine F, Herrera D, Scherpenzeel C, Kalmus P, McCoy F, Smulski S, et al. Different European perspectives on the treatment of clinical mastitis in lactation. *Antibiotics.* (2022) 11:1107. doi: 10.3390/antibiotics11081107

30. NMC. *Bovine Mastitis Pathogens and Trends in Resistance to Antibacterial Drugs*. (2004). Available online at: https://www.nmconline.org/wp-content/uploads/2020/09/Bovine-Mastitis-Pathogens-and-Trends-in-Resistance-to-Antibacterial-Drugs-FINAL-May-2020.pdf (accessed 2022).

31. Swiss Antibiotic Resistance Report 2020. *Usage of Antibiotics and Occurrence of Antibiotic Resistance in Switzerland*. (2020). 2020-OEG-64.

32. Molineri AI, Camussone C, Zbrun MV, Suarez Archilla G, Cristiani M, Neder V, et al. Antimicrobial resistance of *Staphylococcus aureus* isolated from bovine mastitis: systematic review and meta-analysis. *Prev Vet Med.* (2021) 188:105261. doi: 10.1016/j.prevetmed.2021.105261

33. Ivanovic I, Boss R, Romano A, Guedon E, Le-Loir Y, Luini M, et al. Penicillin resistance in bovine *Staphylococcus aureus*: genomic evaluation of the discrepancy between phenotypic and molecular test methods. *J Dairy Sci.* (2023) 106:462–75. doi: 10.3168/jds.2022-22158

34. Lowy FD. Antimicrobial resistance: the example of *Staphylococcus aureus*. *J Clin Invest.* (2003) 111:1265–73. doi: 10.1172/JCI18535

35. Llarrull LI, Toth M, Champion MM, Mobashery S. Activation of Blar1 protein of methicillin-resistant *Staphylococcus aureus*, its proteolytic processing, and recovery from induction of resistance. *J Biol Chem.* (2011) 286:38148–58. doi: 10.1074/jbc.M111.288985

36. Clarke SR, Dyke KGH. The signal transducer (Blari) and the repressor (Blai) of the *Staphylococcus aureus*?-lactamase operon are inducible. *Microbiology.* (2001) 147:7. doi: 10.1099/00221287-147-4-803

37. *Breakpoint Tables for Interpretation of Mics and Zone Diameters*. EUCAST (2022) (accessed January 01, 2022).

38. CLSI. *M100-Performance Standards for Antimicrobial Susceptibility Testing*. Wayne, PA: Informational Supplements ECaLSI (2012).

39. R Core Team. *R: A Language and Environment for Statistical Computing. R 766 Foundation for Statistical Computing V. A Language and Environment for Statistical Computing*. In: 766 R, editor. Vienna: Austria: Foundation for Statistical Computing (2021).

40. Yule GU. On the methods of measuring association between two attributes. *J R Stat Soc.* (1912) 75:73. doi: 10.2307/2340126

41. Cohen J. A coefficient of agreement for nominal scales. *Educ Psychol Measur.* (1960) 20:9. doi: 10.1177/001316446002000104

42. Gazzola A, Maisano AM, Bianchini V, Vezzoli F, Romanò A, Graber HU, et al. Short communication: characterization of staphylococcus aureus from bulk tank milk of dairy cattle in Lombardy (Northern Italy). *J Dairy Sci.* (2020) 103:2685. doi: 10.3168/jds.2019-17181

43. Malachowa N, DeLeo FR. Mobile genetic elements of *Staphylococcus aureus*. *Cell Mol Life Sci.* (2010) 67:3057–71. doi: 10.1007/s00018-010-0389-4

44. Manyi-Loh C, Mamphweli S, Meyer E, Okoh A. Antibiotic use in agriculture and its consequential resistance in environmental sources: potential public health implications. *Molecules.* (2018) 23:795. doi: 10.3390/molecules23040795

45. Mann A, Nehra K, Rana JS, Dahiya T. Antibiotic resistance in agriculture: perspectives on upcoming strategies to overcome upsurge in resistance. *Curr Res Microb Sci.* (2021) 2:100030. doi: 10.1016/j.crmicr.2021.100030

46. Ruegg PL. Making antibiotic treatment decisions for clinical mastitis. *Vet Clin North Am Food Anim Pract.* (2018) 34:413–25. doi: 10.1016/j.cvfa.2018.06.002

47. El Garch F, Youala M, Simjee S, Moyaert H, Klee R, Truszkowska B, et al. Antimicrobial susceptibility of nine udder pathogens recovered from bovine clinical mastitis milk in Europe 2015-2016: vetpath results. *Vet Microbiol.* (2020) 245:108644. doi: 10.1016/j.vetmic.2020.108644

48. Cargill CF, Bootes BW. Field observations on the control of Staphylococcal Bovine Mastitis. *Aust Vet J.* (1970) 46:249–52. doi: 10.1111/j.1751-0813.1970.tb15769.x

49. *Third Joint Inter-Agency Report on Integrated Analysis of Consumption of Antimicrobial Agents and Occurrence of Antimicrobial Resistance in Bacteria from Humans and Food-Producing Animals in the Eu/Eea: Jiacra Iii 2016-2018*. (2021). Available online at: https://www.ncbi.nlm.nih.gov/pubmed/34221148 (accessed October 01, 2022).

50. *Umsichtiger Einsatz Von Antibiotika Bei Rindern, Schweinen, Kleinen Wiederkauern Und Neuweltkameliden*. BLV (2022). Available online at: https://www.blv.admin.ch/blv/de/home/tiere/tierarzneimittel/antibiotika/nationale-strategie-antibiotikaresistenzen--star--/sachgemaesser-antibiotikaeinsatz.html (accessed March, 2022).

51. Prestinaci F, Pezzotti P, Pantosti A. Antimicrobial resistance: a global multifaceted phenomenon. *Pathog Glob Health.* (2015) 109:309–18. doi: 10.1179/2047773215Y.0000000030

52. Guo Y, Song G, Sun M, Wang J, Wang Y. Prevalence and therapies of antibiotic-resistance in *Staphylococcus aureus*. *Front Cell Infect Microbiol.* (2020) 10:107. doi: 10.3389/fcimb.2020.00107

53. *Critically Important Antimicrobials for Human Medicine (6th Revision) 2018—Ranking of Medically Important Antimicrobials for Risk Management of Antimicrobial Resistance Due to Non-Human Use* (2018).

54. World Health Organization. *WHO Access, Watch, Reserve (AWaRe) Classification of Antibiotics for Evaluation and Monitoring of Use, 2021*. World Health Organization (2021). Available online at: https://apps.who.int/iris/handle/10665/345555

55. Toxikologie IfVu. *Wirkstoff: Trimethoprim - Präparate*. (2022). Available online at: https://www.vetpharm.uzh.ch/Zubereitungen/000000000073/8705_99.html (accessed October 01, 2022).

56. *Resistance Data Human Medicine ANRESIS*. (2022). Available online at: https://www.anresis.ch/antibiotic-resistance/resistance-data-human-medicine (accessed October 01, 2022).

57. Monistero V, Barberio A, Biscarini F, Cremonesi P, Castiglioni B, Graber HU, et al. Different distribution of antimicrobial resistance genes and virulence profiles of *Staphylococcus aureus*. strains isolated from clinical mastitis in six countries. *J Dairy Sci.* (2020) 103:3431–46. doi: 10.3168/jds.2019-17141

58. Murnane D. A preliminary report on the treatment of clinical and subclinical streptococcal and staphylococcal infection of bovine udder with penicillin. *Austral Vet J.* (1945) 21:9. doi: 10.1111/j.1751-0813.1945.tb06422.x

frontiers | Frontiers in Veterinary Science

TYPE Review
PUBLISHED 15 June 2023
DOI 10.3389/fvets.2023.1158588

OPEN ACCESS

EDITED BY
João Pedro Rueda Furlan,
University of São Paulo, Brazil

REVIEWED BY
Fábio Sellera,
University of São Paulo, Brazil
Benjamin Andrew Evans,
University of East Anglia, United Kingdom

*CORRESPONDENCE
Alma L. Guerrero-Barrera
✉ lilian.guerrero@edu.uaa.mx

RECEIVED 04 February 2023
ACCEPTED 23 May 2023
PUBLISHED 15 June 2023

CITATION
Ramírez-Castillo FY, Guerrero-Barrera AL and Avelar-González FJ (2023) An overview of carbapenem-resistant organisms from food-producing animals, seafood, aquaculture, companion animals, and wildlife.
Front. Vet. Sci. 10:1158588.
doi: 10.3389/fvets.2023.1158588

An overview of carbapenem-resistant organisms from food-producing animals, seafood, aquaculture, companion animals, and wildlife

Flor Y. Ramírez-Castillo[1], Alma L. Guerrero-Barrera[1]* and Francisco J. Avelar-González[2]

[1]Laboratorio de Biología Celular y Tisular, Departamento de Morfología, Centro de Ciencias Básicas, Universidad Autónoma de Aguascalientes, Aguascalientes, Ags, Mexico, [2]Laboratorio de Estudios Ambientales, Departamento de Fisiología y Farmacología, Centro de Ciencias Básicas, Universidad Autónoma de Aguascalientes, Aguascalientes, Ags, Mexico

Carbapenem resistance (CR) is a major global health concern. CR is a growing challenge in clinical settings due to its rapid dissemination and low treatment options. The characterization of its molecular mechanisms and epidemiology are highly studied. Nevertheless, little is known about the spread of CR in food-producing animals, seafood, aquaculture, wildlife, their environment, or the health risks associated with CR in humans. In this review, we discuss the detection of carbapenem-resistant organisms and their mechanisms of action in pigs, cattle, poultry, seafood products, companion animals, and wildlife. We also pointed out the One Health approach as a strategy to attempt the emergency and dispersion of carbapenem-resistance in this sector and to determine the role of carbapenem-producing bacteria in animals among human public health risk. A higher occurrence of carbapenem enzymes in poultry and swine has been previously reported. Studies related to poultry have highlighted *P. mirabilis*, *E. coli*, and *K. pneumoniae* as NDM-5- and NDM-1-producing bacteria, which lead to carbapenem resistance. OXA-181, IMP-27, and VIM-1 have also been detected in pigs. Carbapenem resistance is rare in cattle. However, OXA- and NDM-producing bacteria, mainly *E. coli* and *A. baumannii,* are cattle's leading causes of carbapenem resistance. A high prevalence of carbapenem enzymes has been reported in wildlife and companion animals, suggesting their role in the cross-species transmission of carbapenem-resistant genes. Antibiotic-resistant organisms in aquatic environments should be considered because they may act as reservoirs for carbapenem-resistant genes. It is urgent to implement the One Health approach worldwide to make an effort to contain the dissemination of carbapenem resistance.

KEYWORDS

carbapenem resistance, One Health approach, food-producing animals, carbapenemase producers, transmission

1. Introduction

Carbapenems are broad-spectrum beta (β)-lactam antimicrobials primarily used to treat severe human infections. These antibiotics are considered one of the most reliable drugs and the last line of therapy for infections caused by multidrug-resistant Gram-negative and Gram-positive bacteria. Carbapenems possess a broad-spectrum antibacterial activity and

have a structure defined by a carbapenem coupled with a β-lactam ring. In addition, these antibiotics contain a carbon instead of a sulfone in the fourth position of the thiazolidine moiety β-lactam ring, which confers protection against most β-lactamases (1).

The widespread use of these antibiotics has increased to a worldwide emergence of carbapenem-resistant organisms (CROs), which constitute a critical growing public health threat, mainly in hospital settings, as their prescription has escalated in recent years and used for treating life-threatening infections. Carbapenem-resistant *Enterobacteriaceae* (CRE) [i.e., Carbapenem-resistant *Klebsiella pneumoniae* (CR-Kp), Carbapenem-resistant *Escherichia coli* (CREc), *Enterobacter* spp., *Serratia* spp., and *Proteus* spp.] are some of the most critical CROs because they are associated with infections that lead to high mortality and have the potential to spread carbapenem resistance via mobile genetic elements (2). In addition, non-fermenting bacteria such as carbapenem-resistant *Acinetobacter baumannii* (CRAB) and carbapenem-resistant *Pseudomonas aeruginosa* (CRPA) have also emerged as critical CROs (3–5).

The four significant carbapenem mechanisms of resistance include the presence of β-lactamase enzymes called carbapenemases, which hydrolyze carbapenem antibiotics encoded on chromosomal or plasmid genes, the synergistic effect of other β-lactamases with bacterial cell membrane permeability due to alterations or mutations in porins, the low affinity of penicillin-binding proteins (PBPs) in different species, and the increased efflux pumps (6, 7).

Among carbapenem-producing (CP) microorganisms, different classes of carbapenemases are found under the Ambler classification (Table 1). Class A or serine carbapenemases can hydrolyze all β-lactams, carbapenems, cephalosporins, penicillins, and aztreonam but are inhibited by clavulanate and tazobactam. Additionally, a combination of the newly cephalosporin antibiotic, ceftaroline, and avibactam (ceftaroline/avibactam) has been shown to produce activity against *Enterobacteriaceae* KPC producers (19). *Klebsiella pneumoniae* carbapenemase (KPC), not metalloenzyme carbapenemase (NMC-A), imipenem-hydrolyzing beta-lactamase (IMI), and *Serratia marcescens* enzyme (SME) are representative of this class. KPC enzymes confer resistance to all β-lactamases and other types of antibiotics, such as quinolones and aminoglycosides. They are only partially inhibited by β-lactamase inhibitors such as clavulanic acid, tazobactam, and boronic acid (1–7).

Class B or metallo-β-lactamases (MBLs) use a zinc ion (Zn^{2+}) to hydrolyze the β-lactam ring. They confer resistance to all β-lactam antibiotics but are susceptible to aztreonam and β-lactam inhibitors such as ethylenediaminetetraacetic acid (EDTA). Most clinically important MBLs belong to the six different families [imipenem (IMP), Verona integron-encoded metallo-β-lactamase (VIM), New Delhi metallo-β-lactamase (NDM), São Paulo metallo-β-lactamases (SPM), German imipenemase (GIM), and Seoul imipenemase (SIM)]. This type of enzyme has been identified in clinically relevant species such as Enterobacterales, *Acinetobacter*, and *P. aeruginosa*. It is commonly expressed from mobile elements such as integrons, plasmids, and transposons (1–7).

Class D serine oxacillinases (OXAs) have been commonly detected worldwide. They have hydrolytic activity against β-lactams, high activity against penicillin, and weak activity against extended-spectrum cephalosporins and carbapenems. OXA-23 and OXA-48 are variants widely dispersed globally. OXA-23 is almost strictly restricted to *Acinetobacter* spp., wily OXA-48 was found among *K. pneumoniae* and Enterobacterales (20).

CROs are usually only susceptible to polymyxins (e.g., colistin), fosfomycin, and tigecycline, while colistin resistance in carbapenem-resistance *K. pneumoniae* (CR-Kp) isolates has been recently reported (21–24). Thus, colistin combination therapy is more frequently used to treat drug-resistant bacteria with significantly lower treatment failure rates (24).

Carbapenems such as imipenem, meropenem, ertapenem, doripenem, biapenem, faropenem, and panipenem have been approved for use in human clinical settings (6). However, carbapenems are not licensed in livestock or veterinary fields; therefore, carbapenem resistance is not commonly tested in animals. However, extended-spectrum beta-lactamases (ESBL), including ceftiofur, cefquinome, cefpodoxime, cefoperazone, and cefovecin, are commonly found in this sector. Additionally, ceftiofur, a third-generation cephalosporin, is the main cephalosporin used in veterinary fields and has been approved for treating bacterial infections in food-producing animals (i.e., pneumonia, arthritis, septicemia, meningitis, metritis, and polyserositis) (25). For this reason, these antibiotics could provide selection pressure that favors the expression of carbapenem-resistant (CR) strains.

Indeed, the author's statement in the Scientific Opinion on carbapenem resistance in food-animal ecosystems that "diagnostic isolates of veterinary origin classified as microbiologically resistant to third- and fourth-generation cephalosporins based on epidemiological cutoff values, should be subjected to phenotypic testing for carbapenem resistance and carbapenemase production and subsequent molecular identification and characterization of carbapenemase production genes because they favor the emergence of carbapenem-resistant isolates" (26).

Many studies have reported CROs in livestock, seafood, companion animals, wildlife, and their environments (7, 27–30). As animals have been identified as a relevant source of multidrug-resistant (MDR) bacteria, they can serve as reservoirs for carbapenem-resistant bacteria, and foodborne routes and emission in the environment through the excreta and the subsequent exposure of humans via the environment serve as transmission pathways for carbapenem-resistance genes from animals to humans and vice versa. Furthermore, their incidence may be underestimated because there is usually no surveillance, which dismisses the potential risks to human health. This review aimed to summarize the occurrence and molecular mechanisms of resistance in carbapenem-resistant organisms in food-producing animals, seafood, companion animals, and wildlife to address the importance of antimicrobial resistance surveillance in these sectors, resistance dissemination to the environment and humans, and potential public health risks.

2. Carbapenem-resistant from seafood and aquaculture

Carbapenem-resistance genes have been identified in isolates from aquatic environments. Generally, resistance to carbapenems in bacteria from aquatic systems such as *Vibrio* and *Shewanella* spp.,

TABLE 1 Carbapenem-enzymes among carbapenem-resistance organisms on Ambler classification.

Ambler class		Class	Encoded		References
			Chromosomal	Plasmid	
Serin carbapenemases	Uses the amino acid serine for beta-lactam hydrolysis by forming an acyl enzyme	A	NmcA (Non-metallo carbapenemase A)	KPC (*K. pneumoniae* carbapenemases), 23 variants	(8–13)
			SME (*Serratia marcescens* enzyme)	GES (Guiana extended spectrum **β**-lactamase), 26 variants	
			IMI (Imipenem-hydrolyzig **β**-lactamase)	IMI 1–6	
			SFC-1 (*Serratia fonticola* carbapenemase)	VCC-1 (from *Vibrio cholerae*)	
			BIC-1 (Bicêtre Carbapenemae)	FRI-1 (from *Enterobacter cloacae*)	
			PenA (penicilinase from *Pseudomonas cepacia*)		
			FPH-1 (from *Francisella philomiragia*)		
			SHV (natural class A β-lactamase of *K. pneumoniae*)		
			KPC (*K. pneumoniae* carbapenemase)	1–3	
			GES (Guiana extended spectrum **β**-lactamase)	20 variants	
		D	OXA	400 enzymes classified in 12 subgroups: (a) OXA-23, (b) OXA-24/40, (c) OXA-48, (d) OXA-51, (e) OXA-58, (f) OXA-134a, (g) OXA-143, (h) OXA-211, (i) OXA-213, (j) OXA-214, (k) OXA-229, and (l) OXA-235, OXA-181 and OXA-497	(9)
Metallo-carbapenemases	Requires at least one active-site zinc ions to facilitate beta-lactam hydrolysis	B	NDMs (New Delhi metallo-beta-lactamases)	NDMs (New Delhi metallo-**β**-lactamases), 17 variants	(14)
				VIM (Verona integron-encoded metallo-**β**-lactamase), 14 variants	
				IMP-type (Imipenem resistant *Pseudomonas*), 55 variants	
				VMB-1, VMB-2 (*Vibrio* MBL)	(15)
				GIM (German imipenemase)	(13, 14)
				SIM (Seoul imipenase)	(16)
				VAM-1 (*V. alginolyticus* metallo-**β**-lactamase)	(17)
				SPM (São Paulo MBL)	(14)
				DIM-1 (Dutch imipenemase-1)	(18)
				KHM-1 (Kyorin University Hospital MBL-1)	(13)
				TMB (Tripoli MBL-1)	(13)
				FIM (Florence imipenemase)	(18)
				AIM (Adelaide imipenemase)	(18)
				SFH-1 (*Serratia fonticola* carbapenem hydrolase)	(13)
				LMB-1 (Linz metallo-β-lactamase)	(13)

as well as Enterobacterales, is mainly mediated by the production of carbapenemases encoded by chromosomal genes or by plasmids. However, carbapenemases are variable, with Class B enzymes and the enzymes described only on aquatic species such as VMB-1 from *Vibrio alginolyticus* (17, 31) and VMB-2 (32) from *Vibrio diabolicus* on shrimps; as well as the Class A, VCC-1 from non-toxigenic *Vibrio cholerae* on shrimp (8).

In 2017, in Canada, Brouwer et al. isolated the *E. cloacae* complex from shrimp (*Litopenaeus vannamei*) originating in India. The isolated had a ST previously described in companion animals in Japan, ST813, and was positive for the $bla_{\text{IMI}-2}$ gene, which is located in a plasmid p3443-IMI2, which is closely related to IncFII plasmids and pIMI-6, which was described in an *E. cloacae* complex clinical isolate from Canada and carries the carbapenemase $bla_{\text{IMI}-6}$ (33). The same strain displayed the plasmid p3442-FLC-1 that carries the gene encoding a novel class A carbapenemase FLC-1 with close sequence similarity to $bla_{\text{FRI}-1,}$ previously described in imipenem-resistant *E. cloacae* recovered from a clinical patient in France (33). In 2013, OXA-23-producing *A. baumannii,* on fish *Pagellus acarne* harvested in the Mediterranean Sea in Algeria, was reported. The isolate belonged to the widespread sequence type 2 (ST2)/international clone II and harbored aminoglycoside-modifying enzymes [*aac(6′)-Ib* and *aac(3′)-I* genes] as well as the naturally occurring

bla$_{OXA-51-like}$ gene. However, the isolates differed from human clinical strains previously isolated from France and Algeria (34). In 2010, in Brazil, a high percentage of resistance to imipenem (71.43%) in *E. coli* isolated from aquaculture was detected, including isolates from pond water, shrimp tissues, and pond sediment (35). In 2014 and 2015, the occurrence of VIM-2-producing *Pseudomonas fluorescens* isolated from squid in Canada (imported from South Korea) and OXA-48-producing bacteria in seafood from China and Korea were described on the bacterial species *Stenotrophomonas maltophilia*, *Myroides odoratimimus*, *Stenotrophomonas* spp., and *Pseudomonas putida* (36, 37). In 2015, carbapenem-resistant *Enterobacter* spp., derived from imported retail seafood in Canada were detected, including two *Enterobacter cloacae* isolated from shrimp imported from Vietnam harboring *bla*$_{IMI-1}$; one *Enterobacter aerogenes* harboring *bla*$_{IMI-2}$ isolated from shrimp imported from Bangladesh, three *E. cloacae* harboring *bla*$_{IMI-1}$ isolated from clam imported from Vietnam, and two *E. cloacae* harboring *bla*$_{NDM-1}$, *bla*$_{TEM}$, and *bla*$_{OXA-1}$ from clam samples from Vietnam (38). *bla*$_{IMI-2}$ gene was plasmid-mediated; the plasmid contained the IncFII (Yp) replicon, while *bla*$_{NDM-1}$ plasmid contained IncHI2, IncFIb, and IncFII replicons. Six different sequence types of *E. cloacae* were assigned (ST479, ST373, ST477, ST478, ST411, and ST412). The authors showed that the human-source *E. cloacae* ST373 isolate harboring *bla*$_{IMI-1}$ shared >75% similarity with the *E. cloacae* IMI-1 positive isolated from clam. In 2016, VCC-1-producing *Vibrio cholerae* isolated from retail shrimp imported from Canada was also identified (8). In 2016, in Italy, one VIM-1 carbapenemase-producing *E. coli* (ST10) was isolated from a Venus's clam (*Ruditapes philippinarum*) harvested in the Mediterranean Sea with *bla*$_{VIM-1}$ as part of the variable region of a class I integron embedded in a Tn*3*-like transposon that also contained the fluoroquinolone resistance gene *qnrS1*. Interestingly, *E. coli* ST10 is widespread among clinical and animal samples (39). In 2018, six *bla*$_{NDM}$-harboring *Enterobacteriaceae* (four *K. pneumoniae* strains and two *E. coli* strains) from the retail fish market were detected in India, including the variants *bla*$_{NDM-5}$, *bla*$_{NDM-2,}$ and *bla*$_{NDM-1}$. The *bla*$_{NDM}$-positive *E. coli* isolates belonged to the multidrug-resistant widespread ST131 clone, representing extra-intestinal pathogenic *E. coli*. ST131 clone is widely distributed among human clinical isolates from urinary tract infections (UTIs). Moreover, they found that all the isolates were resistant to all ***β***-lactam antibiotics, quinolones, trimethoprim-sulfamethoxazole, chloramphenicol, and tetracycline (40). In China, several isolates of *Vibrio* spp. were found to be resistant to imipenem and meropenem. Isolates from shrimps of seafood carried the genes *bla*$_{NDM-1}$, *bla*$_{VIM-1}$ (31), and *bla*$_{VMB-2}$ identified on a plasmid-borne composite transposon IS*Shfr9*-IS*CR1*-*bla*$_{VMB-2}$-*bla*$_{CARB-12}$-*aadA1*-IS*Shfr9*, where IS*Shfr9* was found to be disseminated in multidrug-resistant (MDR) pathogens (32); as well as producing the enzymes VMB-1 encoded by a gene *bla*$_{VMB-1}$ located in an integron bearing, highly transmissible IncC type plasmid, namely pVB1796 (15), and VAM-1 located in a conjugative plasmid, namely, pC1579 (17). Other studies in Korea have reported carbapenem-resistant *Vibrio* spp., isolates from shrimp (41), cockles (42), or hard-shell mussels harboring *bla*$_{OXA}$ genes (43). In Europe, in 2018 and 2017, Italy reported *V. cholerae* resistant to meropenem (44) and *V. vulnificus* isolated from shellfish resistant to imipenem and meropenem (45). KPC-3-producing *E. coli* in mussels (*Mytilus galloprovincialis*) and OXA-23-producing *A. baumannii* ST2 isolates from mussels and oysters (*Crassostrea gigas*) were also reported in Tunisia in 2016 (46, 47). The *bla*$_{KPC-3}$ gene was identified on an ∼180 kb IncFII plasmid carrying Tn*4401d* transposon and belonged to the ST167 phylogroup A of the ST10 complex. Interestingly, the authors mention that the predominance of *bla*$_{KPC-3}$ in Portugal was also associated with the spread of an IncF plasmid carrying Tn*4401d*. The ST10 complex was reported previously in a hospital from the US to spread *bla*$_{KPC}$ genes. In 2018, France reported the isolation of NDM-1-producing *V. parahaemolyticus* ST864 from a shelled shrimp tail imported from Vietnam, which harbored the epidemic plasmid IncA/C (48). In South America and Ecuador in 2015, *Vibrio* spp., resistant to imipenem, was isolated from shrimp from seawater (49). In 2020, *E. coli*, *Enterobacter cloacae* complex, and *K. pneumoniae* were found in tilapia fish from Egyptian fish farms carrying *bla*$_{KPC}$, *bla*$_{OXA-48}$, and *bla*$_{NDM}$ (50). In 2018, in Taiwan, carbapenem-resistant *Shewanella algae* were isolated from small abalone (*Haliotis diversicolor*) harboring genes encoding OXA-55 and multiple antibiotic-resistance genes including *dfrA3* (trimethoprim resistance), *tet* (35) (tetracycline resistance), and *qnrA3* (quinolone resistance); and the *pmrCAB* operon, which has been shown to mediate resistance to colistin (51). In Taiwan in 2019, carbapenem-resistant *S. algae* carrying *bla*$_{OXA-55}$ with multiple genes encoding efflux pumps was detected in Asian hard clam (*Meretrix lusoria*) (52). In 2020 in Italy, OXA-55-like producing *S. algae* was found (53).

All the previous information demonstrated the presence of CROs in seafood and aquaculture. Even when bacteria in aquatic environments are mainly non-pathogenic, their occurrence highlights the relevance of the food production chain in the global spread of antibiotic-resistance genes. Moreover, it is crucial to consider the seafood market, where countries can consume a specific product imported from a separate region by another country, which could have different regulations for antimicrobial resistance surveillance in food. Canada is one example of this since shrimp consumed by Canadians are imported from Asian countries. However, carbapenemase genes have been detected in these products. Examples include the isolation of *Enterobacter cloacae* or *Enterobacter aerogenes* harboring *bla*$_{IMI-1}$, *bla*$_{IMI-2}$, or *bla*$_{NDM-1}$ genes in retail seafood (39, 54).

In addition, it is remarkably the high amount of antibiotics used and their multiple classes in aquaculture for prophylactic purposes or metaphylactic treatment. Quinolones, tetracyclines, amphenicols, and sulfonamides are the most commonly used classes (55); however, they also include aminoglycosides, antimycobacterial (rifampin), beta-lactams (aminopenicillins and cephalosporins), and polymyxins. This factor could promote selective pressure for the emergence of antimicrobial resistance and the selection of multidrug-resistant organisms among seafood animals. Indeed, global antimicrobial consumption is estimated to reach 13,600 tons by 2030 (55). Additionally, antibiotic residues are difficult to eliminate by water treatment plants and can be discharged into water flows, thus being a source of antimicrobial resistance genes.

Moreover, fish do not effectively metabolize antibiotics; thus, the active substance passes into the environment in the feces. Indeed, it has been suggested that ∼70–80% of the antibiotics applied in aquaculture are dispersed into water systems (37), which might provide a selection and enrichment mechanism for resistant bacteria (22). Studies reporting the occurrence of carbapenem-resistant genes worldwide in seafood and aquaculture are summarized in Table 2.

3. Occurrence of carbapenem-producing bacteria in terrestrial food-producing animals

Intensive farming has frequently been associated with the excessive use of antimicrobials and drug-resistant microorganisms isolated from food-producing animals that can be transmitted to humans via direct contact with animals or ingestion of derived food products (9). By 2030, global antimicrobial use from human, terrestrial, and aquatic food-producing animal sectors will reach 236,757 tons annually, with an estimated proportion of terrestrial food-producing animal use of 174,549 tons, representing 73.7% of the global consumption of antimicrobials (55). This intensive use of antibiotics creates selective pressure for the emergence of antimicrobial resistance among farmers and the environment. In addition, antibiotics continue to be used in livestock production as prophylaxis and animal growth support (9), which may lead to the emergence of antimicrobial resistance.

The transmission of antimicrobial resistance between food-producing animals and humans can occur via the food chain, by consuming food products contaminated with antimicrobial resistance genes or antimicrobial-resistant bacteria, through direct contact between humans and animals, or through shared environmental sources such as contaminated water (56, 57). In addition, resistance can be transmitted to livestock from environmental sources (i.e., hospital sewage, wastewater treatment plants contaminating water and soil, surface water flow, and wildlife) and biological vectors, such as flies and wild birds (21, 58). Moreover, livestock growth promotion antibiotics may interact with the animal gut microbiota and introduce increased variation in antimicrobial resistance genes (ARGs) in the gut (59), thus increasing their dispersion. Studies reporting the occurrence of carbapenem-resistant genes worldwide in terrestrial food-producing animals are summarized in Table 3.

3.1. Carbapenem resistance in swine

In pigs, carbapenem resistance has been observed in microorganisms from different bacterial species, including *E. coli*, *Salmonella*, *P. aeruginosa*, and *A. baumannii*. In 2011, VIM-1-producing *E. coli* ST88 and *Salmonella* Infantis harboring bla_{VIM-1}IncHI2 plasmids were reported in Germany (60, 61). ST88 was also previously identified among chickens, cattle, and humans in Germany. Moreover, class 1 integron harbored by an IncH12 plasmid was found in human strains (60). In addition, during the sampling period in the same year (2011), 35 isolates were positive for bla_{VIM-1}, indicating that carbapenemase-producing bacteria may persist in livestock farms. Another study reported VIM-1-producing *Salmonella* Infantis in Germany in 2017 (59). The encoded genes bla_{VIM-1} and bla_{NDM-5} have reported resistance to third-generation cephalosporins used in animal husbandry. Moreover, *Salmonella* Infantis is one of the leading causes of human salmonellosis in Europe and a zoonotic pathogen commonly transferred via contaminated food products (59). In 2017, Pulss et al. (30) reported a porcine *E. coli* isolated carrying OXA-181 carbapenemase and the coexistence of *mcr-1* (mobilized colistin resistance gene) and acquired carbapenemase gene $bla_{OXA-48-like}$ on isolates originated from Italy farms. $bla_{OXA-181}$ gene was located on al IncX3 plasmid (pEcIHIT31346-OXA-181), which presented high nucleotide similarity >99% to previously published plasmids from human sources (plasmid pOXA181_14828 of an *E. coli* isolated from a human patient in China) and also carried *qnrS1* (plasmid-media quinolone resistance gene), thus providing evidence of the possible link between human- and animal-derived carbapenem resistance. In 2015, bla_{IMP-27} was detected in *Proteus mirabilis*, *Morganella morganii*, *Providencia rettgeri*, *Proteus vulgaris*, *Enterobacter cancerogenus*, *Citrobacter braakii*, *Enterobacter cloacae*, *Citrobacter* spp., *Citrobacter farmeri*, *Citrobacter koseri*, and *Klebsiella oxytoca* in the United States, within an IncQ1 plasmid recovered from the nursery and farrowing barns of a swine production system (57). In 2013 in China, *A. baumannii* harboring bla_{NDM-1} genes isolated from lung samples of pigs with pneumonia and sepsis were identified (62). Meropenem-resistant *P. aeruginosa* strains carrying $bla_{OXA-486}$, $bla_{OXA-396}$, bla_{OXA-50}, and bla_{PAO} were also found in Italy in 2018, as were meropenem-resistant isolates of *Pseudomonas oryzihabitans* and *P. aeruginosa* (9). Three other isolates of *P. aeruginosa* carrying bla_{PAO}, bla_{OXA-50}, $bla_{OXA-486}$, and $bla_{OXA-488}$ were detected in animals reared on different farms (85). Interestingly, two isolates of *P. aeruginosa* ST938 carrying bla_{PAO} and $bla_{OXA-396}$ and the resistance genes to aminoglycosides [*aph(3ı)-IIb*], fosfomycin (*fosA4)*, and chloramphenicol (*catB7*) were detected, one in a pig and another one in 83-year-old patients. However, no epidemiological links were demonstrable between the animal and the patient. Other sequence types found were ST274, ST782, and ST885. The presence of bla_{OXA-50} is concerning because this variant confers a decreased susceptibility to ampicillin, ticarcillin, and meropenem. In addition, the OXA-50 family also comprises $bla_{OXA-396}$, $bla_{OXA-486}$, and $bla_{OXA-488}$ genes (9). bla_{OXA48}-like contained no plasmid, and $bla_{OXA-181}$-carrying IncX plasmid has also been reported in *E. coli* isolated from Italian fattening pigs (64). In the study, the authors recovered samples from fattening pigs, cattle, and workers from slaughterhouses. Twenty-four isolates were positive for $bla_{OXA-181}$ and one for bla_{OXA-48}. The isolates presented high ST diversity within ST5229 with higher prevalence. Different plasmid replicons were present in the isolates, with IncX1 and IncX3, and IncF types being the most represented. OXA-48-producing isolates did not contain any plasmid replicon. Furthermore, the authors detected an OXA-181-producing *E. coli* belonging to ST410 isolated in two fecal samples from fattening pigs, described as a high-risk clone associated with $bla_{OXA-181}$ in human patients. Moreover, in China

TABLE 2 Occurrence of carbapenemase-encoding genes in seafood and aquaculture.

Year	Animal origen	Country	Bacteria	Carbapenemase-encoding genes	References
2013	Fish	Algeria	*A. baumannii*	bla_{OXA-23}	(34)
2014	Squid	Canada	*Pseudomonas fluorescens*-like	bla_{VIM-2}	(37)
2015	Squid, sea squirt, seafood medley, clam	Canada	*Stenotrophomonas maltophilia, Myroides odoratimimus, Stenotrophomonas* spp., and *P. putida*	bla_{OXA-48}	(36)
	Retail seafood	Originated from Korea and China			
2015	Shrimp	Canada	*E. cloacae*	bla_{IMI-1}	(38)
	Retail seafood			bla_{NDM-1}	
				bla_{OXA-1}	
2015	Shrimp	Canada	*E. aerogenes*	bla_{IMI-2}	(38)
	Retail seafood				
2016	Shrimp	Canada	*V. cholerae*	bla_{VCC-1}	(39)
	Retail seafood				
2016	Venus clam	Italy	*E. coli* ST10	bla_{VIM-1}	(40)
2016	Oyster	Tunisia	*A. baumannii*	bla_{OXA-23}	(47)
2016	Fish	Algeria	*A. baumannii*	bla_{OXA-23}	(34)
2016	Bivalves (oyster)	Tunisia	*A. baumannii*	bla_{OXA-23}	(46)
2018	Fish	India	*Enterobacteriaceae (K. pneumoniae, E. coli)*	bla_{NDM-1}	(40)
	Retail seafood			bla_{NDM-2}	
				bla_{NDM-5}	
2018	Shirmp	France	*V. parahaemolyticus*	bla_{NDM-1}	(48)
2018	Abalone	Taiwan	*S. algae*	bla_{OXA-55}	(51)
2019	Hard clam	Taiwan	*S. algae*	bla_{OXA-55}	(52)
2019	White shrimp	The Netherlands	*E. cloacae* complex	bla_{IMI-2}	(33)
	Retail seafood	Originated in India		bla_{FLC-2}	
2019	Shrimp	China	*V. alginolyticus*	bla_{VIM-1}	(31)
	Retail seafood		*V. parahaemolyticus*	bla_{NDM-1}	
			V. vulnificus	bla_{NDM-1}	
2019	Hard-shelled mussel	Korea	*Vibrio* spp.	bla_{OXA}	(43)
	Retail seafood				
2020	Tilapia fish	Egypt	*E. coli*	bla_{OXA-48}	(50)
	Fish farm		*E. cloacae* complex	bla_{NDM}	
			K. pneumoniae	bla_{KPC}	
2020	Fish	Italy	*S. algae*	bla_{OXA-55}	(53)
	Aquaculture farms				
2020	Shrimp	China	*V. alginolyticus*	bla_{VMB-1}	(15)
	Retail seafood				
2021	Shrimp	China	*V. diabolicus*	bla_{VMB-2}	(32)
	Retail seafood				
2021	Shrimp	China	*V. alginolyticus*	bla_{VAM-1}	(17)

TABLE 3 Occurrence of carbapenemase-encoding genes in terrestrial food-producing animals.

Year	Country	Carbapenemase-encoding genes	Bacteria	Source		References
2011	Germany	bla_{VIM-1}	*E. coli*	Pig	Pig farm	(60)
2011	Germany	bla_{VIM-1}	*S. enterica* subsp. *enterica* serovar Infantis	Pig	Pig farm	(61)
2013	China	bla_{NDM}	*A. baumannii*	Pig	Lung sample	(62)
2015	China	bla_{NDM-5}	*E. coli*	Pig	Commercial pig farm	(63)
2016	US	bla_{IMP-27}	*E. coli*, *P. mirabilis*, *Morganella morganii*, *Providencia rettgeri*, *Proteus vulgaris*, *Enterobacter cancerogenus*, *Citrobacter braakii*, *E. cloacae*, *Citrobacter* spp., *Citrobacter farmeri*, *Citrobacter koseri*, and *Klebsiella oxytoca*	Environmental and fecal samples on pig farms	Pig farm (nursery rooms)	(57)
2016	Germany	bla_{VIM-1}	*Salmonella* Infantis	Pig	Sick piglet	(61)
2017	Germany and Italy	$bla_{OXA-181}$, $bla_{OXA-48-like}$	*E. coli*	Pig	Fecal samples	(30)
2018	Italy	$bla_{OXA-486}$, $bla_{OXA-396}$, bla_{OXA-50}	*P. oryzihabitans, P. aeruginosa*	Pig	Slaughter	(9)
2022	Italy	$bla_{OXA-181}$	*E. coli*	Pig	Fattening	(64)
2012	China	bla_{NDM-1}	*Acinetobacter lwoffii*	Broiler	Poultry	(65)
2016	Egypt	bla_{OXA-48}, bla_{KPC}, bla_{NDM}	*K. pneumoniae*	Chicken	Broiler-poultry farming	(60)
2017	Egypt	bla_{NDM-1}, bla_{NDM-2}	*E. coli*	Retail chicken carcasses	Supermarkets, poultry slaughterhouses, and butcher shops	(66)
2020	China	bla_{NDM-5}, bla_{NDM-1}, bla_{NDM-9}, bla_{NLM}	*Enterobacteriaceae* (*K. pneumoniae, E. coli*), *Morganellaceae, Alcaligenes faecalis, P. putida*	Poultry farm	Chicken	(21)
2021	China	bla_{NDM}, bla_{OXA-1}, bla_{OXA-10}	*P. mirabilis*	Chicken	Slaughterhouses/ chicken farm	(67)
2021	China	bla_{NDM-1}	*P. mirabilis*	Broiler	Chicken	(68)
2015–2021	China	bla_{NDM}	*E. coli*	Broiler farm	Chicken feces	(58)
2021	Egypt	bla_{OXA-1}, $bla_{KPC,}$ bla_{NDM}	*P. mirabilis*	Ducks	Farm	(69)
2021	China	bla_{NDM}, bla_{VIM}, bla_{KPC}, $bla_{OXA-like}$		Broiler	Cooperative broiler feedlot	(70)
2023	China	bla_{NDM}, bla_{OXA}	*P. mirabilis, E. coli, K. pneumoniae*	Broiler	Fattening farm	(71)
2010	France	bla_{OXA-23}	*A. baumannii*	Cattle	Dairy farm	(72)
2016	US	$bla_{OXA-497}$	*A. baumannii*	Cattle	Dairy cattle	(73)
2016	Egypt	bla_{OXA-48}, $bla_{OXA-181}$, bla_{OXA-7}, bla_{OXA-10}	ESBL-producing *E. coli*	Cattle	Dairy cattle farms	(29)
2016	India	bla_{NDM-5}	*E. coli*	Cattle	Bovine	(74)
2016	Algeria	bla_{NDM-5}	*E. coli*	Cattle	Raw milk	(75)
2017	China	bla_{NDM-5}	*K. pneumoniae*	Cattle	Dairy cows	(76)
2017	Germany	bla_{OXA-23}	*Acinetobacter indicus*-like	Cattle	Calves	(77)
2018	US	bla_{KPC-2}	*K. pneumoniae*	Cattle	Beef cattle	(78)
2019	India	bla_{VIM}	*E. coli*	Cattle	Calves	(79)

(Continued)

TABLE 3 (Continued)

Year	Country	Carbapenemase-encoding genes	Bacteria	Source		References
2019	Egypt	bla_{KPC}, bla_{OXA-48}, bla_{NDM}	*P. aeruginosa*	Buffaloes and cattle	Farm	(80)
2020	South Africa	bla_{KPC}, bla_{NDM}, bla_{GES}, bla_{OXA-48}, bla_{VIM}, bla_{OXA-23}	*E.coli, K. pneumoniae, P. mirabilis, Salmonella*	Cattle	Beef cattle	(81)
2022	Spain	bla_{NDM-1}	*E. coli*	Cattle	Dairy calves	(82)
2022	Italy	$bla_{OXA-181}$, bla_{OXA-48}	*E. coli*	Cattle	Bovine beef	(64)
2022	Pakistan	bla_{OXA-23}, bla_{OXA-51}, bla_{NDM-1}, bla_{IMP}	*A. baumannii*	Cattle	Dairy cattle and beef cattle	(83)
2023	Tunisia	bla_{OXA-48}, bla_{IMP}	ESBL-producing *E. coli*	Cattle	Diarrheic calves	(84)

in 2017, *E. coli* harboring a carbapenem-resistance gene bla_{NDM-5} and *mcr-1* were detected on IncX3 plasmid with a high degree of diversity of ST. ST156 was also previously reported in a Chinese hospital (63).

3.2. Carbapenem resistance in poultry

Among broiler farms, an increased prevalence of CRE has been shown, mainly in *E. coli*, *K. pneumoniae*, and *P. mirabilis*, harboring bla_{NDM} genes, with bla_{NDM-1} and bla_{NDM-5} being the most predominant in chicken farm environments. Specifically, farming presented a higher prevalence of CRO among the studies, mainly attributed to the heavy use of antimicrobials on farms, transportation activities, and inadequate farm disinfection and management. Similarly, bla_{NDM} genes are usually carried by the IncX3 plasmid, which is clinically significant because it contributes to disseminating various bla_{NDM} variant genes (86).

In 2010, carbapenem-resistant isolates were detected in eight chicken farms, six duck farms, and one pig slaughterhouse in China. One of these isolates, *Acinetobacter lwoffii,* was identified as positive for bla_{NDM-1}, particularly on a 270 kb plasmid (65). In 2021, NDM-producing *P. mirabilis* was reported in broiler chickens (68). The isolate harbors a plasmid named pSNYG35, a pPrY2001-like plasmid that shares high nucleotide identity with pHFK418-NDM and an NDM-1-encoding plasmid from clinical *P. mirabilis*. Recently, Su et al. (87) reported isolation rates of 3.57% for carbapenem-resistant *E. coli*, 10% for carbapenem-resistant *P. mirabilis*, and 3.03% for carbapenem-resistant *K. pneumoniae* in six broiler fattening farms in China. Among carbapenem-resistant isolates, six *E. coli* carried class I integron, one carried class II integron, four *P. mirabilis* carried class I or II integrons, and one *K. pneumoniae* carried class 1 integron. All of these isolates harbor bla_{NDM} and bla_{OXA} genes. In 2016 in Egypt, carbapenem-producing *K. pneumoniae* (CR-Kp) in broiler poultry farming was reported. The authors found that 42% of the isolates from poultry samples carried bla_{NDM} (11 isolates carried bla_{KPC}, bla_{OXA-48}, and bla_{NDM}; four isolates carried bla_{KPC}, bla_{NDM} or bla_{OXA-48} and one isolate carried bla_{NDM} alone) (70). Interestingly, the authors collected 49 fecal samples from workers and veterinarians working in the poultry farm; 56% of the samples were CR-Kp-positive, with all strains carrying the three carbapenemase genes bla_{KPC}, bla_{OXA-48}, and bla_{NDM}, and 5% of them displayed all the carbapenemase-encoding genes at the same time. Furthermore, the prevalence was higher in farm workers (67%) compared to veterinarians (33%), indicating that transmission could be facilitated by close contact between broilers and humans since the workers are in continuous contact with the animals and lived on the farm during the fattening program. However, the study did not compare clones or plasmids; non-genetic relationships between humans and chickens were found (70). Lately, in China in 2023, He et al. (58) observed the transmission of bla_{NDM}-bearing plasmids of *E. coli* isolated from chickens between different farms and detected carbapenem-resistant isolates in farmlands, vegetable fields, and the environment of chicken farms. The authors performed a longitudinal study from 2015 to 2021 that demonstrated that the prevalence of bla_{NDM}-positive clones and plasmids varied in different years, which suggested that new strains and plasmids are constantly being introduced into the farms. In 2020 in China, Zhai et al. (21) reported 279 NDM-producing bacteria, including *Enterobacteriaceae* (*K. pneumoniae*, *E. coli*), *Morganellaceae*, *Alcaligenes faecalis*, and *Pseudomonas putida*, with the variants NDM-5, NDM-1, and NDM-9 as well as a novel NDM-like-metallo-β-lactamase (NLM) within IncX3, IncA/C2, and IncFII as major bla_{NDM}-carrying plasmid types among isolates. Moreover, they found the coexistence of *mcr-1* or *mcr-8* on *K. pneumoniae* positive for bla_{NDM-1}. The authors identified 14 sequence types among the *E. coli* isolates, with ST6751 being the most prevalent. ST6716, ST156, ST69, ST48, and ST10 were also found. STs 6751, 10, 125, and 746 were recovered from chicken and environmental samples (sewage trenches, corridor floors, drooping boards, nipple drinkers, and air). Most of the *K. pneumoniae* isolates were ST37, followed by ST3410 and ST726. Additionally, in China, Shi et al. (71) reported the presence of the resistance genes bla_{NDM}, bla_{VIM}, bla_{KPC}, and $bla_{OXA-like}$ on broiler, layer, and pig farms with a significative higher relative abundance on $bla_{OXA-like}$ genes from 2016 to 2019. The authors detected a prevalence of 20–30% for bla_{KPC} and bla_{VIM} genes, respectively, and a prevalence of 75% for bla_{NDM}, reflecting the great incidence of carbapenemase-producing genes in farming. Moreover, the study also found the coexistence of colistin resistance gene *mcr-1* and bla_{NDM} with pig and chicken farms displaying high prevalence. In 2020 in Egypt, 155 meropenem-resistant isolates were obtained from retail chicken meat, indicating that carbapenem-producing

bacteria may enter the food chain. The study reported a single *K. pneumoniae* ST147 and a single *E. coli* ST648 producing NDM-1 and NDM-5. This last isolated carried also bla_{OXA-1}, bla_{TEM-1}, $bla_{CTX-M-3}$, and *aac(6′)-Ib-cr*, while the *K. pneumoniae* harbored the bla_{SHV-1}, $bla_{CTX-M-15}$, and *aac(6′)-Ib-cr* genes (71). NDM-producing ST648 *E. coli* has been reported in clinical isolates in India, the United Kingdom, and Australia. NDM-1-producing ST147 *K. pneumoniae* clone has been reported previously in Iraq, Oman, Tunisia, and Egypt from hospitalized patients (66). A study conducted in China in 2019 reported NDM-1-producing *P. mirabilis* recovered from commercial broilers in slaughterhouses (67). In 2021 in Egypt, *P. mirabilis* harbored bla_{NDM-1}, bla_{OXA-1}, and bla_{KPC} was isolated from ducks on a duck farm (69).

3.3. Carbapenem resistance in cattle

Carbapenem-resistant bacteria are rare in cattle. However, since 2012, more studies have reported CROs in cattle with OXA- and NDM-producing bacteria leading to carbapenem resistance. In 2010, nine OXA-23-producing *Acinetobacter* genomospecies 15TU were reported in France, with a Tn*2008* as a vehicle for the spread (72). In 2016 in the United States, a novel $bla_{OXA-497}$ gene was detected in *A. baumannii*, which is part of the OXA-51-like enzyme group and displays resistance to ertapenem; however, these enzymes are naturally occurring in *A. baumannii* (73, 88). In 2017, bla_{OXA-23} harboring *Acinetobacter indicus*-like strains that displayed imipenem, meropenem, and doripenem resistance were isolated from nasal swabs of two calves in Germany. bla_{OXA-23} was localized on the chromosome and surrounded by interrupted Tn*2008* transposon structures. In addition, genetic relatedness between bovine isolates and *Acinetobacter indicus* type strains $A648^T$ and human clinical *A. indicus* isolates were found (77). In 2022, 27.7% of CRAB bacteria in Pakistan were reported to harbor bla_{OXA-23} and bla_{OXA-51} within 17 isolates carrying bla_{IMP} and one isolate carrying bla_{NDM-1}. The typical sequence types found were ST642 and the international clone ST2 (83). In Egypt in 2019, carbapenem-resistant *P. aeruginosa* (CRPA) was reported in buffaloes and cattle with a prevalence of 60 and 59% (50 total samples) within isolates harboring bla_{KPC}, bla_{OXA-48}, and bla_{NDM}. The authors also found carbapenem-resistance genes from drinking water within 67% of prevalence and from stool human samples within 80% of prevalence. Additionally, phylogenetic analysis showed that cattle and water sequences were in one cluster and more related to each other than to human isolates (80). Similarly, in Egypt in 2014, five *E. coli* carrying bla_{OXA-48}, and one *E. coli* carrying $bla_{OXA-181}$ were reported in dairy cattle (29). In South Africa, 28–42% of carbapenem resistance was found in isolates such as *E. coli*, *K. pneumoniae*, *P. mirabilis*, and *Salmonella* spp., carrying bla_{KPC}, bla_{NDM}, bla_{OXA-23}, bla_{VIM}, bla_{OXA-48} and bla_{GES} with different prevalence (81). In Italy, in 2021, the EU harmonized antimicrobial resistance (AMR) monitoring program reported that units of fattening pigs (21/301) and bovines (4/310) were positive for OXA-48-like *E. coli* (n = 24 OXA-181, n = OXA-48) (64). Most recently, in Tunisia, one isolate of ESBL-producing *E. coli* from calves with diarrhea carrying bla_{OXA-48} and bla_{IMP} were reported (84).

Antimicrobial resistance mediated through NDM enzymes is present in cattle. In 2013 in India, *E. coli* harboring the bla_{NDM-5} gene was detected in milk samples of dairy cattle suffering mastitis (74). NDM-5-producing *E. coli* isolates from raw milk collected in a dairy farm in Algeria and India in 2016 were again found (75, 89). bla_{NDM-1} gene in *E. coli* isolated from cattle, carried in an IncC plasmid, was reported in 2022 in Spain. The IncC plasmid also carried genes for aminoglycoside, sulphonamide, and trimethoprim resistance (82). *K. pneumoniae* carrying bla_{NDM-5} located on IncX3 plasmid was isolated from dairy cows in China in 2017 (76). The presence of IncX3 plasmid is highly relevant since it mediates the spread of genes encoding resistance to clinically relevant antibiotics. It has been reported to encode *qnrB7*, *qnrS*, $bla_{CTX-M-3}$, bla_{SHV-12}, bla_{KPC-2}, bla_{KPC-3}, bla_{NDM-1}, bla_{NDM-4}, bla_{NDM-5}, bla_{NDM-7}, bla_{NDM-13}, bla_{NDM-17}, and $bla_{OXA-181}$ (86). The authors found that the *K. pneumoniae* bla_{NDM-5} positive belonged to five STs, within ST1661 and ST2108, which were the most prevalent. The bla_{NDM-5} gene was located on the ∼46 kb IncX3 plasmid. The plasmid shared a similar genetic context and was nearly identical to the human *K. pneumoniae* plasmid (pNDM-MGR194) previously reported in India. Among beef cattle in the United States in 2018, isolates of *K. pneumoniae* carrying bla_{KPC-2} from feces were detected in 72 samples (78). bla_{VIM} gene located on an Incl1 plasmid of a novel sequence type (ST 297) from *E. coli* was well-detected among calves from India in 2019 (79).

4. Occurrence of carbapenem resistance in wildlife

Antimicrobial resistance genes can colonize wild animals following contact with sewage, human waste, or animal manure (90). Human feces and manure runoff are the primary sources of AMR in wild animals, as intake water polluted with feces could directly or indirectly contaminate other animals and the surrounding environment. Moreover, AMR genes, such as carbapenemase genes, could originate from environmental bacteria, such as the OXA-48 family of enzymes, which occurs naturally in *Shewanella* spp., a genus that inhabits lake sediments (91, 92), and OXA-23 enzymes, which are almost entirely restricted to *A. baumannii* and originate from the environmental species *Acinetobacter radioresistens* (93). Chickens have been proposed as a source of carbapenemase-producing *Salmonella enterica* in livestock (85).

Several carbapenemase-producing bacteria have been reported worldwide in wildlife including the NDM, IMP, VIM, and OXA enzymes. In Germany, *Salmonella corvallis* carried bla_{NDM-1} belonging to ST1541 isolated from black kites (*Milvus migrans*) were detected in 2013 (94). bla_{NDM-1} gene was located in the IncA/C conjugative plasmid pRH-1738 and contained a fosfomycin-resistance gene (*fosA3* gen) (95). In 2016 in Australia, a high prevalence of *Salmonella* and IMP-4-producing *Enterobacteriaceae* was reported in silver gulls (96). The authors detected 120 carbapenem-resistant *Enterobacteriaceae* strains of 10 species, mainly *E. coli* carrying the bla_{IMP-4}, bla_{IMP-38}, and bla_{IMP-26} genes, with a prevalence of 40% in the gulls. bla_{IMP} gene was carried by conjugative plasmids of variable sizes and diverse replicons, including HI2-N, HI2, A/C, A/C-Y, L/M, I1,

and non-typeable plasmids. The authors showed that isolates from gulls have significant similarities with clinical isolates from Australia, suggesting the human origin of the isolates. In 2017, France reported 22 carbapenem-resistant VIM-1-producing *E. coli* in yellow-legged gull (*Larus michahellis*) isolated in 2012 (97). Interestingly, gulls live in close contact with humans; thus, wildlife may be an important transmission route of AMR. In 2018, carbapenem-producing *Enterobacteriaceae* isolates (two *E. coli* ST635 and one *K. pneumoniae* ST13) were reported in fecal samples from wild boars in Algeria, Africa. OXA-48-producing isolates were also resistant to amoxicillin, amoxicillin-clavulanate, tobramycin, ertapenem, and meropenem (98). In 2019, China reported a high frequency of carbapenemase producer isolates (350 isolates) in migratory birds (*Anser indicus*, *Phalacrocorax*, and *Larus ichthyaetus*), while 233 *Klebsiella* spp. and 2 *E. coli* isolates were NDM-5-carriers (99). In 2019 in Korea, zoonotic *Aeromonas* spp., resistant to imipenem and meropenem, were isolated from the nutria (*Myocastor coypus*). These isolates also carried the *cphA* gene (*Aeromonas hydrophila* gene) coding for a carbapenem-hydrolyzing metallo-β-lactamase (100). In the same year, in Algeria, carbapenemase-producing *K. pneumoniae* was reported in bat guano with OXA-48 and KPC-3 enzymes present in the isolates, as well as the resistance genes $bla_{\text{TEM}-1}$ (ampicillin resistance) and *aac(6ı)-lb* (aminoglycoside resistance) (101). In 2019, 13 carbapenem-resistant *K. pneumoniae* were isolated from Barbary deer (*Cervus elaphus barbarus*) in Akfadou Forest in Algeria. The resistome of these isolates revealed the presence of $bla_{\text{NDM}-1}$, $bla_{\text{CTX}-\text{M}-15}$, $bla_{\text{SHV}-182}$, $bla_{\text{DHA}-1}$, $bla_{\text{OXA}-1}$, *aac(3)-IIa*, *aac(3)-IId* (aminoglycoside resistance), *aac(6ı)-Ib-cr* (aminoglycoside-fluoroquinolone resistance), *rmtC* (rRNA methyltransferase with high-level resistance to aminoglycosides), *sul1* (sulfonamides resistance), *qnrB9* (plasmid-mediated quinolone resistance), *fosA* (fosfomycin resistance), *tetA* (tetracycline resistance), *dfrA14* (trimethoprim resistance), *catA2*, *catB3* (chloramphenicol resistance), and *mphA* (macrolide-resistant phosphotransferase) genes. Five different plasmids, IncA/C2, IncFIA/(HI1), IncFIB(K), IncFII(K), and ColRNAI, were also found (102). Similarly, in 2020, carbapenem-resistant *K. pneumoniae* carried $bla_{\text{OXA}-48}$ on an incompatible group L/M plasmid found in seals (*Phoca vitulina*) (103), reflecting anthropogenic pollution as a source of AMR genes. In 2021, a genomic comparison was performed between *E. coli* carrying KPC-2 and *K. pneumoniae* containing KPC-3 isolated from gulls and humans in Alaska. The authors found varying levels of genetic similarity at discrete genetic loci with no evidence of direct transmission of bla_{KPC} between people and gulls; however, the conserved genetic elements surrounding bla_{KPC} suggest a possible exchange between species (104). In 2021 in India, five carbapenem-resistant *E. coli* were reported isolated from rescued sloth bear (*Melursus ursinus*). The isolates were positive for bla_{NDM} (60%, 3/5) carbapenemase gene and efflux pump-mediated carbapenem resistance (40%, 2/5), and co-harbor AMR genes $bla_{\text{TEM}-1}$, *bla*AmpC, *qnrA*, *qnrB*, *qnrS*, *tetA*, *tetB,* and *sulI* (105). In 2022, carbapenem-resistant *P. aeruginosa* strains were recovered from the feces of a red deer (*Cervus elaphus*) from Portugal, which resulted in a high-risk clone belonging to ST274 and co-harboring the genes bla_{PAO}, $bla_{\text{PDC}-24}$, $bla_{\text{OXA}-486}$, *aph(3ı)-lb* (aminoglycoside resistance), *fosA* (fosfomycin resistance), and *catB7* (chloramphenicol resistance), which are phenotypically resistant to imipenem and intermediate resistance to meropenem and doripenem (106). In 2022, a high diversity of carbapenem-resistance genes was found in wild birds sampled from Alaska, Chile, Spain, Ukraine, Turkey, and Pakistan. The authors found carbapenemase genes in diverse isolates, including *K. pneumoniae* carrying KPC, NDM, OXA, and VIM, as well as in hypervirulent CR-Kp isolates from gulls in Spain and Ukraine. Some isolates harbored antimicrobial resistance to up to 10 antibiotic classes, including colistin. OXA-48-producing *E. coli* in gulls in Alaska and Turkey and CRE from Chile and Spain also harbored colistin-resistance genes. Similarly, the authors found evidence of global temporal and spatial dissemination (107). In 2022 in Brazil, NDM-1-producing *E. coli* ST162 infecting a pygmy sperm whale (*Kogia breviceps*) was reported (108). Moreover, the resistome of the isolate carried genes conferring resistance to β-lactams ($bla_{\text{NDM}-1}$, $bla_{\text{TEM}-1}$, and $bla_{\text{OXA}-1}$), aminoglycosides [*aph(3ı)-lb*, *aph(3ı)-VI*], macrolide (*ermB*, *mdfA*, and *mphA*), rifamycin (*arr-3*), [*aac(6′)-Ib-cr*, and *qnrB6*], phenicols (*catB3* and *floR*), sulfonamide (*sul1* and *sul2*), and tetracycline (*tetA*), and plasmid replicons IncFIB and IncA/C2 were also detected. All the previous studies shown here demonstrate that wild animals are reservoirs of carbapenem-resistant bacteria. They provide a biological mechanism for spreading antibiotic-resistance genes and can facilitate their transmission to humans and livestock.

On the other hand, rivers and water flow are also an environment from the emergence of CROs. For example, *Pseudomonas fluorescens* was recovered from the Seine River (Paris, France) in 2010, which expressed PF-1, a novel Ambler class A carbapenemase (109). In 2022 in Poland, 301 carbapenem-resistant *Acinetobacter* strains were isolated from municipal wastewater and river water (110). In 2005, carbapenem-resistant bacteria were reported on water bodies in the United States (111, 112). In 2019, carbapenem-resistant bacteria on water bodies were isolated, including *Enterobacter asburiae*, *Aeromonas veronii*, *Cupriavidus gilardii*, *Pseudomonas*, and *Stenotrophomonas* spp. This study found that most strains were carbapenemase producers, and all the isolates of *Enterobacter asburiae* carried the $bla_{\text{IMP}-2}$ gene (111). Other studies have also reported the presence of carbapenem-resistant strains in seawater, stormwater, and surface runoff water at Costa locations in Sydney, Australia, in 2020 (113). Therefore, water environments are an important reservoir of bacteria resistant to carbapenems and other antibiotics, including bacteria carrying intrinsic and acquired carbapenemase genes.

5. Occurrence of carbapenem resistance in companion animals

Carpabenemase-producing Enterobacterales (CPE) and non-fermenting bacteria have also been reported in companion animals. As we pointed out before, carbapenems are not approved for veterinary use. The prescription is restricted to treating urinary tract infections and respiratory tract infections in dogs and cats originating from multidrug-resistant (MDR) *E. coli*, *K. pneumoniae*, and *P. aeruginosa* bacteria. Additionally, the treatment must be supported by a veterinarian specializing in infectious disease, and by a pharmacologist (114), even though

the continuous evidence of carbapenem-producing bacteria in companion animals has been increasing.

Companion animals can acquire carbapenemase-producing bacteria through direct contact with colonized hosts and the through contaminated environments such as veterinary hospitals (115, 116). In this regard, the human-pet bond has favored the silent transmission of carbapenem-producing bacteria to companion animals by a reverse zoonotic route called zooanthroponosis (115–117). Indeed, in Finland in 2015, identically isolates from dogs (with a long history of recurrent *otitis externa* without carbapenem prescription) and human family members with NDM-5-producing multidrug-resistant ST167 *E. coli* were reported. In addition, the same family carried an identical extended-spectrum beta-lactamase (ESBL) CTX-M-group 9 *E. coli* ST69, indicating interspecies transmission (118). In 2018 in Brazil, six VIM-2 carbapenemase-producing *P. aeruginosa* ST233 isolates were recovered from an infected dog, its owner (with a history of hospitalization), and its domestic environment (sofa, balcony, and water cooler) (116). ST233 has been reported as an international high-risk clone associated with carbapenemase production with resistance to all antimicrobial drugs. It has generally been restricted to human hospital settings (119–121), suggesting a zooanthroponotic transmission of this clone after the patient's hospital discharge. More recently, in 2022 in Guangzhou, China, a large-scale investigation on the prevalence of bla_{NDM}-positive *E. coli* isolates from companion animals and their healthcare providers in clinical veterinary settings revealed the clonal spread bla_{NDM}-positive ST453 *E. coli* isolates between both species (122). In France in 2022, OXA-48-producing *K. pneumoniae* were isolated from companion animals (dogs, cats, horses, cattle, and birds) with 56.2% (59/105 isolates) of the isolates belonging to the human-associated MDR ST11, ST15, and ST307 lineages, suggesting that numerous human-associated clones could infect the animal host (123).

Among carbapenemases on companion animals, NDM-5 and OXA-48-like carbapenemases are the most frequently described enzymes, with *E. coli* and *K. pneumoniae* being the main carbapenem-producing Enterobacterales, along with the non-fermenting bacteria *A. baumannii* (124). OXA-48 has been identified in *Enterobacteriaceae* from dogs and cats in different countries, such as Germany (2013) and the United States (2009–2013) (27, 125), as well as in an ST38 *E. coli* isolated from fowl (*Gallus domesticus*) in 2015 in Lebanon (28). In 2012, in Belgium, two OXA-23-producing *Acinetobacter* spp. were detected in fecal samples from 20 hospitalized horses, both resistant to imipenem and presented resistance to tetracyclines, sulfonamides, trimethoprim, and gentamicin but were still susceptible to colistin (126).

On the other hand, the KPC enzyme has also been reported. In 2018 in Brazil, in *K. pneumoniae* and *E. coli* from dogs, the bla_{KPC-2} gene was found in Tn*4401* transposons contained in IncN plasmids, which also carried $bla_{CTX-M-15}$, and other clinically significant resistance determinants conferring resistance to aminoglycosides (*aadA5*), quinolines (*qnrS1*), macrolides [*mph(A)* and *erm(B)*], sulfonamides (*sul1*), tetracycline [*tet(B)*], and trimethoprim (*dfrA17*), and point of mutation conferring quinolone resistance (127). In Brazil 2021, the KPC-2-producing *K. pneumoniae* belonging to the high-risk international clone ST11/CG258 in a dog with urinary tract infection carrying the IncN plasmid assigned to ST15 was reported (128). The bla_{KPC-4} gene was detected in 2016 in Ohio, US, in an IncHI2 plasmid in the context of the Tn*4401b* transposon in *Enterobacter xiangfangensis* isolated from a clinical dog sample with ST171, which has been responsible for major clusters of human CRE infections in the northeastern and upper-midwestern of the United States (129, 130). IMP-4 has been reported in *Salmonella enterica* serovar Typhimurium isolated from cats in Australia in 2016 (131). NDM-1 was isolated in 2013 in the United States from dogs and cats from *E. coli* that also carried $bla_{CTX-M-15}$ and belonged to ST167 (132), as well as in China, with *Acinetobacter* species carrying bla_{NDM-1} and bla_{OXA-23} (133), and in Italy from *A. radioresistens* (134). Recently, in 2022 in China, five bla_{NDM-5} harboring *E. coli* were reported in dogs and cats, all of them multidrogo resistant. The bla_{NDM-5} gene was located on 46 kb IncX3 plasmids in the five strains. Additionally, one strais coharbored bla_{NDM-5}-encoding-IncX3 plasmid along with an *mcr-1*-IncX4 hybrid plasmid (135). OXA-48 has mainly been described in dogs, cats, and horses and mostly from infections such as urinary tract infections (UTIs) isolates from *E. coli*, *K. pneumoniae*, *E. cloacae*, and *K. oxytoca* (136, 137). VIM-1 and VIM-2 were also reported in dogs infected with *K. pneumoniae* and *P. aeruginosa* in Spain and Korea in 2016 and 2018, respectively (138, 139), as well as OXA-181-producing extra-intestinal pathogenic *E. coli* ST410 from a dog in Portugal in 2020 (140), OXA-23-mediated carbapenem-resistance *A. baumannii* ST2 from a cat (141), and OXA-66-producing *A. baumannii* isolated from cats (124, 142).

6. Transmission between animals and humans

Among transmission between animals and humans, few studies have investigated the evidence for established links between human- and animal-derived carbapenem resistance. In 2022, Shen et al. (143) reported 29,799 *E. coli* isolates recovered from patients at 30 hospitals in China, as well as 61 pig farms and 45 chicken farms in 2017. From human clinical isolates, 631 were defined as carbapenem-resistant *E. coli* (CREc, 2.1%) with 195 NDM-positive. For livestock production, bla_{NDM} was detected in 73.8% (n = 45) and 62.2% (n = 28) of pig and chicken farms, respectively. Furthermore, they found that human NDM-positive *E. coli* isolates shared 15 (n = 111), 11 (n = 90), and 10 (n = 96) STs with those from chickens, pigs, and flies, respectively. NDM-positive isolates belonging to ST167, ST206, ST10, and ST48 were recovered from all four origins. Furthermore, the authors found that large proportions of bla_{NDM} genes (>70%) were associated with IncX3 plasmid in both animals (pig, chicken, and fly isolates) and humans. The authors also predicted the origins of 463 NDM-positive isolates. They found that 19% (n = 24), 8.1% (n = 10), and 1.6% (n = 2) of chicken NDM-positive *E. coli* isolates (n = 123) were predicted to originate from humans, pigs, and flies, respectively. In contrast, 27.3% (27/99) of pig NDM-positive *E. coli* isolates were predicted to originate from humans. Similarly, 53.8% (n = 105) and 14.9% (n = 29) of human isolates were predicted to have originated from chickens and pigs. All fly-derived isolates (n = 46) were predicted to have originated from humans (n = 5, 10.9%), chickens (n = 22, 47.8%), and pigs (n = 19, 41.3%). These results indicated

positive associations and transmission of CREc between animals and humans. Indeed, the authors hypothesize that "CREc first arose in clinical settings and was then introduced into livestock animals, which are favorable hosts for the persistence of CREc. This led to the circulation of CREc between humans and animals, either via the food chain or through environmental vectors".

In 2019, Li et al. (144) sampled 12 villages in China used as pig production farms [using the household as a single surveillance unit (resident and their backyard animals, including farm and companion animals)] and two commercial pig farms near the villages. The authors collected flies, fecal samples from humans, pigs, chickens, cattle, goats, ducks, one donkey, dogs, and cats across the villages, and additional fecal samples from pigs and farm workers at the two commercial farms. They obtained 88 CREC isolates that contained the bla_{NDM} carbapenemase gene, 17 from humans, 44 from pigs, 12 from chickens, 12 from flies, two from dogs, and one from cattle. No CREC isolates were recovered from workers of pigs at the two nearest commercial pig farms. The authors detected bla_{NDM-5}, bla_{NDM-1}, and bla_{NDM-9}, with most of these bla_{NDM}-genes likely located on IncX3-type plasmids. Indeed, the bla_{NDM}-carrying regions/plasmid (IncX3) in CRE isolates from humans exhibited >99% nucleotide sequence identity to those in isolates from backyard animals and flies. MLST showed that six human CRE-NDM-positive isolates displayed ST48, ST10, ST1114, or ST6910 shared by animal isolates. ST48 was the most prevalent and was associated with isolates from pigs, humans, chickens, and flies. Furthermore, they found that two human isolates displayed only three single-nucleotide polymorphisms (SNPs) with two pig isolates from the same village. They also reported that CREC isolates from flies have human and dog origins, while chicken isolates had a predominant origin from pigs and dogs. In addition, the single cattle-derived isolate was clustered with the chicken isolates. Therefore, many CRE isolates from humans, backyard animals, and flies originated from hosts other than those included in the study.

In 2017, Wang et al. (145) recovered 245 CRE from poultry (chicken farms, slaughterhouses, and supermarkets), dogs, sewage, wild birds, flies, and farmers. The authors identified bla_{NDM} in 21.8% ($n = 161$) of the *E. coli* isolates, 7.4% on *K. pneumoniae*, and 3.9% in *E. cloacae*, with bla_{NDM-5}, bla_{NDM-9}, bla_{NDM-1}, and bla_{NDM-7} variants. Importantly, 23% of CREC isolates were also positive for *mcr-1*. High rates of CREC were found in dogs' feces (82.4%), flies (25.8%), wild birds' nests (40%), and anal swabs of farmers (50%). The most prevalent STs among bla_{NDM}-positive isolates were ST101, ST156, and ST746. Moreover, MLST analysis showed commonality between strains from chicken farms, slaughterhouses, supermarkets, and humans, typified by genotypes ST10 and ST156. The authors confirm the commonality of ST156 isolates among disparate samples by core-genome single-nucleotide polymorphism (SNP)-based phylogenetic analysis. Additionally, bla_{NDM}-carrying contigs gave three main genomic backbone profiles. The type II backbone included the contigs from 84 isolates derived from chicken cloacae ($n = 37$), flies ($n = 21$), dog feces ($n = 12$), chicken meat from supermarkets ($n = 5$), sewage from the farm ($n = 1$), chicken caeca from a slaughterhouse ($n = 1$), feces from farmers ($n = 3$), swallows ($n = 3$), and sewage from a slaughterhouse ($n = 1$). Type II genomic backbone was found in 26 *E. coli* isolates and shares >99.9% nucleotide sequence identity with the corresponding region of a 46,253bp IncX3 plasmid pJEG027 from *K. pneumoniae* isolated from an Australian traveler who was repatriated to Sydney from Myanmar.

The studies above showed a positive association between livestock production and human CREC infections since they identified a close relationship between the genomic profile of carbapenem-resistant isolates from humans and animals. High similarities between isolates from different sources were found. However, the studies only focused on NDM-positive isolates and not on other carbapenemases-encoding genes. Further studies are needed to elucidate the link between humans and animals.

7. One health approach for antimicrobial resistance

Different strategies to combat antimicrobial resistance have been developed, including the One Health approach, the EU Harmonized AMR Monitoring Program conducted in Italy in 2021, the National Action Plan for Combating Antibiotic-Resistant Bacteria (CARB) by the US (2020–2025), and the implementation of antimicrobial risk assessment.

One Health approach is a term recognized in the EU in 2016 by the United Nations Political Declaration on Antimicrobial Resistance (AMR), which states that human health, animal health, and the environment are interconnected and that disease is transmitted from humans to animals and vice versa. Furthermore, the environment could be a potential source of new resistant microorganisms; therefore, AMR should be addressed in all scenarios (25, 146, 147).

Strictly, One Health is defined as "a collaborative, multisectoral, and trans-disciplinary approach—working at local, regional, national, and global levels—to achieve optimal health (and wellbeing) outcomes recognizing the interconnections between people, animals, plants and their shared environment" (146). Therefore, a multidisciplinary approach is required to prevent the spread and emergence of antimicrobial resistance. Antimicrobial resistance (AMR) surveillance using the One Health approach has been implemented in Europe, the UK, and the US to mitigate the crisis. However, the lack of implementation in most developing countries resulted in the underestimation of the burden of AMR on terrestrial and aquatic animals and the environment (147).

Improper management of antimicrobials, such as inadequate control of infection, use of antimicrobials as growth promoters (long-term, low-dose mass medication), prophylaxis in livestock, farmed fish in aquaculture systems, agricultural debris, environmental pollutants from sewage, pharmaceutical industry waste, manure runoff from farms, use of heavy metals, use of disinfectants, and migration of people and animals infected with resistant bacteria, facilitate the spread of resistance between humans and animals (25, 148). Consequently, the One Health approach is fundamental since it is a multidisciplinary approach that tries to prevent, predict, detect, and respond to AMR (25, 148).

Critical strategies for addressing AMR from the One Health perspective includes: (1) conduct a global campaign to raise awareness of antimicrobial resistance and the damage caused by the overuse and misuse of antibiotics, (2) improve hygiene measures and prevent the spread of infections (i.e., decrease missing of

animals from different sources, stress of transport, unsanitary or crowded conditions), (3) reduce the use of antimicrobials in agriculture and their dissemination to the environment (including third-generation cephalosporins, fluoroquinolones, colistin, tetracyclines, and macrolides; i.e., growth promoters such as colistin has been banned in Europe, Canada, Denmark, United States, and other countries) (24), (4) improve global surveillance of drug resistance in order to understand and clarify the new mechanisms of resistance acquisition and predict future threats, (5) promote new and rapid clinical diagnoses, (6) promote the development and use of vaccines and alternatives to antibiotics (i.e., phage therapy, probiotics, antibodies, lysins, among others), (7) improve the number of studies in the field, (8) generated a global innovation fund for early-stage research on new treatments, (9) promote investment in new drugs and in the improvement of existing drugs, and finally, (10) build a global coalition for real action against AMR (25, 148, 149).

All previous studies have highlighted the urgent need to establish a One Health AMR surveillance system to understand the magnitude of the AMR problem, specifically the carbapenem-resistance problem, identify trends, and determine how all scenarios are linked and establish settings to content the widespread carbapenem-resistant organisms and genes. This approach requires the integration of human healthcare, livestock, aquaculture, and the environment, as well as other variety of disciplines and fields (149). Furthermore, the role of infections caused by antimicrobial-resistant organisms in wildlife may also have to be addressed, along with resistant organisms from aquatic environments, as they could possess intrinsic resistance and the possibility of being transmitted horizontally. In addition, carbapenem-resistant in companion animals has to take seriously since the human-pet bond might favor the silent transmission of clinically significant multidrug-resistant bacteria through zooanthroponosis (148).

Moreover, antimicrobial residues in fish products can persist in aquatic environments through excreta. For example, testing foodstuffs for carbapenem-resistant bacteria is not a legal requirement in any country; however, even a low prevalence of carbapenem-resistant genes has been detected in imported shrimp and salmon. In addition, studies have shown that aquaculture and terrestrial farms exhibit significant differences in drug consumption, with the aquaculture sector exhibiting the lowest. However, commensal bacterial flora can act as reservoirs of AMR genes, which may be transferred to microorganisms capable of causing human and animal diseases. Furthermore, it has been documented that animals excrete a significant percentage (75–90%) of antimicrobials without being metabolized and dispersed into the environment (145), which could be taken up by wild animals and function as a reservoir for antimicrobial resistance genes (149).

8. Discussion

The reports on carbapenem-resistant organisms published from seafood and aquaculture are still low. Most of these reports must include information on potential sources or transmission between humans, animals, and their environment. Similarly, some of the microorganisms found are of clinical importance. Some examples are carbapenem-producing *Vibrio alginolyticus*, which causes vibriosis, wound infection, and ear infection; *Vibrio cholerae*, which causes cholera; *Vibrio parahaemolyticus*, that cause acute, self-limiting gastroenteritis (150); *Shewanella algae*, a potential foodborne zoonotic agent in humans that causes necrotizing fasciitis, discitis, meningitis, biliary infection, pneumonia, and endocarditis (151); and *Enterobacter cloacae* complex that is common in nosocomial settings and capable of producing several infections such as pneumonia, urinary tract infections, and septicemia (152).

On livestock, carbapenem resistance has been observed in microorganisms from different bacterial species, critical in human settings, and associated with significant public health concerns worldwide, including *E. coli*, *Salmonella*, *P. mirabilis*, *P. aeruginosa*, and *A. baumannii*. In swine and poultry settings, VIM-1-producing *Salmonella* Infantis was found. This bacterium is a zoonotic pathogen commonly transferred via contaminated food products that have been implicated in human salmonellosis and foodborne outbreaks associated with egg and chicken meat (153). For instance, *A. baumannii* is an important opportunistic pathogen for hospital-acquired infections commonly associated with multi-drug resistance. The mortality rate of *A. baumannii* infection has been estimated to be over 50% (154). In livestock such as cattle and pigs, *A. baumannii* causes mastitis, pneumonia, and sepsis. In companion, animals cause urinary tract infections (155–157). Whether the presence of OXA-23-producing *A. baumannii* poses a substantial public health threat is unclear, but the presence of NDM-1 producers in *A. lwoffii and A. baumannii* isolated from poultry, swine, and cattle, which are clinically relevant to humans, is worrying. Similarly, NDM-producing *E. coli* and *K. pneumoniae*, isolated from poultry and cattle, are worrisome since they have been reported in clinical isolates worldwide.

The carbapenem-resistance determinants in wild animals need to be better understood. Wild animals may act as potential environmental reservoirs for bacterial resistance. VIM, NDM, OXA-48, and KPC-producing *K. pneumoniae* are the more frequent carbapenemases reported, followed by IMP, and NDM-producing *E. coli*. Contaminated food and water are the main routes of transmission of carbapenem-resistance bacteria to wild animals (158). However, anthropogenic pressure plays an essential role in the emergence of resistance, particularly in this setting. Interestingly, migrating birds (i.e., gulls) have been proposed to serve as a vehicle for disseminating carbapenem-resistance genes (159).

Among food-producing animals, the link between farming practices, animal health, carbapenem-resistant organisms' development and spread to farmers, and the presence of carbapenem-resistant organisms in foodstuffs requires much more investigation. Three studies positively associated livestock production with human CREC infections (143–145). These associations are mainly based on the observation that bla_{NDM}-carrying IncX3 plasmid isolated from humans exhibited between 75 and 99% nucleotide sequence identity to those in isolates from other sources, including chicken, pigs, and fly isolates. Moreover, one article identified a close relationship between the core-genome sequences of NDM-positive *E. coli* from humans and animals. The source-tracing analysis revealed indistinct boundaries between

human- and animal-derived NDM-positive *E. coli* (143). Several studies have found genetic similarities between carbapenem-producing bacteria from animal and human sources, including the detection of a porcine *E. coli* isolated carrying OXA-181 carbapenemase located on al IncX3 plasmid with high nucleotide similarity (99%) to previously published plasmid from human sources (30); *E. cloacae* IMI-1 positive isolated from clam and human-source *E. cloacae* ST373 isolate harboring bla_{IMI-1} sharing >75% similarity (8), and the detection of varying levels of genetic similarity at discrete genetic loci between *E. coli* carrying KPC-2 and *K. pneumoniae* containing KPC-3 isolated from gulls and humans in Alaska (104).

In companion animals, carbapenem resistance has been reported. The enzymes NDM-5, VIM, KPC, OXA-48-like, and OXA-23 were detected in *E. coli*, *K. pneumoniae*, *P. aeruginosa,* and *A. baumannii* (124–142). In this setting, the evidence suggests that zooanthroponosis is the main route of transmission of carbapenem-producing bacteria from humans to companion animals, indicating a cross-species transmission (115–123). Remarkably, in carbapenem-resistant organisms isolated from all the sources presented here (food-producing animals, seafood, aquaculture, wildlife, and companion animals), NDM-1 enzymes are occurring. These enzymes can hydrolyze all β-lactam antibiotics and have a high potential for rapid dissemination, thus, may constitute a public health risk (159).

These examples demonstrated that direct anthropozoonotic or zooanthroponotic transmission might be possible for CRE. However, to estimate the public health relevance of this transmission, more studies are needed to elucidate the problem. The addition of high-throughput technology, such as whole-genome sequencing and next-generation sequencing (NGS), has been permitted to determine the genetic relationship among CRO from different species at gene, plasmid, and strain levels. Similarly, introducing discriminant analysis of principal components (DAPCs) is helpful for tracing carbapenem-producing strains' potential origins.

9. Conclusion

The data presented in this review confirm the widespread of carbapenemase-producing bacteria and encoding genes in food-producing animals, seafood, aquaculture, companion animals, and wildlife as a cause of representing a severe problem for human and animal health.

Several studies have shown genetic similarities between human and animal carbapenem-resistance isolates, thus, demonstrating the possible cross-species transmission. Nonetheless, epidemiologic and genotypic analysis studies are needed to understand better the dynamics of antimicrobial drug resistance transmission between humans, animals, and the environment. In addition, the presence of CROs in the food chain compromises food safety and security and increases the chance of cross-border transmission of these bacteria.

One Health approach can help to implement global monitoring programs and establish antimicrobial risk assessments for the zoonotic and environmental sectors to address AMR emergencies. It is essential to identify and share best practices and policies globally. Collaboration between governments is needed to address cross-border health threats of AMR.

Author contributions

AG-B conceived and designed the idea for the manuscript. FR-C and AG-B wrote the manuscript. AG-B, FR-C, and FA-G reviewed the manuscript. All authors read and approved the final manuscript.

Funding

FR-C received a research grant from CONACYT (grant number: 373176). This study was supported by the internal research project PIB19-3 of the Autonomous University of Aguascalientes.

Acknowledgments

The authors are grateful to the Universidad Autonoma de Aguascalientes for supporting this study and the Master in Veterinary Science María Magdalena Soto Perezchica for their advice and point of view about the use of antibiotics in livestock. The authors are grateful to the financial support of the National Council of Science and Technology (CONACyT) for the scholarship awarded in the Call for Postdoctoral Stays for Mexico.

Conflict of interest

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.

Publisher's note

References

1. Nordmann P, Dortet L, Poirel L. Carbapenem resistance in *Enterobacteriaceae*: here is the storm! *Trends Mol Med.* (2012) 18:263–72. doi: 10.1016/j.molmed.2012.03.003

2. Gupta N, Limbago BM, Patel JB, Kallen AJ. Carbapenem-resistant *Enterobacteriaceae*: epidemiology and prevention. *Clin Infect Dis.* (2011) 53:60–7. doi: 10.1093/cid/cir202

3. Aurilio C, Sansone P, Barbarisi M, Pota V, Giaccari LG, Coppolino F, et al. Mechanisms of action of carbapenem resistance. *Antibiotics.* (2022) 11:421. doi: 10.3390/antibiotics11030421

4. Codjoe FS, Donkor ES. Carbapenem resistance: a review. *Med Sci.* (2017) 6. doi: 10.3390/medsci6010001

5. Meletis G, Protonotariou E, Papadopoulou D, Skoura L. Comment on: the carbapenemase menace: do dual mechanisms code for more resistance? *Infect Control Hosp Epidemiol.* (2016) 37:1392–4. doi: 10.1017/ice.2016.197

6. G.Taggar, Attiq Rheman M, Boerlin P, Diarra MS. Molecular epidemiology of carbapenemases in *Enterobacteriales* from humans, animals, food and the environment. *Antibiotics.* (2020) 9:693. doi: 10.3390/antibiotics9100693

7. Anderson REV, Boerlin P. Carbapenemase-producing *Enterobacteriaceae* in animals and methodologies for their detection. *Can J Vet Res.* (2020) 84:3–17.

8. Mangat CS, Boyd D, Janecko N, Martz SL, Desruisseau A, Carpenter M, et al. Characterization of VCC-1, a novel Ambler Class A carbapenemase from *Vibrio cholerae* isolated from imported retail shrimp sold in Canada. *Antimicrob Agents Chemother.* (2016) 60:1819–25. doi: 10.1128/AAC.02812-15

9. Bonardi S, Cabassi CS, Manfreda G, Parisi A, Fiaccadori E, Sabatino A, et al. Survey on carbapenem-resistant bacteria in pigs at slaughter and comparison with human clinical isolates in Italy. *Antibiotics.* (2022) 11:777. doi: 10.3390/antibiotics11060777

10. Walther-Rasmussen J, Høiby N, Class A. carbapenemases. *J Antimicrob Chemother.* (2007) 60:470–82. doi: 10.1093/jac/dkm226

11. Queenan AM, Bush K. Carbapenemases: the versatile beta-lactamases. *Clin Microbiol Rev.* (2007) 20:440–58. doi: 10.1128/CMR.00001-07

12. Hammoudi Halat D, Ayoub Moubareck C. The current burden of carbapenemases: review of significant properties and dissemination among gram-negative bacteria. *Antibiotics.* (2020) 9:186. doi: 10.3390/antibiotics9040186

13. Bonnin RA, Jousset AB, Emeraud C, Oueslati S, Dortet L, Naas T. Genetic diversity, biochemical properties, and detection methods of minor carbapenemases in *Enterobacterales*. *Front Med.* (2021) 7:616490. doi: 10.3389/fmed.2020.616490

14. Castanheira M, Toleman MA, Jones RN, Schmidt FJ, Walsh TR. Molecular characterization of a beta-lactamase gene, *bla*GIM-1, encoding a new subclass of metallo-beta-lactamase. *Antimicrob Agents Chemother.* (2004) 48:4654–61. doi: 10.1128/AAC.48.12.4654-4661.2004

15. Zheng Z, Cheng Q, Chan EW, Chen S. Genetic and biochemical characterization of VMB-1, a novel metallo-β-Lactamase encoded by a conjugative, broad-host range IncC plasmid from *Vibrio* spp. *Adv Biosyst.* (2020) 4:e1900221. doi: 10.1002/adbi.201900221

16. Lee K, Yum JH, Yong D, Lee HM, Kim HD, Docquier JD, et al. Novel acquired metallo-beta-lactamase gene, *bla*SIM-1, in a class 1 integron from *Acinetobacter baumannii* clinical isolates from Korea. *Antimicrob Agents Chemother.* (2005) 49:4485–91. doi: 10.1128/AAC.49.11.4485-4491.2005

17. Cheng Q, Zheng Z, Ye L, Chen S. Identification of a novel metallo-β-lactamase, VAM-1, in a foodborne *Vibrio alginolyticus* isolate from China. *Antimicrob Agents Chemother.* (2021) 65:e0112921. doi: 10.1128/AAC.01129-21

18. Zhao WH, Hu Z-Q. Acquired metallo-$\boldsymbol{\beta}$-lactamses and their genetic association with class 1 integrons and ISCR elements in Gram-negative bacteria. *Fut Microbiol.* (2015) 10. doi: 10.2217/fmb.15.18

19. Castanheira M, Sader HS, Farrell DJ, Mendes RE, Jones RN. Activity of ceftaroline-avibactam tested against Gram-negative organism populations, including strains expressing one or more β-lactamases and methicillin-resistant *Staphylococcus aureus* carrying various staphylococcal cassette chromosome mec types. *Antimicrob Agents Chemother.* (2012) 9:4779–85. doi: 10.1128/AAC.00817-12

20. Hansen GT. Continuous evolution: perspective on the epidemiology of carbapenemase resistance among Enterobacterales and other Gram-negative bacteria. *Infect Dis Ther.* (2021) 10:75–92. doi: 10.1007/s40121-020-00395-2

21. Zhai R, Fu B, Shi X, Sun C, Liu Z, Wang S, et al. Contaminated in-house environment contributes to the persistence and transmission of NDM-producing bacteria in a Chinese poultry farm. *Environ Int.* (2020) 139:105715. doi: 10.1016/j.envint.2020.105715

22. Nordmann P, Cuzon G, Naas T. The real threat of *Klebsiella pneumoniae* carbapenemase-producing bacteria. *Lancet Infect Dis.* (2009) 9:228–36. doi: 10.1016/S1473-3099(09)70054-4

23. van Duin D, Doi Y. Outbreak of colistin-resistant, carbapenemase-producing *Klebsiella pneumoniae*: are we at the end of the road? *J Clin Microbiol.* (2015) 53:3116–7. doi: 10.1128/JCM.01399-15

24. Shein AMS, Wannigama DL, Higgins PG, Hurst C, Abe S, Hongsing P, et al. High prevalence of *mgrB*-mediated colistin resistance among carbapenem-resistant *Klebsiella pneumoniae* is associated with biofilm formation, and can be overcome by colistin-EDTA combination therapy. *Sci Rep.* (2022) 12:12939. doi: 10.1038/s41598-022-17083-5

25. McEwen SA, Collignon PJ. Antimicrobial resistance: a one health perspective. *Microbiol Spectr.* (2018) 6. doi: 10.1128/microbiolspec.ARBA-0009-2017

26. EFSA Panel on Biological Hazards (BIOHAZ). Scientific Opinion on Carbapenem resistance in food animal ecosystems. *EFSA J.* (2013) 11:3501. doi: 10.2903/j.efsa.2013.3501

27. Liu X, Thungrat K, Boothe DM. Occurrence of OXA-48 carbapenemase and other β-lactamase genes in ESBL-producing multidrug resistant *Escherichia coli* from dogs and cats in the United States, 2009-2013. *Front Microbiol.* (2016) 7:1057. doi: 10.3389/fmicb.2016.01057

28. Al Bayssari C, Olaitan AO, Dabboussi F, Hamze M, Rolain JM. Emergence of OXA-48-producing *Escherichia coli* clone ST38 in fowl. *Antimicrob Agents Chemother.* (2015) 59:745–6. doi: 10.1128/AAC.03552-14

29. Braun SD, Ahmed MF, El-Adawy H, Hotzel H, Engelmann I, Weiss D, et al. Surveillance of extended-spectrum beta-lactamase-producing *Escherichia coli* in dairy cattle farms in the Nile Delta, Egypt. *Front Microbiol.* (2016) 7:1020. doi: 10.3389/fmicb.2016.01020

30. Pulss S, Semmler T, Prenger-Berninghoff E, Bauerfeind R, Ewers C. First report of an *Escherichia coli* strain from swine carrying an OXA-181 carbapenemase and the colistin resistance determinant MCR-1. *Int J Antimicrob Agents.* (2017) 50:232–6. doi: 10.1016/j.ijantimicag.2017.03.014

31. Zheng Z, Ye L, Chan EW, Chen S. Identification and characterization of a conjugative *bla*VIM-1-bearing plasmid in *Vibrio alginolyticus* of food origin. *J Antimicrob Chemother.* (2019) 74:1842–7. doi: 10.1093/jac/dkz140

32. Liu M, Zhang W, Peng K, Wang Z, Li R. Identification of a novel plasmid-mediated carbapenemase-encoding gene, *bla*VMB-2, in *Vibrio diabolicus*. *Antimicrob Agents Chemother.* (2021) 65:e0020621. doi: 10.1128/AAC.00206-21

33. Brouwer MSM, Tehrani HME, Rapallini M, Geurts Y, Kant A, Harders F, et al. Novel carbapenemases FLC-1 and IMI-2 encoded by an *Enterobacter cloacae* complex esolated from food products. *Antimicrob Agents Chemother.* (2019) 63:e02338–18. doi: 10.1128/AAC.02338-18

34. Brahmi S, Touati A, Cadiere A, Djahmi N, Pantel A, Sotto A, et al. First description of two sequence Type 2 *Acinetobacter baumannii* isolates carrying OXA-23 Carbapenemase in *Pagellus acarne* fished from the Mediterranean Sea near Bejaia, Algeria. *Antimicrob Agents Chemother.* (2016) 60:2513–5. doi: 10.1128/AAC.02384-15

35. Vieira RH, Carvalho EM, Carvalho FC, Silva CM, Sousa OV, Rodrigues DP. Antimicrobial susceptibility of *Escherichia coli* isolated from shrimp (*Litopenaeus vannamei*) and pond environment in northeastern Brazil. *J Environ Sci Health B.* (2010) 45:198–203. doi: 10.1080/03601231003613526

36. Morrison BJ, Rubin JE. Carbapenemase producing bacteria in the food supply escaping detection. *PLoS ONE.* (2015) 10:e0126717. doi: 10.1371/journal.pone.0126717

37. Rubin JE, Ekanayake S, Fernando C. Carbapenemase-producing organism in food, 2014. *Emerg Infect Dis.* (2014) 20:1264–5. doi: 10.3201/eid2007.140534

38. Janecko N, Martz SL, Avery BP, Daignault D, Desruisseau A, Boyd D, et al. Carbapenem-resistant *Enterobacter* spp. in retail seafood imported from Southeast Asia to Canada. *Emerg Infect Dis.* (2016) 22:1675–7. doi: 10.3201/eid2209.160305

39. Roschanski N, Guenther S, Vu TTT, Fischer J, Semmler T, Huehn S, et al. VIM-1 carbapenemase-producing *Escherichia coli* isolated from retail seafood, Germany 2016. *Euro Surveill.* (2017) 22:17-00032. doi: 10.2807/1560-7917.ES.2017.22.43.17-00032

40. Das UN, Singh AS, Lekshmi M, Nayak BB, Kumar S. Characterization of *bla*(NDM)-harboring, multidrug-resistant *Enterobacteriaceae* isolated from seafood. *Environ Sci Pollut Res Int.* (2019) 26:2455–63. doi: 10.1007/s11356-018-3759-3

41. Kim DH, Rajapaksha LG, Gunasekara CW, Wimalasena SH, Pathirana HN, Kim SR, et al. Phylogenetic relationships and antibiotic resistance of *Vibrio parahaemolyticus* isolates related to acute hepatopancreatic necrosis disease in Korea. *Aquaculture.* (2021) 545:737253. doi: 10.1016/j.aquaculture.2021.737253

42. Dahanayake PS, Hossain S, Wickramanayake M, Heo GJ. Prevalence of virulence and extended-spectrum beta-lactamase (ESBL) genes harbouring *Vibrio* spp. isolated from cockles (*Tegillarca granosa*) marketed in Korea. *Lett Appl Microbiol.* (2020) 71:61–9. doi: 10.1111/lam.13232

43. Hossain S, Wickramanayake M, Dahanayake PS, Heo GJ. Occurrence of virulence and extended-spectrum beta-lactamase leterminants in *Vibrio* spp. Isolated from marketed hard-shelled mussel (*Mytilus coruscus*). *Microb Drug Resist.* (2020) 26:391–401. doi: 10.1089/mdr.2019.0131

44. Ottaviani D, Medici L, Talevi G, Napoleoni M, Serratore P, Zavatta E, et al. Molecular characterization and drug susceptibility of non-O1/O139 *V. cholerae* strains of seafood, environmental and clinical origin, Italy. *Food Microbiol.* (2018) 72:82–8. doi: 10.1016/j.fm.2017.11.011

45. Serratore P, Zavatta E, Fiocchi E, Serafini E, Serraino A, Giacometti F, et al. Preliminary study on the antimicrobial susceptibility pattern related to the genotype of *Vibrio vulnificus* strains isolated in the north-western Adriatic Sea coastal area. *Ital J Food Saf.* (2017) 6:6843. doi: 10.4081/ijfs.2017.6843

46. Mani Y, Mansour W, Mammeri H, Denamur E, Saras E, Boujâafar N, Bouallègue O, J-Madec Y, Haenni M, KPC-3-producing ST167 *Escherichia coli* from mussels bought at a retail market in Tunisia. *J Antimicrob Chemotherapy.* (2017) 72:2403–4. doi: 10.1093/jac/dkx124

47. Mani Y, Mansour W, Lupo A, Saras E, Bouallègue O, Madec JY, et al. Spread of *bla*CTX-M-15-producing *Enterobacteriaceae* and OXA-23-producing *Acinetobacter baumannii* sequence type 2 in Tunisian seafood. *Antimicrob Agents Chemother.* (2018) 62:e00727–18. doi: 10.1128/AAC.00727-18

48. Briet A, Helsens N, Delannoy S, Debuiche S, Brisabois A, Midelet G, et al. NDM-1-producing *Vibrio parahaemolyticus* isolated from imported seafood. *J Antimicrob Chemother.* (2018) 73:2578–9. doi: 10.1093/jac/dky200

49. Sperling L, Alter T, Huehn S. Prevalence and antimicrobial resistance of *Vibrio* spp. in retail and farm shrimps in Ecuador. *J Food Prot.* (2015) 78:2089–92. doi: 10.4315/0362-028X.JFP-15-160

50. Hamza D, Dorgham S, Ismael E, Siel-Moez A, Elhariri M, Elhelw R, et al. Emergence of β-lactamase- and carbapenemase-producing *Enterobacteriaceae* at integrated fish farms. *Antimicrob Resist Infect Control.* (2020) 9. doi: 10.1186/s13756-020-00736-3

51. Huang YT, Cheng JF, Chen SY, Hong YK, Wu ZY, Liu PY. Draft genome sequence of carbapenem-resistant *Shewanella algae* strain AC isolated from small abalone (*Haliotis diversicolor*). *J Glob Antimicrob Resist.* (2018) 14:65–7. doi: 10.1016/j.jgar.2018.06.005

52. Lee YH, Tung KC, Cheng JF, Wu ZY, Chen SY, Hong YK. Genomic characterization of carbapenem-resistant Shewanella algae isolated from Asian hard clam (*Meretrix lusoria*). *Aquaculture.* (2019) 500:300–4. doi: 10.1016/j.aquaculture.2018.10.028

53. Zago V, Veschetti L, Patuzzo C, Malerba G, MLleo M. *Shewanella algae* and *Vibrio* spp. strains isolated in Italian aquaculture farms are reservoirs of antibiotic resistant genes that might constitute a risk for human health. *Mar Pollut Bull.* (2020) 154:111057. doi: 10.1016/j.marpolbul.2020.111057

54. Loest D, Uhland FC, Young KM, Li XZ, Mulvey MR, Reid-Smith R, et al. Carbapenem-resistant *Escherichia coli* from shrimp and salmon available for purchase by consumers in Canada: a risk profile using the Codex framework. *Epidemiol Infect.* (2022) 150:e148. doi: 10.1017/S0950268822001030

55. Schar D, Klein EY, Laxminarayan R, Gilbert M, Van Boeckel TP. Global trends in antimicrobial use in aquaculture. *Sci Rep.* (2020) 10:21878. doi: 10.1038/s41598-020-78849-3

56. Mollenkopf DF, Kleinhenz KE, Funk JA, Gebreyes WA, Wittum TE. *Salmonella enterica* and *Escherichia coli* harboring *bla*CMY in retail beef and pork products. *Foodborne Pathog Dis.* (2011) 8:333–6. doi: 10.1089/fpd.2010.0701

57. Mollenkopf DF, Stull JW, Mathys DA, Bowman AS, Feicht SM, Grooters SV, et al. Carbapenemase-producing *Enterobacteriaceae* recovered from the environment of a swine farrow-to-finish operation in the United States. *Antimicrob Agents Chemother.* (2017) 61:e01298-16. doi: 10.1128/AAC.01298-16

58. He W, Gao M, Lv L, Wang J, Cai Z, Bai Y, et al. Persistence and molecular epidemiology of *bla*(NDM)-positive Gram-negative bacteria in three broiler farms: A longitudinal study (2015-2021). *J Hazard Mater.* (2023) 446:130725. doi: 10.1016/j.jhazmat.2023.130725

59. Borowiak M, Szabo I, Baumann B, Junker E, Hammerl JA, Kaesbohrer A, et al. VIM-1-producing Salmonella Infantis isolated from swine and minced pork meat in Germany. *J Antimicrob Chemotherapy.* (2017) 72:2131–3. doi: 10.1093/jac/dkx101

60. Fischer J, San José M, Roschanski N, Schmoger S, Baumann B, Irrgang A, et al. Spread and persistence of VIM-1 Carbapenemase-producing *Enterobacteriaceae* in three German swine farms in 2011 and 2012. *Vet Microbiol.* (2017) 200:118–23. doi: 10.1016/j.vetmic.2016.04.026

61. Fischer J, Rodríguez I, Schmoger S, Friese A, Roesler U, Helmuth R, et al. *Salmonella enterica* subsp. enterica producing VIM-1 carbapenemase isolated from livestock farms. *J Antimicrob Chemother.* (2013) 68:478–80. doi: 10.1093/jac/dks393

62. Zhang WJ, Lu Z, Schwarz S, Zhang RM, Wang XM, Si W, et al. Complete sequence of the *bla*NDM-1-carrying plasmid pNDM-AB from *Acinetobacter baumannii* of food animal origin. *J Antimicrob Chemother.* (2013) 68:1681–2. doi: 10.1093/jac/dkt066

63. Kong LH, Lei CW, Ma SZ, Jiang W, Liu BH, Wang YX, et al. Various sequence types of *Escherichia coli* isolates coharboring *bla*NDM-5 and *mcr-1* genes from a commercial swine farm in China. *Antimicrob Agents Chemother.* (2017) 61:e02167–16. doi: 10.1128/AAC.02167-16

64. Carfora V, Diaconu EL, Ianzano A, Di Matteo P, Amoruso R, Dell'Aira E, et al. The hazard of carbapenemase OXA-181-producing *Escherichia coli* spreading in pig and veal calf holdings in Italy in the genomics era: Risk of spill over and spill back between humans and animals. *Front Microbiol.* (2022) 13:1016895. doi: 10.3389/fmicb.2022.1016895

65. Wang Y, Wu C, Zhang Q, Qi J, Liu H, Wang Y, et al. Identification of New Delhi metallo-β-lactamase 1 in *Acinetobacter lwoffii* of food animal origin. *PLoS ONE.* (2012) 7:e37152. doi: 10.1371/journal.pone.0037152

66. Sadek M, Poirel L, Nordmann P, Nariya H, Shimamoto T, Shimamoto T. Genetic characterisation of NDM-1 and NDM-5-producing Enterobacterales from retail chicken meat in Egypt. *J Glob Antimicrob Resist.* (2020) 23:70–1. doi: 10.1016/j.jgar.2020.07.031

67. Zhu X, Zhang Y, Shen Z, Xia L, Wang J, Zhao L, et al. Characterization of NDM-1-Producing Carbapenemase in *Proteus mirabilis* among Broilers in China. *Microorganisms.* (2021) 9:2443. doi: 10.3390/microorganisms9122443

68. Xie X, Zhang J, Wang HN, Lei CW. Whole genome sequence of a New Delhi metallo-β-lactamase 1-producing *Proteus mirabilis* isolate SNYG35 from broiler chicken in China. *J Glob Antimicrob Resist.* (2021) 24:266–9. doi: 10.1016/j.jgar.2020.12.023

69. Algammal AM, Hashem HR, Alfifi KJ, Hetta HF, Sheraba NS, Ramadan H, et al. *atpD* gene sequencing, multidrug resistance traits, virulence-determinants, and antimicrobial resistance genes of emerging XDR and MDR-*Proteus mirabilis*. *Sci Rep.* (2021) 11:9476. doi: 10.1038/s41598-021-88861-w

70. Hamza E, Dorgham SM, Hamza DA. Carbapenemase-producing *Klebsiella pneumoniae* in broiler poultry farming in Egypt. *J Glob Antimicrob Resist.* (2016) 7:8–10. doi: 10.1016/j.jgar.2016.06.004

71. Shi X, Li Y, Yang Y, Shen Z, Cai C, Wang Y, et al. High prevalence and persistence of carbapenem and colistin resistance in livestock farm environments in China. *J Hazard Mater.* (2021) 406:124298. doi: 10.1016/j.jhazmat.2021.125951

72. Poirel L, Berçot B, Millemann Y, Bonnin RA, Pannaux G, Nordmann P. Carbapenemase-producing *Acinetobacter* spp. in Cattle, France. *Emerg Infect Dis.* (2012) 18:523–5. doi: 10.3201/eid1803.111330

73. Webb HE, Bugarel M, den Bakker HC, Nightingale KK, Granier SA, Scott HM, et al. Carbapenem-resistant bacteria recovered from faeces of dairy cattle in the High Plains Region of the USA. *PLoS ONE.* (2016) 11:e0147363. doi: 10.1371/journal.pone.0147363

74. Ghatak S, Singha A, Sen A, Guha C, Ahuja A, Bhattacharjee U, et al. Detection of New Delhi metallo-beta-lactamse and extended-spectrum beta-lactamse genes in *Escherichia coli* isolated from mastitic milk samples. *Transbound Emerg Dis.* (2013) 60:385–9. doi: 10.1111/tbed.12119

75. Yaici L, Haenni M, Saras E, Boudehouche W, Touati A, Madec JY. *bla*NDM-5-carrying IncX3 plasmid in *Escherichia coli* ST1284 isolated from raw milk collected in a dairy farm in Algeria. *J Antimicrob Chemother.* (2016) 71:2671–2. doi: 10.1093/jac/dkw160

76. He T, Wang Y, Sun L, Pang M, Zhang L, Wang R. Occurrence and characterization of *bla*NDM-5-positive *Klebsiella pneumoniae* isolates from dairy cows in Jiangsu, China. *J Antimicrob Chemother.* (2017) 72:90–4. doi: 10.1093/jac/dkw357

77. Klotz P, Göttig S, Leidner U, Semmler T, Scheufen S, Ewers C. Carbapenem-resistance and pathogenicity of bovine *Acinetobacter indicus*-like isolates. *PLoS ONE.* (2017) 12:e0171986. doi: 10.1371/journal.pone.0171986

78. Vikram A, Schmidt JW. Functional bla(KPC-2) Sequences are present in US beef cattle feces regardless of antibiotic use. *Foodborne Pathog Dis.* (2018) 15:444–8. doi: 10.1089/fpd.2017.2406

79. Murugan MS, Sinha DK, Vinodh Kumar OR, Yadav AK, Pruthvishree BS, Vadhana P, et al. Epidemiology of carbapenem-resistant *Escherichia coli* and first report of *bla*VIM carbapenemases gene in calves from India. *Epidemiol Infect.* (2019) 147:e159. doi: 10.1017/S0950268819000463

80. Elshafiee EA, Nader SM, Dorgham SM, Hamza DA. Carbapenem-resistant *Pseudomonas aeruginosa* originating from farm animals and people in Egypt. *J Vet Res.* (2019) 63:333–7. doi: 10.2478/jvetres-2019-0049

81. Tshitshi L, Manganyi MC, Montso PK, Mbewe M, Ateba CN. Extended spectrum beta-Lactamase-resistant determinants among carbapenem-resistant *Enterobacteriaceae* from beef cattle in the North West Province, South Africa: a critical assessment of their possible public health implications. *Antibiotics.* (2020) 9:820. doi: 10.3390/antibiotics9110820

82. Tello M, Oporto B, Lavín JL, Ocejo M, Hurtado A. Characterization of a carbapenem-resistant *Escherichia coli* from dairy cattle harbouring *bla*NDM-1 in an IncC plasmid. *J Antimicrob Chemother.* (2022) 77:843–5. doi: 10.1093/jac/dkab455

83. Alam M, Rasool MH, Khan I, Khurshid M, Aslam B. Multilocus sequence typing of carbapenem-resistant *Acinetobacter baumannii* isolates harboring *bla*OXA-23 and *bla*IMP in cattle from Punjab, Pakistan. *Microb Drug Resist.* (2022) 28:997–1002. doi: 10.1089/mdr.2022.0083

84. Ben Haj Yahia A, Tayh G, Landolsi S, Maamar E, Galai N, Landoulsi Z„ et al. First report of OXA-48 and IMP genes among extended-spectrum beta-lactamase-producing *Escherichia coli* isolates from diarrheic calves in Tunisia. *Microb Drug Resist.* (2023) 29:150–62. doi: 10.1089/mdr.2022.0129

85. Girlich D, Naas T, Nordmann P. Biochemical characterization of the naturally occurring oxacillinase OXA-50 of *Pseudomonas aeruginosa*. *Antimicrob Agents Chemother.* (2004) 48:2043–8. doi: 10.1128/AAC.48.6.2043-2048.2004

86. Liakopoulos A, van der Goot J, Bossers A, Betts J, Brouwer MSM, Kant A, et al. Genomic and functional characterisation of IncX3 plasmids encoding *bla*SHV-12 in *Escherichia coli* from human and animal origin. *Sci Rep.* (2018) 8:7674. doi: 10.1038/s41598-018-26073-5

87. Su Y, Xin L, Zhang F, Peng C, Li Z, Liu C, et al. Drug resistance analysis of three types of avian-origin carbapenem-resistant *Enterobacteriaceae* in Shandong Province, China. *Poult Sci.* (2023) 102:102483. doi: 10.1016/j.psj.2023.102483

88. Evans BA, Amyes SG. OXA β-lactamases. *Clin Microbiol Rev.* (2014) 27:241–63. doi: 10.1128/CMR.00117-13

89. Purkait D, Ahuja A, Bhattacharjee U, Singha A, Rhetso K, Dey TK, et al. Molecular characterization and computational modelling of New Delhi metallo-β-lactamase-5 from an *Escherichia coli* Isolate (KOEC3) of bovine origin. *Indian J Microbiol.* (2016) 56:182–9. doi: 10.1007/s12088-016-0569-5

90. Pesapane R, Ponder M, Alexander KA. Tracking pathogen transmission at the human-wildlife interface: banded mongoose and *Escherichia coli*. *Ecohealth.* (2013) 10:115–28. doi: 10.1007/s10393-013-0838-2

91. Poirel L, Héritier C, Nordmann P. Chromosome-encoded ambler class D beta-lactamase of *Shewanella oneidensis* as a progenitor of carbapenem-hydrolyzing oxacillinase. *Antimicrob Agents Chemother.* (2004) 48:348–51. doi: 10.1128/AAC.48.1.348-351.2004

92. Potron A, Poirel L, Nordmann P. Origin of OXA-181, an emerging carbapenem-hydrolyzing oxacillinase, as a chromosomal gene in *Shewanella xiamenensis*. *Antimicrob Agents Chemother.* (2011) 55:4405–7. doi: 10.1128/AAC.00681-11

93. Poirel L, Figueiredo S, Cattoir V, Carattoli A, Nordmann P. *Acinetobacter radioresistens* as a silent source of carbapenem resistance for *Acinetobacter* spp. *Antimicrob Agents Chemother.* (2008) 52:1252–6. doi: 10.1128/AAC.01304-07

94. Fischer J, Schmoger S, Jahn S, Helmuth R, Guerra B. NDM-1 carbapenemase-producing *Salmonella enterica* subsp. enterica serovar Corvallis isolated from a wild bird in Germany. *J Antimicrob Chemother.* (2013) 68:2954–6. doi: 10.1093/jac/dkt260

95. Qin S, Fu Y, Zhang Q, Qi H, Wen JG, Xu H, et al. High incidence and endemic spread of NDM-1-positive *Enterobacteriaceae* in Henan Province, China. *Antimicrob Agents Chemother.* (2014) 58:4275–82. doi: 10.1128/AAC.02813-13

96. Dolejska M, Masarikova M, Dobiasova H, Jamborova I, Karpiskova R, Havlicek M, et al. High prevalence of *Salmonella* and IMP-4-producing *Enterobacteriaceae* in the silver gull on Five Islands, Australia. *J Antimicrob Chemother.* (2016) 71:63–70. doi: 10.1093/jac/dkv306

97. Vittecoq M, Laurens C, Brazier L, Durand P, Elguero E, Arnal A, et al. VIM-1 carbapenemase-producing *Escherichia coli* in gulls from southern France. *Ecol Evol.* (2017) 7:1224–32. doi: 10.1002/ece3.2707

98. Bachiri T, Bakour S, Lalaoui R, Belkebla N, Allouache M, Rolain JM, et al. Occurrence of carbapenemase-producing *Enterobacteriaceae* isolates in the wildlife: first report of OXA-48 in wild boars in Algeria. *Microb Drug Resist.* (2018) 24:337–45. doi: 10.1089/mdr.2016.0323

99. Liao X, Yang RS, Xia J, Chen L, Zhang R, Fang LX, et al. High colonization rate of a novel carbapenem-resistant *Klebsiella* lineage among migratory birds at Qinghai Lake, China. *J Antimicrob Chemotherapy.* (2019) 74:2895–903. doi: 10.1093/jac/dkz268

100. Lim SR, Lee D-H, Park SY, Lee S, Kim HY, Lee M-S, et al. Wild Nutria (*Myocastor coypus*) is a potential reservoir of carbapenem-resistant and zoonotic *Aeromonas* spp. in Korea. *Microorganisms.* (2019) 7:224. doi: 10.3390/microorganisms7080224

101. Gharout-Sait A, Touati A, Ahmim M, Brasme L, Guillard T, Agsous A, de Champs C. Occurrence of carbapenemase-producing *Klebsiella pneumoniae* in bat guano. *Microbial Drug Resist.* (2019) 25:1057–62. doi: 10.1089/mdr.2018.0471

102. Mairi A, Barraud O, Muggeo A, de Champs C, Touati A. Genomic analysis of a multidrug-resistant *Klebsiella pneumoniae* ST11 strain recovered from barbary deer (*Cervus elaphus barbarus*) in Akfadou forest, Algeria. *J Global Antimicrob Resist*. (2019) 22:300–4. doi: 10.1016/j.jgar.2020.04.027

103. Duff JP, AbuOun M, Bexton S, Rogers J, Turton J, Woodford N, et al. Resistance to carbapenems and other antibiotics in *Klebsiella pneumoniae* found in seals indicates anthropogenic pollution. *Vet Rec.* (2020) 187:154. doi: 10.1136/vr.105440

104. Ahlstrom CA, Frick A, Pongratz C, Spink K, Xavier C, Bonnedahl J, et al. Genomic comparison of carbapenem-resistant *Enterobacteriaceae* from humans and gulls in Alaska. *J Glob Antimicrob Resist.* (2021) 25:23–5. doi: 10.1016/j.jgar.2021.02.028

105. VinodhKumar OR, Karikalan M, Ilayaraja S, Sha AA, Singh BR, Sinha DL, et al. Multi-drug resistant (MDR), extended spectrum beta-lactamase (ESBL) producing and carbapenem resistant *Escherichia coli* in rescued Sloth bears (*Melursus ursinus*), India. *Vet Res Commun.* (2021) 45:163–70. doi: 10.1007/s11259-021-09794-3

106. Torres RT, Cunha MV, Ferreira H, Fonseca C, Palmeira JD, A. high-risk carbapenem-resistant *Pseudomonas aeruginosa* clone detected in red deer (*Cervus elaphus*) from Portugal. *Sci Total Environ.* (2022) 829:154699. doi: 10.1016/j.scitotenv.2022.154699

107. Ahlstrom CA, Woksepp H, Sandegren L, Mohsin M, Hasan B, Muzyka D, et al. Genomically diverse carbapenem resistant *Enterobacteriaceae* from wild birds provide insight into global patterns of spatiotemporal dissemination. *Sci Total Environ.* (2022) 824:153632. doi: 10.1016/j.scitotenv.2022.153632

108. Sellera FP, Cardoso B, Fuentes-Castillo D, Esposito F, Sano E, Fontana H, et al. Genomic analysis of a highly virulent NDM-1-producing *Escherichia coli* ST162 infecting a pygmy sperm whale (Kogia breviceps) in South America. *Front Microbiol.* (2022) 13:915375. doi: 10.3389/fmicb.2022.915375

109. Girlich D, Poirel L, Nordmann P. Novel ambler class A carbapenem-hydrolyzing beta-lactamase from a *Pseudomonas fluorescens* isolate from the Seine River, Paris, France. *Antimicrob Agents Chemother.* (2010) 54:328–32. doi: 10.1128/AAC.00961-09

110. Hubeny J, Korzeniewska E, Buta-Hubeny M, Zieliński W, Rolbiecki D, Harnisz M. Characterization of carbapenem resistance in environmental samples and *Acinetobacter* spp. isolates from wastewater and river water in Poland. *Sci Total Environ.* (2022) 822:153437. doi: 10.1016/j.scitotenv.2022.153437

111. Harmon DE, Miranda OA, McCarley A, Eshaghian M, Carlson N, Ruiz C. Prevalence and characterization of carbapenem-resistant bacteria in water bodies in the Los Angeles-Southern California area. *Microbiol Open.* (2019) 8:e00692. doi: 10.1002/mbo3.692

112. Aubron C, Poirel L, Ash RJ, Nordmann P. Carbapenemase-producing *Enterobacteriaceae*, US rivers. *Emerg Infect Dis.* (2005) 11:260–4. doi: 10.3201/eid1102.030684

113. Dewi D, Götz B, Thomas T. Diversity and genetic basis for carbapenem resistance in a coastal marine environment. *Appl Environ Microbiol.* (2020) 86. doi: 10.1128/AEM.02939-19

114. Sellera FP, Da Silva LCBA, Lincopan N. Rapid spread of critical priority carbapenemase-producing pathogens in companion animals: a One Health challenge for a post-pandemic world. *J Antimicrob Chemotherapy.* (2021) 76:2225–9. doi: 10.1093/jac/dkab169

115. Sellera FP, Lincopan N. Zooanthroponotic transmission of high-risk multi-drug-resistant pathogens: a neglected public health issue. *J Infect Public Health.* (2019) 12:294–5. doi: 10.1016/j.jiph.2018.12.013

116. Fernandes MR, Sellera FP, Moura Q, Carvalho MP, Rosato PN, Cerdeira L, et al. Zooanthroponotic transmission of drug-resistant *Pseudomonas aeruginosa*, Brazil. *Emerg Infect Dis.* (2018) 24:1160–2. doi: 10.3201/eid2406.180335

117. Smith A, Wayne AS, Fellman CL, Rosenbaum MH. Usage patterns of carbapenem antimicrobials in dogs and cats at a veterinary tertiary care hospital. *J Vet Intern Med.* (2019) 33:1677–85. doi: 10.1111/jvim.15522

118. Grönthal T, Österblad M, Eklund M, Jalava J, Nykäsenoja S, Pekkanen K, et al. Sharing more than friendship – transmission of NDM-5 ST167 and CTX-M-9 ST69 *Escherichia coli* between dogs and humans in a family, Finland 2015. *Euro Surveill.* (2018) 23:1700497. doi: 10.2807/1560-7917.ES.2018.23.27.1700497

119. Zafer MM, Al-Agamy MH, El-Mahallawy HA, Amin MA, Ashour EDS. Dissemination of VIM-2 producing *Pseudomonas aeruginosa* ST233 at tertiary care hospitals in Egypt. *BMC Infect Dis.* (2015) 15:122. doi: 10.1186/s12879-015-0861-8

120. Wright LL, Turton JF, Livermore DM, Hopkins KL, Woodford N. Dominance of international 'high-risk clones' among metallo-β-lactamase producing *Pseudomonas aeruginosa* in the UK. *J Antimicrob Chemother.* (2015) 70:103–10. doi: 10.1093/jac/dku339

121. Sader HS, Reis AO, Silbert S, Gales AC. IMPs, VIMs and SPMs: the diversity of metallo-β-lactamases produced by carbapenem-resistant *Pseudomonas aeruginosa* in a Brazilian hospital. *Clin Microbiol Infect.* (2005) 11:73–6. doi: 10.1111/j.1469-0691.2004.01031.x

122. Wang MG, Fang C, Liu KD, Wang LL, Sun RY, Zhang RM, et al. Transmission and molecular characteristics of *bla*NDM-producing *Escherichia coli* between companion animals and their healthcare providers in Guangzhou, China. *J Antimicrob Chemother.* (2022) 77:351–5. doi: 10.1093/jac/dkab382

123. Garcia-Fierro R, Drapeau A, Dazas M, Saras E, Rodrigues C, Brisse S, et al. Comparative phylogenomics of ESBL-, AmpC- and carbapenemase-producing Klebsiella pneumoniae originating from companion animals and humans. *J Antimicrob Chemotherapy.* (2022) 77:1263–71. doi: 10.1093/jac/dkac041

124. Silva JMD, Menezes J, Marques C, Pomba CF. Companion animals-an overlooked and misdiagnosed reservoir of carbapenem resistance. *Antibiotics.* (2022) 11:533. doi: 10.3390/antibiotics11040533

125. Stolle I, Prenger-Berninghoff E, Stamm I, Scheufen S, Hassdenteufel E, Guenther S, et al. Emergence of OXA-48 carbapenemase-producing *Escherichia coli* and *Klebsiella pneumoniae* in dogs. *J Antimicrob Chemother.* (2013) 68:2802–8. doi: 10.1093/jac/dkt259

126. Smet A, Boyen F, Pasmans F, Butaye P, Martens A, Nemec A, et al. OXA-23-producing *Acinetobacter* species from horses: a public health hazard? *J Antimicrob Chemother.* (2012) 67:3009–10. doi: 10.1093/jac/dks311

127. Sellera FP, Fernandes MR, Ruiz R, Falleiros ACM, Rodrigues FP, Cerdeira L, et al. Identification of KPC-2-producing *Escherichia coli* in a companion animal: a new challenge for veterinary clinicians. *J Antimicrob Chemother.* (2018) 73:2259–61. doi: 10.1093/jac/dky173

128. Sellera FP, Fuga B, Fontana H, Esposito F, Cardoso B, Konno S, et al. Detection of IncN-pST15 one-health plasmid harbouring *bla*KPC-2 in a hypermucoviscous *Klebsiella pneumoniae* CG258 isolated from an infected dog, Brazil. *Transbound Emerg Dis.* (2021) 68:3083–8. doi: 10.1111/tbed.14006

129. Gomez-Simmonds A, Hu Y, Sullivan SB, Wang Z, Whittier S, Uhlemann AC. Evidence from a New York City hospital of rising incidence of genetically diverse carbapenem-resistant *Enterobacter cloacae* and dominance of ST171, 2007-14. *J Antimicrob Chemother.* (2016) 71:2351–3. doi: 10.1093/jac/dkw132

130. Daniels JB, Chen L, Grooters SV, Mollenkopf DF, Mathys DA, Pancholi P, et al. *Enterobacter cloacae* complex Sequence Type 171 Isolates Expressing KPC-4 Carbapenemase Recovered from Canine Patients in Ohio. *Antimicrob Agents Chemother.* (2018) 62:e01161-18. doi: 10.1128/AAC.01161-18

131. Abraham S, O'Dea M, Trott DJ, Abraham RJ, Hughes D, Pang S, et al. Isolation and plasmid characterization of carbapenemase (IMP-4) producing *Salmonella enterica* Typhimurium from cats. *Sci Rep*. (2016) 6:35527. doi: 10.1038/srep35527

132. Shaheen BW, Nayak R, Boothe DM. Emergence of a New Delhi metallo-β-lactamase (NDM-1)-encoding gene in clinical *Escherichia coli* isolates recovered from companion animals in the United States. *Antimicrob Agents Chemother*. (2013) 57:2902–3. doi: 10.1128/AAC.02028-12

133. Cui L, Lei L, Lv Y, Zhang R, Liu X, Li M, et al. bla(NDM-1)-producing multidrug-resistant *Escherichia coli* isolated from a companion dog in China. *J Glob Antimicrob Resist*. (2018) 13:24–7. doi: 10.1016/j.jgar.2017.10.021

134. Gentilini F, Turba ME, Pasquali F, Mion D, Romagnoli N, Zambon E, et al. Hospitalized pets as a source of carbapenem-resistance. *Front Microbiol*. (2018) 9:2872. doi: 10.3389/fmicb.2018.02872

135. Kuang X, Yang R, Ye X, Sun J, Liao X, Liu Y, et al. NDM-5-Producing *Escherichia coli* co-harboring *mcr-1* gene in companion animals in China. *Animals*. (2022) 12:1310. doi: 10.3390/ani12101310

136. Pulss S, Stolle I, Stamm I, Leidner U, Heydel C, Semmler T, et al. Multispecies and clonal dissemination of OXA-48 carbapenemase in *Enterobacteriaceae* from companion animals in Germany, 2009-2016. *Front Microbiol*. (2018) 9:1265. doi: 10.3389/fmicb.2018.01265

137. Yousfi M, Touati A, Muggeo A, Mira B, Asma B, Brasme L, et al. Clonal dissemination of OXA-48-producing *Enterobacter cloacae* isolates from companion animals in Algeria. *J Glob Antimicrob Resist*. (2018) 12:187–91. doi: 10.1016/j.jgar.2017.10.007

138. González-Torralba A, Oteo J, Asenjo A, Bautista V, Fuentes E, Alós JI. Survey of carbapenemase-producing *Enterobacteriaceae* in companion dogs in Madrid, Spain. *Antimicrob Agents Chemother*. (2016) 60:2499–501. doi: 10.1128/AAC.02383-15

139. Hyun JE, Chung TH, Hwang CY. Identification of VIM-2 metallo-β-lactamase-producing *Pseudomonas aeruginosa* isolated from dogs with pyoderma and otitis in Korea. *Vet Dermatol*. (2018) 29:186–e68. doi: 10.1111/vde.12534

140. Brilhante M, Menezes J, Belas A, Feudi C, Schwarz S, Pomba C, et al. OXA-181-Producing extraintestinal pathogenic *Escherichia coli* Sequence Type 410 isolated from a dog in Portugal. *Antimicrob Agents Chemother*. (2020) 64:e02298-19. doi: 10.1128/AAC.02298-19

141. Pomba C, Endimiani A, Rossano A, Saial D, Couto N, Perreten V. First report of OXA-23-mediated carbapenem resistance in sequence type 2 multidrug-resistant *Acinetobacter baumannii* associated with urinary tract infection in a cat. *Antimicrob Agents Chemother*. (2014) 58:1267–8. doi: 10.1128/AAC.02527-13

142. Ewers C, Klotz P, Leidner U, Stamm I, Prenger-Berninghoff E, Göttig S, et al. OXA-23 and IS*Aba1*-OXA-66 class D β-lactamases in *Acinetobacter baumannii* isolates from companion animals. *Int J Antimicrob Agents*. (2017) 49:37–44. doi: 10.1016/j.ijantimicag.2016.09.033

143. Shen Y, Hu F, Wang Y, Yin D, Yang L, Chen Y, et al. Transmission of carbapenem resistance between human and animal NDM-positive *Escherichia coli* strains. *Engineering*. (2022) 15:24–33. doi: 10.1016/j.eng.2021.07.030

144. Li J, Bi Z, Ma S, Chen B, Cai C, He J, et al. Inter-host transmission of carbapenemase-producing *Escherichia coli* among humans and backyard animals. *Environ Health Perspect*. (2019) 127:107009. doi: 10.1289/EHP5251

145. Wang Y, Zhang R, Li J, Wu Z, Yin W, Schwarz S, et al. Comprehensive resistome analysis reveals the prevalence of NDM and MCR-1 in Chinese poultry production. *Nat Microbiol*. (2017) 6:16260. doi: 10.1038/nmicrobiol.2016.260

146. One Health High-Level Expert Panel (OHHLEP), Adisasmito WB, Almuhairi S, Behravesh CB, Bilivogui P, Bukachi SA, et al. (One Health: A new definition for a sustainable and healthy future. *PLoS Pathog*. (2022) 18:e1010537. doi: 10.1371/journal.ppat.1010537

147. Das S. The crisis of carbapenemase-mediated carbapenem resistance across the human-animal-environmental interface in India. *Infect Dis Now*. (2023) 53:104628. doi: 10.1016/j.idnow.2022.09.023

148. Velazquez-Meza ME, Galarde-López M, Carrillo-Quiróz B, Alpuche-Aranda CM. Antimicrobial resistance: One Health approach. *Vet World*. (2022) 15:743–9. doi: 10.14202/vetworld.2022.743-749

149. Ferri M, Ranucci E, Romagnoli P, Giaccone V. Antimicrobial resistance: a global emerging threat to public health systems. *Crit Rev Food Sci Nutr*. (2017) 57:2857–76. doi: 10.1080/10408398.2015.1077192

150. Ottaviani D, Leoni F, Serram Serracca L, Decastelli L, Rocchegiani E, Masini L, et al. Nontoxigenic *Vibrio parahemolyticus* strains causing acute gastroenteritis. *J Clin Microbiol*. (2012) 50:4141–3. doi: 10.1128/JCM.01993-12

151. Bauer MJ, Stone-Garza KK, Croom D, Andreoli C, Woodson P, Graf PC-F, et al. *Shewanella algae* infections in United States naval special warfare trainees. *Open Forum Infect Dis*. (2019) 6:ofz442 doi: 10.1093/ofid/ofz442

152. Annavajhala MK, Gomez-Simmonds A, Uhlemann A-C. (2019). Multidrug-resistant enterobacter cloacae complex emerging as a global, diversifying threat. *Front. Microbiol*. 10:44. doi: 10.3389/fmicb.2019.00044

153. Pillai SD. Source tracing in Thailand following contamination of dog chew toys with Salmonella. In: Hoorfar J, editor. *Woodhead Publishing Series in Food Science, Technology and Nutrition, Case Studies in Food Safety and Authenticity*. Woodhead Publishing. p. 123–9. doi: 10.1533/9780857096937.2.123

154. Bulens SN Yi SH, Walters MS, Jacob JT, Bower C, Reno J, et al. Carbapenem-nonsusceptible *Acinetobacter baumannii*, 8 US metropolitan areas, 2012–2015. *Emerg Infect Dis*. (2018) 24:727–34. doi: 10.3201/eid2404.171461

155. Müller S, Janssen T, Wieler LH. Multidrug resistant Acinetobacter baumannii in veterinary medicine - emergence of an underestimated pathogen? *BMTW*. (2014) 127:435–46.

156. van der Kolk JH, Endimiani A, Graubner C, Gerber V, Perreten V. *Acinetobacter* in Veterinary Medicine with emphasis on *A. baumannii*. *J Glob Antimicrob Resist*. (2018) 16:59–71. doi: 10.1016/j.jgar.2018.08.011

157. Wareth G, Neubauer H, Sprague LD. *Acinetobacter baumannii* – a neglected pathogen in veterinary and environmental health in Germany. *Vet Res Commun*. (2019) 43:1–6. doi: 10.1007/s11259-018-9742-0

158. Cole D, Drum DJ, Stalknecht DE, White DG, Lee MD, Ayers S, et al. Free-living Canada geese and antimicrobial resistance. *Emerg Infect Dis*. (2005) 11:935–8. doi: 10.3201/eid1106.040717

159. Radhouani H, Silva N, Poeta P, Torres C, Correia S, Igrejas G. Potential impact of antimicrobial resistance in wildlife, environment, human health. *Front Microbiol*. (2014) 5:23. doi: 10.3389/fmicb.2014.00023

frontiers | Frontiers in Veterinary Science

TYPE Original Research
PUBLISHED 24 July 2023
DOI 10.3389/fvets.2023.1127819

OPEN ACCESS

EDITED BY
Eliana Guedes Stehling,
University of São Paulo, Ribeirão Preto, Brazil

REVIEWED BY
Timothy Frana,
Boehringer Ingelheim, United States
Olivia S. K. Chan,
The University of Hong Kong,
Hong Kong SAR, China
Noelle Robertson Noyes,
University of Minnesota Morris, United States

*CORRESPONDENCE
Ken Katsuda
katsuda@affrc.go.jp

RECEIVED 20 December 2022
ACCEPTED 10 July 2023
PUBLISHED 24 July 2023

CITATION
Kobayashi S, Tamamura-Andoh Y, Yamane I, Kusumoto M and Katsuda K (2023) The association between farm-level antimicrobial usage and resistance of *Staphylococcus spp.*, as the major genus isolated from aerosol samples, in Japanese piggeries.
Front. Vet. Sci. 10:1127819.
doi: 10.3389/fvets.2023.1127819

The association between farm-level antimicrobial usage and resistance of *Staphylococcus spp.*, as the major genus isolated from aerosol samples, in Japanese piggeries

Sota Kobayashi[1], Yukino Tamamura-Andoh[1], Itsuro Yamane[1], Masahiro Kusumoto[1] and Ken Katsuda[2]*

[1]Enteric Pathogen Group, Division of Zoonosis Research, National Institute of Animal Health, NARO, Tsukuba, Ibaraki, Japan, [2]Division of Hygiene Management Research, National Institute of Animal Health, NARO, Tsukuba, Ibaraki, Japan

Bacteria are the dominant particulate matter in livestock houses and can threaten animal and public health. Antimicrobial resistance (AMR) is a crucial concern worldwide, and nationwide measures established based on the One Health approach are being implemented in many countries. This requires multidisciplinary perspectives and collaboration among the human, animal, and environmental sectors. However, information on the AMR risk in livestock house aerosol is limited, especially its association with antimicrobial usage (AMU). Therefore, this study was conducted to reveal the AMR profile of *Staphylococcus*, the major bacterial genus in the aerosol of the piggeries of Japanese farms, and the association between farm-level AMU and AMR. The investigation at 10 farrow-to-finish pig farms revealed that regardless of the sampling season and the piggery group, the resistance rate of isolated staphylococci for oxacillin, erythromycin, and lincomycin was more than 40% of the median and tended to be higher than that for other antimicrobials. The AMU adjusted by the defined daily dose (DDD-adjusted AMU) in the fattening piggery group was significantly higher than that in the sow piggery group ($p < 0.05$). Finally, for the fattening piggery group, the generalized linear mixed model revealed that the AMR rate for oxacillin, erythromycin, tetracycline, and chloramphenicol was positively associated with the corresponding class-based DDD-adjusted AMU of penicillins (odds ratio (OR) = 2.63, $p = 0.03$), macrolides (OR = 6.89, $p = 0.0001$), tetracyclines (OR = 2.48, $p = 0.04$), and amphenicols (OR = 3.22, $p = 0.03$), respectively. These significant positive associations observed in this study imply that the resistance rate for these antimicrobials may decrease by reducing the corresponding antimicrobials' use. In addition, the resistance rates for erythromycin and chloramphenicol also displayed a positive association with the AMU of antimicrobial classes other than macrolides and amphenicols, respectively. The mechanism underlying these phenomena is unclear; therefore, further evaluation will be needed. As limited studies have reported staphylococci in piggery aerosol and its AMR with quantitative AMU, these results based on on-farm investigations are expected to aid in establishing countermeasures for AMR of aerosol bacteria in pig farms.

KEYWORDS

antimicrobial resistance, antimicrobial usage, staphylococci, aerosol, piggery

1. Introduction

Particulate matter in aerosol is an essential indicator of air pollution (1). Toxic and harmful substances, including microorganisms and bacteria, constitute air pollution (2, 3), dominating livestock farm aerosols (4). Therefore, it is rational that bacteria from the environment and animals threaten both animal and public health. For instance, the increased density of animals in piggeries under an intensive production system often results in poor air quality (5). This phenomenon increases the risk of various opportunistic infections, unless ventilation is appropriately managed. Moreover, pig house farmers are at higher risk of respiratory diseases than chicken, cattle, or sheep farmers (6, 7).

Moreover, as the world faces multiple health challenges, antimicrobial resistance (AMR) is a crucial concern listed among the top 10 global health threats (8, 9). A recent worldwide estimation revealed approximately 4.95 million deaths associated with AMR in 2019 (10). Excessive and inappropriate antimicrobial usage (AMU) has been primary reason; therefore, nationwide measures based on the action plan of each country, established based on the Global Action Plan with the One Health approach, are being taken (11). Thus, the human, animal, and environmental sectors need to have multidisciplinary perspectives and collaborate by sharing the insights obtained from each sector.

In Japan, the total quantity of antimicrobials based on the weight of active substances was 1,761.4 tons in 2018. Among those, 36.7 and 12.3% accounted for the livestock sector and feed additives, respectively. Moreover, 74.5% of those for the livestock sector were used in pig production, with tetracyclines, penicillins, sulfonamides, and macrolides as the major classes (12).

Although information on bacterial AMR in the piggery aerosol is available (13), that on its association with AMU is limited. Therefore, this study revealed the AMR characteristics of staphylococci, including animal and human pathogens. We also aimed to evaluate the association between farm-level AMU and AMR of staphylococci. This study's findings would aid in establishing better countermeasures for AMR in piggeries for animal and public health.

2. Materials and methods

2.1. Farm recruitment and sampling frame

With the cooperation of the field veterinarians from The Japanese Association of Swine Veterinarians,[1] consent for participation in this observational study was obtained from ten farrow-to-finish pig farms on a convenient basis. Between November 2017 and July 2020, each farm was visited twice in the warm (spring and summer) and cold (autumn and winter) seasons, respectively, except for farm E (visited only once in the cold season). Brief descriptions of these farms are presented in Table 1 with the varied farm size of 70–1,790, based on sow number. At each visit, aerosol samples were collected from five pig houses of different life stages, including sow stall and farrowing houses as the sow piggery group and the weaners, growers, and finishers houses as the fattening piggery group. Using a commercial air sampler (CORIOLIS MICRO, Bertin Technologies SAS, France) placed at the center of each piggery, 3,000 L of air was passed into 10 mL of sterilized phosphate-buffered saline (PBS, Dulbecco's PBS (−) "Nissui" Nissui Pharmaceutical Co., Ltd., Tokyo, Japan) for 10 min. These PBS samples were brought to the National Institute of Animal Health for laboratory investigations.

In total, 19 sampling visits were made in ten cold and nine warm seasons. Samples were obtained from both piggery groups during all nine warm season visits, meaning nine sow and nine fattening piggeries. However, during the ten cold season visits, due to the technical condition, samples were collected from only the fattening piggery group of Farm D, which meant nine sow and ten fattening piggeries were targeted. This sampling frame is summarized in Figure 1.

2.2. Isolation and identification of bacteria

Within 20 h after the on-farm sampling, 100 µL of the PBS sample obtained from each piggery was inoculated on 5% sheep blood in trypticase soy agar (TSA) (BD Trypticase Soy Agar with 5% Sheep Blood, Nippon Becton, Dickinson, and Company, Japan) and mannitol salt agar (MSA) (Mannitol Salt Agar "Nissui," Nissui Pharmaceutical Co., Ltd., Tokyo, Japan) and aerobically cultured for 16–20 h at 37°C. MSA was used as gram-positive bacteria selective agar, especially, salt-tolerant bacteria, which included some members of the *Staphylococcus* genus. Then, 10 isolates were randomly selected from each medium and stored in 10% glycerol-added Muller Hinton broth (Difco; BD, New Jersey, United States) at −80°C until identification.

All the isolates were identified using species-specific PCR for staphylococci, assumed as the dominant genus by the authors, following previously established procedures (14, 15). For those not identified using this PCR, partial gap gene sequencing (16) or 16S rRNA partial sequencing using a commercial kit (Bacterial 16S rDNA PCR Kit, Takara Bio Inc., Shiga, Japan) was applied following the manufacturer's instructions. In addition, sequence data were analyzed to determine the most likely species, referring to the EzBioCloud Database.[2]

The identified isolates' distribution by genus was summarized. In particular, the Chi-square test statistically evaluated the proportion of staphylococci among all the bacterial isolates in each farm by the seasons and the piggery groups.

2.3. Antimicrobial susceptibility test

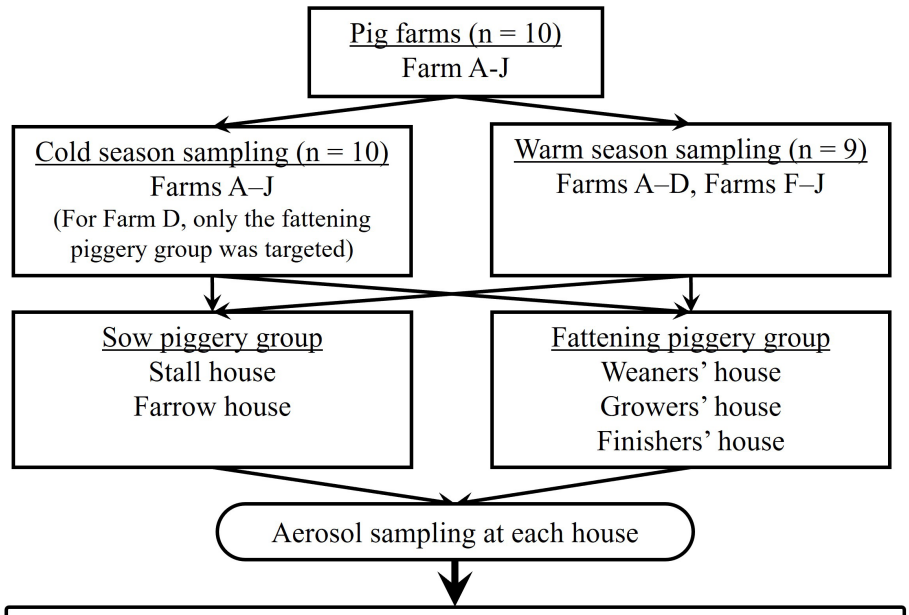
For all the isolated staphylococci, the minimum inhibitory concentration (MIC) values of the 11 antimicrobials below were determined using a commercial kit (Dry Plate "Eiken," Eiken Chemical Co., Ltd., Japan). Antimicrobial phenotypes were interpreted based on the breakpoints provided by the CLSI guidelines: 0.5 µg/mL for oxacillin (OXA), 0.5 µg/mL for ampicillin (AMP), 8.0 µg/mL for

1 http://www.e-jasv.com/

2 https://www.ezbiocloud.net/

TABLE 1 Brief description of the ten recruited pig farms.

ID	Number of sites	Sows (head)	Annual shipment (head)	Workers	All-in all-out in operation
A	1	70	2,200	3	No
B	2	620	16,200	16	Yes
C	1	1,790	40,700	34	Yes
D	1	510	12,500	11	Yes
E	1	800	18,200	15	Yes
F	2	1,260	21,900	18	Yes
G	2	490	13,000	7	Yes
H	2	320	7,600	7	Yes
I	1	90	1,700	3	No
J	1	240	4,100	6	Yes



Number of data for evaluations of the resistance rate, antimicrobial usage, and their statistical modeling

	Sow piggery group	Fattening piggery group	Total
Cold season sampling (n = 10)	9	10	19
Warm season sampling (n = 9)	9	9	18
Total	18	19	37

FIGURE 1
Flowchart of the sampling process in this study based on farm visits by season and piggery group.

cefazoline (CFZ), 16.0 μg/mL for kanamycin (KAN), 16.0 μg/mL for gentamycin (GEN), 8.0 μg/mL for erythromycin (ERY), 16.0 μg/mL for tetracycline (TET), 32.0 μg/mL for chloramphenicol (CHL), 32.0 μg/mL for vancomycin (VAN), 8.0 μg/mL for lincomycin (LCM), and 4.0 μg/mL for ciprofloxacin (CIP), respectively (17). In addition, the following quality control strains were also assessed: *Staphylococcus aureus* ATCC 29213, *Enterococcus faecalis* ATCC 29212, *Escherichia coli* ATCC 25923, and *Pseudomonas aeruginosa* ATCC 27853.

The resistance rate (%) for each antimicrobial was defined as the proportion of resistant staphylococci isolates among the staphylococci isolates analyzed using the antimicrobial susceptibility test and the distribution of each antimicrobial based on the sampling seasons [$n = 19$ for "cold"s, and 18 for "warm"s (Figure 1)] and piggery groups [$n = 18$ for "sow"s, and 19 for "fattening"s (Figure 1)] was compared using the Mann–Whitney *U* test.

2.4. Quantification of AMU

Annual antimicrobial product purchases were recorded for the piggery groups in each farm. This study used the previous year's volume as the reference AMU data for a farm visit between January and June. The current year's data were adopted for visits between July and December. Then, the annual mean treatment days (head*day) were estimated as follows:

$$\text{Annual mean treatment days for sows } (head * day) = \frac{\text{weight of active substance of each product / defined daily dose of each product}}{\text{number of sows} \times 240kg}$$

$$\text{Annual mean treatment days for fattening pigs } (head * day) = \frac{\text{weight of active substance of each product / defined daily dose of each product}}{\text{number of fattening pigs} \times 65kg}$$

The defined daily dose (DDD) is the Japanese pig production-specific indicator established previously (18, 19), based on the original concept and definition by the World Health Organization (20). These annual mean treatment days were the annual DDD-adjusted usage of each commercial product and summed up by the antimicrobial classes, which were tetracyclines (TETs), amphenicols (APCs), penicillins (PENs), cephalosporins (CEPs), sulfonamides (SULs), pyrimidines (PMDs), macrolides (MCLs), lincosamides (LCMs), aminoglycosides (AGDs), quinolones (QUIs), polymixins (PMXs), and pleuromutilins (PLMs), respectively. The class-based annual DDD-adjusted AMU was statistically compared by the seasons [$n = 19$ of "cold"s, and 18 of "warm"s (Figure 1)] and piggery groups [$n = 18$ of "sow"s, and 19 of "fattening"s Figure 1)], respectively, using the Mann–Whitney *U* test.

2.5. Statistical modeling to evaluate the association between the resistance rate of each antimicrobial and class-based annual DDD-adjusted AMU

Association between the resistance rate of staphylococci for each antimicrobial and class-based annual DDD-adjusted AMU was explored using the generalized linear mixed model on each piggery group ($n = 18$ for the sow group and $n = 19$ for the fattening group). Considering the difference in the number of staphylococci successfully obtained on each sampling visit, raw data used for resistance rate calculation were incorporated into the model as the dependent variable; both tested and resistant staphylococci isolates were directly employed. Moreover, with the various farms cooperating, as presented in Table 1, sampling season was forced into the model, and the farm was employed as the random effect. Therefore, the model is described as follows:

$$logit(p) = log\left(\frac{p}{1-p}\right) = \alpha + season + \sum \beta\chi + RF + e$$

Where p in logit (p) of the model outcome represents the resistance rate accounting for the tested and resistant staphylococci isolates, α is the model intercept, *season* is the dichotomous data of cold or warm season, χ is the fixed effect as the dichotomous data classified as "high" or "low" based on the median of the class-based annual DDD-adjusted AMU, β is its coefficient, *RF* is the farm as the random effect, and e is the binomially distributed residual term.

If the resistance rate to an antimicrobial revealed a positive and significant association with "high" class-based annual DDD-adjusted AMU of its class, a multivariable model for the associations with the AMU of other classes was also explored. The final model met the minimum Akaike's Information Criterion (AIC), and statistical significance was set at $p < 0.05$ for the remaining independent variables with positive fixed effects.

The statistical modeling and other tests mentioned above were performed using R version 4.1.0.[3] Primarily, the "glmmML" package version 1.1.3[4] was used for the generalized linear mixed model.

2.6. Ethics statement

Animal ethics approval was not required for this study as the samples consisted of piggery aerosol and were collected in the presence of the veterinarians during their routine farm visits for veterinary care and consultation.

3. Results

3.1. Description of the aerosol bacteria

In total, 915 bacterial isolates were obtained from TSA, and the genus-level description is summarized in Figure 2. The most dominant genus was *Staphylococcus* ($n = 610$, 66.7%), followed by *Aerococcus* ($n = 85$, 9.3%) and *Rothia* ($n = 50$, 5.5%). Finally, 1,113 staphylococci isolated from TSA ($n = 610$) and MSA ($n = 503$) underwent the antimicrobial susceptibility test, respectively.

The proportion of staphylococci exceeded 50% in most sampling visits. Farms A and H had over 70%. Apart from Farm E, which was visited once, no intra-farm significant seasonal difference was observed in the staphylococci proportion (Figure 3A, Chi-square test: $p > 0.05$). Sow and fattening piggeries had over 40% of staphylococci among the isolates. Farm A's sow piggery group and both piggery groups of Farm H had over 80%. Only Farm C and I had significantly

3 https://www.R-project.org/

4 https://CRAN.R-project.org/package=glmmML

higher proportions of staphylococci in the fattening piggery group than in the sow piggery group (Figure 3B, Chi-square test: $p = 0.02$ and 0.03 for Farm C and Farm I, respectively).

Among the 1,113 staphylococci isolates, the most dominant specie was *S. sciuri* (which was renamed *Mammaliicoccus sciuri* in 2020) ($n = 265$, 23.8%), and others had <10% each (Table 2). The top five species *S. sciuri*, *S. cohnii*, *S. saprophyticus*, *S. haemolyticus*, and *S. chromogenes* dominated over 40% of each farm and some over 80% (data not shown).

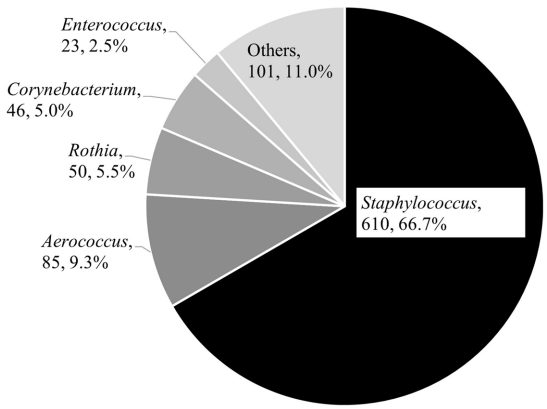


FIGURE 2
Genus description of bacterial isolates from the aerosol in ten pig farms (n = 915 obtained by trypticase soy agar).

3.2. Distribution of AMR rate

For 10 of the 11 tested antimicrobials (all the isolates were susceptible to VAN), datasets of the resistance rate of staphylococci by the seasons (Figure 4A and Supplementary file 1) and piggery groups (Figure 4B and Supplementary file 2) were obtained. Regardless of the seasons and piggery groups, resistance rates for OXA, ERY, and LCM were > 40% of the median and tended to be higher than those of other antimicrobials.

A significant seasonal difference was only identified in the resistance rate for OXA, with the median for the cold and warm seasons being 65.4 and 80.7%, respectively (Figure 4A and Supplementary file 1, $p = 0.03$ as revealed by the Mann–Whitney U test). In contrast, a significant between-piggery group difference was identified in OXA, AMP, ERY, and CHL. The resistance rates for these four antimicrobials in the fattening piggery group were significantly higher than those in the sow piggery group, with a median of 78.8 and 58.0% for OXA, 57.1 and 31.8% for AMP, 82.4 and 48.4% for ERY, and 45.5 and 21.1% for CHL for the fattening and sow piggery groups, respectively (Figure 4B and Supplementary file 2, all $p < 0.05$ as revealed by the Mann–Whitney U test).

3.3. Distribution of AMU

Figure 5 illustrates the distribution of the class-based annual DDD-adjusted AMU. The AMU varied by farm; however, no intra-class difference was identified by season (Figure 5A and

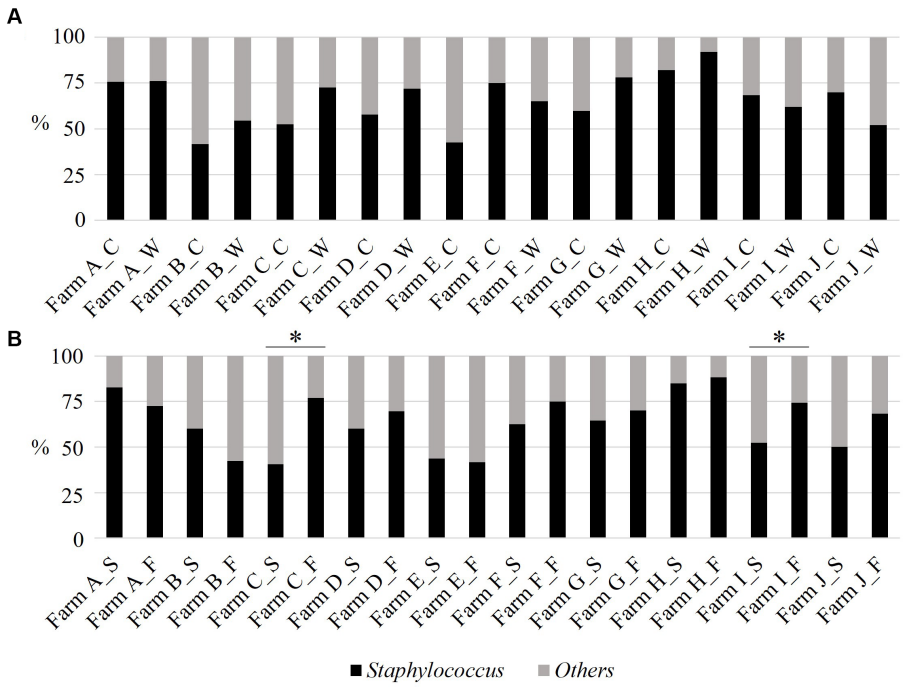


FIGURE 3
The proportion (%) of staphylococci from the aerosol in ten pig farms by sampling season **(A)** and piggery group **(B)**. The last letter of each item denotes the following: C: cold season, W: warm season, S: sow piggery group, and F: fattening piggery group. *: $p < 0.05$ as revealed by the Mann–Whitney U test.

Supplementary file 3, all $p > 0.45$ as revealed by the Mann–Whitney *U* test). In contrast, a between-piggery group difference was identified in the AMU of all the classes, except PMXs and PLMs. Therefore, the fattening piggery group had a significantly higher AMU than the sow piggery group, with zero medians for all classes, except TETs and MCLs (Figure 5B and Supplementary file 4, all $p < 0.05$ as revealed by the Mann–Whitney *U* test).

TABLE 2 Species description of staphylococci isolated from the aerosol in ten pig farms ($n = 1,113$).

Species	Isolates	%
Staphylococcus sciuri	265	23.8
S. cohnii	98	8.8
S. saprophyticus	94	8.4
S. haemolyticus	81	7.3
S. chromogenes	75	6.7
S. cohnii subsp. cohnii	59	5.3
S. aureus	52	4.7
S. simulans	45	4.0
S. epidermidis	36	3.2
S. hyicus	35	3.1
S. nepalensis	34	3.1
S. equorum	31	2.8
Other *Staphylococcus spp.*	208	18.8
Total	1,113	100.0

3.4. Association between AMU and resistance rate

Table 3 presents four final models obtained by statistical modeling from the datasets of the fattening piggery group for the association between the class-based annual DDD-adjusted AMU and resistance rate of staphylococci. Out of the 11 evaluated antimicrobials, the resistance rate for OXA, ERY, TET, and CHL was significantly associated with the AMU of the corresponding PENs, MCLs, TETs, and APCs, respectively.

Regarding OXA, the final model included only PENs, and its "high" usage was associated with a higher resistance rate for OXA [odds ratio (OR) and 95% confidence interval (CI)] = 2.36 (1.11, 5.05), $p = 0.03$). For ERY, the final model included MCLs, APCs, and LCMs. A "high" usage of these three antimicrobial classes was independently associated with a higher resistance rate for ERY

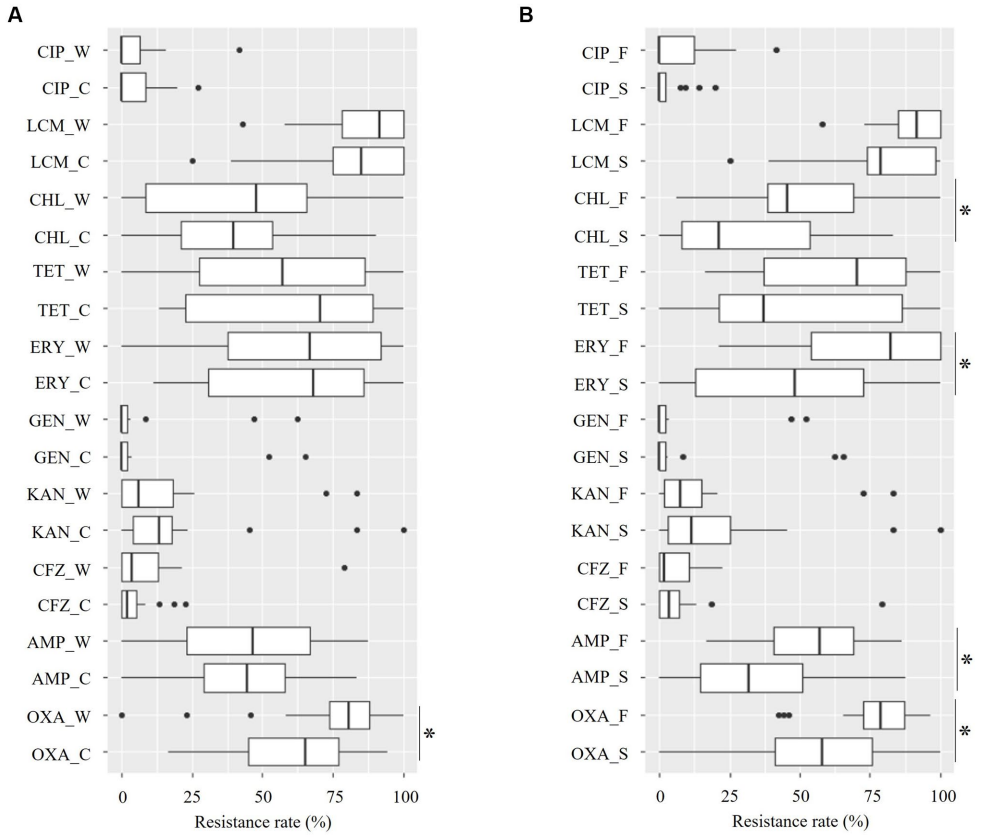


FIGURE 4
Distribution of the resistance rate for ten antimicrobials of staphylococci from the aerosol in ten pig farms by sampling season **(A)** and piggery group **(B)**. The last letter of each item denotes the following: C: cold season, W: warm season, S: sow piggery group, and F: fattening piggery group. OXA, oxacillin; AMP, ampicillin; CFZ, cefazoline; KAN, kanamycin; GEN, gentamycin; ERY, erythromycin; TET, tetracycline; CHL, chloramphenicol; LCM, lincomycin; CIP, ciprofloxacin. *: $p < 0.05$ as revealed by the Mann–Whitney *U* test.

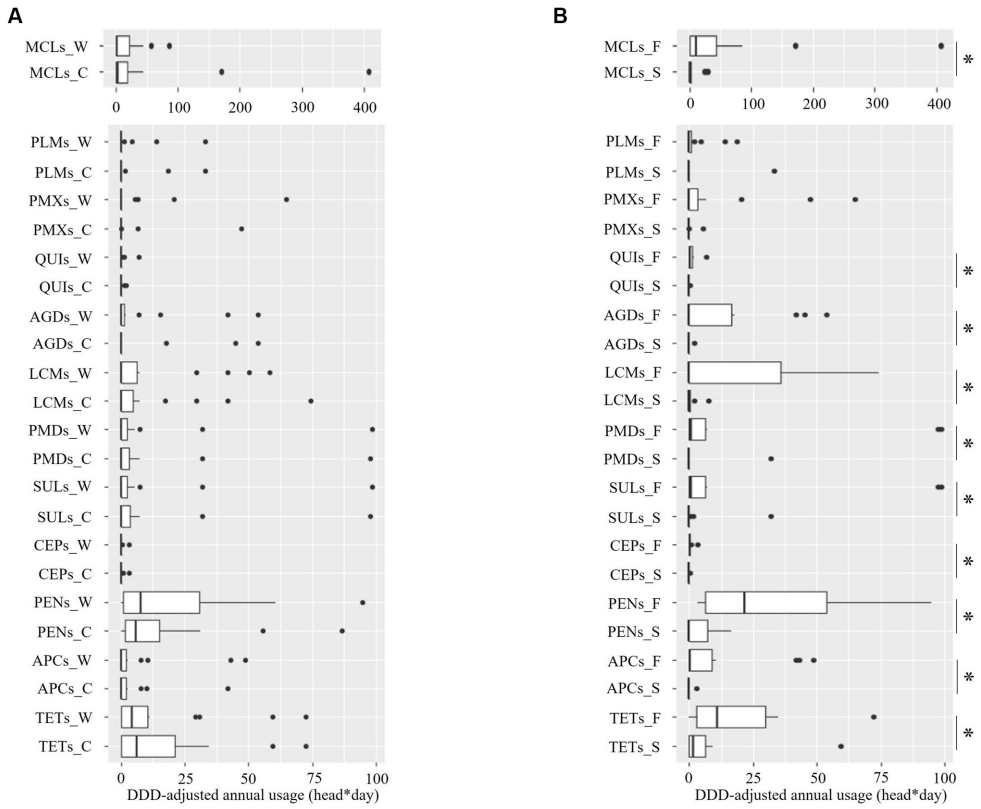


FIGURE 5
Distribution of the defined-daily-dose-adjusted annual usage of 12 antimicrobial classes in ten pig farms by sampling season **(A)** and piggery group **(B)**. The last letter of each item denotes the following: C: cold season, W: warm season, S: sow piggery group, and F: fattening piggery group. TETs, tetracyclines; APCs, amphenicols; PENs, penicillins; CEPs, cephalosporins; SULs, sulfonamides; PMDs, pyrimidines; MCLs, macrolides; LCMs, lincosamides; AGDs, aminoglycosides; QUIs, quinolones; PMXs, polymyxins; and PLMs, pleuromutilins. *: $p < 0.05$ as revealed by the Mann–Whitney *U* test.

TABLE 3 Final models of resistance rate for four antimicrobials of staphylococci from the aerosol in fattening piggeries of ten Japanese pig farms in association with the annual antimicrobial class-based usage.

Model outcome	Significant antimicrobial class	Usage level	Coefficient (SE)	Odds ratio (95% CI)	*p*
Resistance rate of oxacillin	Penicillins	Low	Reference		
		High	0.86 (0.39)	2.36 (1.11, 5.05)	0.03
Resistance rate of erythromycin	Macrolides	Low	Reference		
		High	1.93 (0.51)	6.89 (2.53, 18.73)	0.0001
	Amphenicols	Low	Reference		
		High	1.57 (0.72)	4.81 (1.17, 19.69)	0.03
	Lincosamides	Low	Reference		
		High	3.04 (0.90)	20.91 (3.60, 121.51)	0.001
Resistance rate of tetracycline	Tetracyclines	Low	Reference		
		High	0.91 (0.45)	2.48 (1.03, 5.99)	0.04
Resistance rate of chloramphenicol	Amphenicols	Low	Reference		
		High	1.17 (0.53)	3.22 (1.14, 9.12)	0.03
	Tetracyclines	Low	Reference		
		High	1.22 (0.34)	3.89 (1.73, 6.62)	0.0004

A generalized linear mixed model was used, in which season was forced into each model, and the farm was incorporated as the random effect.
SE, standard error; CI, confidence interval.

(OR (95% CI) = 6.89 (2.53, 18.73), $p = 0.0001$ for MCLs, OR (95% CI) = 4.81 (1.17, 19.69), $p = 0.03$ for APCs, and OR (95% CI) = 20.91 (3.60, 121.51), $p = 0.001$ for LCMs, respectively). For TET, the final model included only TETs, and its "high" usage was associated with a higher resistance rate for TET (OR (95% CI) = 2.48 (1.03, 5.99), $p = 0.04$). In addition, for CHL, the final model included APCs and TETs. A "high" usage of these antimicrobial classes was independently associated with a higher resistance rate for CHL (OR (95% CI) = 3.22 (1.14, 9.12), $p = 0.03$ for APCs, and (OR (95% CI) = 3.39 (1.73, 6.62), $p = 0.0004$ for TETs, respectively). No significant interaction terms were identified in all the final models.

Conversely, analyses of the sow piggery group did not reveal any significantly positive association between the AMU and resistance rate. However, the resistance rates for KAN and TET had a marginally positive association with "high" AGDs and TETs use, respectively ($p = 0.09$ and 0.11, respectively, data not shown).

4. Discussion

Previous studies on aerosol bacteria in piggeries have been limited thus far (21); therefore, White et al. (22) evaluated piggery staphylococci for their viability, capturability, inflammogenicity, and biofilm-forming capacity. Eisenlöffel et al. (23) and Tenzin et al. (24) revealed the impact of dust filtration and decontamination. These studies are relevant; however, once countermeasures are in operation, it is better to understand the extent of bacterial distribution and AMR status in these years to strengthen the rationale of the activities. However, few studies have targeted staphylococci AMR with quantitative AMU in Japan. Therefore, this study evaluated the bacterial profile of aerosol in Japanese piggeries, AMR characteristics, and the association between farm-level AMU and AMR, especially for staphylococci.

The aerobic culture using TSA revealed that most isolates were gram-positive bacteria (Figure 2), including the hazardous genus for animal and public health. The most dominant genus was *Staphylococcus*. In this study, staphylococci exceeded 40% and did not differ by sampling season and piggery group in each farm, with a few exceptions (Figure 3). Seasonal differences in sand dust in the general environment influence the bacterial community during aerosol pollution (25); nonetheless, the bacterial distribution stability observed in this study might be due to the relatively steady and closed state in the piggery based on the firm on-farm management system. These results imply the importance of staphylococci among aerosol bacteria and necessitate the maintenance or improvement of on-farm biosecurity levels, especially ventilation and humidity control in piggeries, to prevent clinical diseases in pigs. Further, workers need the shower-in and-out operation and change to washed and clean clothes and disinfected boots before they start their daily tasks. These procedures would promote animal and occupational health.

Among these staphylococci, the most dominant specie *S. sciuri* is a principally animal-associated bacterial species on the skin and mucosal surfaces of various pets and farm and wild animals. However, its clinical relevance in humans is increasing (26), and this bacterium is ubiquitous in human wound infection (27, 28). *S. hyicus* and *S. aureus* are occasionally involved in pig infections (29). Moreover, *S. hyicus* commonly occurs in the nares and on the hairy cutaneous areas of pigs; therefore, it sporadically induces exudative epidermitis in 5–60 d-old pigs along with other staphylococci, such as *S. chromogenes* and *S. aureus* (30). Livestock-associated methicillin-resistant *S. aureus* is more recognized as a public health concern, mainly associated with pigs. In Japan, its presence has been investigated using nasal swabs from slaughtered pigs (31). Given the present situation, there have been few evaluations on the environmental risks of each specie isolated from piggery aerosols. Therefore, a detailed species-based investigation is highly needed under the rational sampling frame in the future.

AMR was revealed for 11 antimicrobials. A high resistance rate of staphylococci was observed for OXA, ERY, and LCM (Figure 3). The influence of season on the resistance rate was not identified in all antimicrobials, except OXA (Figure 4A). The class-based annual DDD-adjusted AMU did not exhibit seasonal differences (Figure 5A). In contrast, the resistance rate in the fattening piggery group was significantly higher than that in the sow piggery group for OXA and AMP of PENs, ERY of MCLs, and CHL of APCs (Figure 4B) as the AMU of the 10 classes, including PENs, MCLs, and APCs, was also higher in the fattening piggery group (Figure 5B). These results indicated that the AMU of the corresponding class might influence some antimicrobials' resistance compared with environmental conditions. Generally, bacterial survival relies on various factors, such as bacterial species and their burden (32, 33) and environmental conditions, including the type of surface materials, ambient temperature, UV radiation extent, and water and nutrient availability (34, 35). These factors may affect AMR regardless of the bacterial isolates.

From the statistical modeling of the fattening piggery group, the resistance rate for four antimicrobials, including OXA, ERY, TET, and CHL, was positively associated with the AMU of the corresponding class (Table 3). This implies that the resistance rate for these antimicrobials might be decreased by reducing the use of the corresponding antimicrobials.

Moreover, the modeling identified an association between the resistance rate for ERY and the AMU of APCs and LCMs, in addition to MCLs. A similar result was obtained in the association between the resistance rate to CHL and the AMU of TETs, in addition to APCs. The mechanism of these phenomena is unclear; however, Makita et al. (36) suggested that these issues were due to the natural, cross- or co-selection based on analyses of individual pig-originated *Escherichia coli* isolates and qualitative AMU. Further evaluation is strongly needed to validate our study.

In contrast, no significant association between the resistance rate and AMU in the dataset of the sow piggery group was identified. The possible reasons could be the relatively lower AMU in this group, which might be insufficient to establish antimicrobial selection. Moreover, considering that the isolates were from the aerosol, they may include both environmental and pig-origin bacteria. Therefore, the AMR in this group was probably influenced by other factors along with the AMU. However, the resistance rate to KAN and TET displayed a marginally positive association with AGDs and TETs. Among these, TETs with relatively high AMUs in the sow piggery group could be the reason.

Some limitations should be considered in interpreting this study's results. First, as mentioned above, the AMR of aerosol-origin bacteria is influenced by both the AMU and other factors. Therefore, evaluating

the pig-origin (including healthy and diseased ones) staphylococci will help better understand the piggery's AMR risk. Second, this study's statistical modeling was performed using aggregated data on the resistance rate and AMU, which could have an ecological fallacy (37). However, antimicrobials are administered on a herd basis in the general pig industry; hence, this is the best way to assess the on-farm situation quantitatively. Based on these results, it is essential to further evaluate the effect of the countermeasures aimed at decreasing the resistance rate for single antimicrobials at the farm level and clarifying multidrug resistance. Lastly, all the evaluations on the association between the resistance rate and AMU were performed on a genus basis to provide an overview of staphylococci. Therefore, detailed investigations focusing on each species will be more useful for the species-level measures.

In conclusion, the aerosol bacteria in Japanese pig farms included those that could threaten public and animal health, mostly staphylococci. Staphylococci resistance to some antimicrobials was associated with using the corresponding antimicrobial class, implying that reducing such antimicrobials would decrease resistance. These results should help establish countermeasures for the AMR of aerosol bacteria in pig farms.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Author contributions

KK conceptualized the study. KK and SK designed and performed the on-farm investigation, sample collection, and laboratory experiments. IY, YT-A, and SK contributed to the data management of antimicrobial usage. SK analyzed the data and drafted the manuscript in consultation with KK, YT-A, IY, and MK. All authors contributed to the article and approved the submitted version.

Funding

This study was conducted under the research project on "Regulatory Research Projects for Food Safety, Animal Health and Plant Protection (grant JPJ008617.17935699/22682153)" funded by the Ministry of Agriculture, Forestry and Fisheries of Japan.

Acknowledgments

The authors are grateful for the on-farm support by Katsumasa Kure, Hiromichi Ishikawa, and Mitsuo Kagawa from The Japanese Association of Swine Veterinarians as well as for the laboratory support by Yoshie Watanabe.

Conflict of interest

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.

Publisher's note

All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations, or those of the publisher, the editors and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.

Supplementary material

The Supplementary material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fvets.2023.1127819/full#supplementary-material

References

1. Tang Q, Huang K, Liu J, Jin X, Li C. Distribution characteristics of bioaerosols inside pig houses and the respiratory tract of pigs. *Ecotoxicol Environ Saf.* (2021) 212:112006. doi: 10.1016/j.ecoenv.2021.112006

2. Gou H, Lu J, Li S, Tong Y, Xie C, Zheng X. Assessment of microbial communities in PM1 and PM10 of Urumqi during winter. *Environ Pollut*. (2016) 214:202–10. doi: 10.1016/j.envpol.2016.03.073

3. Wang Y, Li W, Gao W, Liu Z, Tian S, Shen R, et al. Trends in particulate matter and its chemical compositions in China from 2013–2017. *Sci China Earth Sci.* (2019) 62:1857–71. doi: 10.1007/s11430-018-9373-1

4. Zucker BA, Trojan S, Müller W. Airborne gram-negative bacterial flora in animal houses. *J Vet Med B Infect Dis Vet Public Health*. (2000) 47:37–46. doi: 10.1046/j.1439-0450.2000.00308.x

5. White JK, Nielsen JL, Madsen AM. Microbial species and biodiversity in settling dust within and between pig farms. *Environ Res*. (2019) 171:558–67. doi: 10.1016/j.envres.2019.01.008

6. Radon K, Danuser B, Iversen M, Jörres R, Monso E, Opravil U, et al. Respiratory symptoms in European animal farmers. *Eur Respir J*. (2001) 17:747–54. doi: 10.1183/09031936.01.17407470

7. Vogelzang PF, van der Gulden JW, Preller L, Heederik D, Tielen MJ, van Schayck CP. Respiratory morbidity in relationship to farm characteristics in swine confinement work: possible preventive measures. *Am J Ind Med*. (1996) 30:212–8. doi: 10.1002/(SICI)1097-0274(199608)30:2<212::AID-AJIM13>3.0.CO;2-#

8. World Health Organization (2019). Ten threats to global health in 2019. Available at: https://www.who.int/news-room/spotlight/ten-threats-to-global-health-in-2019 (Accessed November 29, 2022).

9. World Health Organization (2020). 10 global health issues to track in 2021. Available at: https://www.who.int/news-room/spotlight/10-global-health-issues-to-track-in-2021 (Accessed November 29, 2022).

10. Murray CJ, Ikuta KS, Sharara F, Swetschinski L, Robles Aguilar G, Gray A, et al. Global burden of bacterial antimicrobial resistance in 2019: a systematic analysis. *Lancet*. (2022) 399:629–55. doi: 10.1016/S0140-6736(21)02724-0

11. World Health Organization (2015). Global action plan on antimicrobial resistance. Available at: https://www.who.int/publications/i/item/9789241509763 (Accessed November 29, 2022).

12. Ministry of Health, Labour and Welfare (2021). Nippon AMR one health report (NAOR) 2020. Available at: https://www.mhlw.go.jp/stf/seisakunitsuite/bunya/0000120172.html (Accessed November 29, 2022).

13. Létourneau V, Nehmé B, Mériaux A, Massé D, Cormier Y, Duchaine C. Human pathogens and tetracycline-resistant bacteria in bioaerosols of swine confinement buildings and in nasal flora of hog producers. *Int J Hyg Environ Health*. (2010) 213:444–9. doi: 10.1016/j.ijheh.2010.09.008

14. Shome BR, Das Mitra S, Bhuvana M, Krithiga N, Velu D, Shome R, et al. Multiplex PCR assay for species identification of bovine mastitis pathogens. *J Appl Microbiol*. (2011) 111:1349–56. doi: 10.1111/j.1365-2672.2011.05169.x

15. Campos-Peña E, Martín-Nuñez E, Pulido-Reyes G, Martín-Padrón J, Caro-Carrillo E, Donate-Correa J, et al. Multiplex PCR assay for identification of six different Staphylococcus spp. and simultaneous detection of methicillin and mupirocin resistance. *J Clin Microbiol*. (2014) 52:2698–701. doi: 10.1128/JCM.00918-14

16. Ghebremedhin B, Layer F, König W, König B. Genetic classification and distinguishing of Staphylococcus species based on different partial gap, 16S rRNA, hsp60, rpoB, sodA, and tuf gene sequences. *J Clin Microbiol*. (2008) 46:1019–25. doi: 10.1128/JCM.02058-07

17. Clinical Laboratory Standards Institute. *VET01S, performance standards for antimicrobial disk and dilution susceptibility tests for Bacteria isolated from animals. 3rd* ed. PA, United States: Wayne (2015).

18. Takagi H, Lei Z, Yamane I, Yamazaki H, Kure K, Sugiura K. Establishing DDD values for veterinary antimicrobial products in Japan for measuring antimicrobial use on pig farms. *J Jpn Vet Med Assoc*. (2020) 73:352–5.

19. Fujimoto K, Kawasaki M, Abe R, Yokoyama T, Haga T, Sugiura K. Establishing defined daily doses (DDDs) for antimicrobial agents used in pigs, cattle and poultry in Japan and comparing them with European DDD values. *PLoS One*. (2021) 16:e0245105. doi: 10.1371/journal.pone.0245105

20. WHO Collaborating Centre for Drug Statistics and Methodology (2020). ATCvet Index 2020. Available at: http://www.whocc.no/atcvet/atcvet/ (Accessed November 29, 2022).

21. Cui H, Zhang C, Liu J, Dong S, Zhao K, Chen L, et al. The distribution characteristics of aerosol bacteria in different types of pig houses. *Animals (Basel)*. (2022) 12:1540. doi: 10.3390/ani12121540

22. White JK, Nielsen JL, Larsen CM, Madsen AM. Impact of dust on airborne *Staphylococcus aureus*' viability, culturability, inflammogenicity, and biofilm forming capacity. *Int J Hyg Environ Health*. (2020) 230:113608. doi: 10.1016/j.ijheh.2020.113608

23. Eisenlöffel L, Reutter T, Horn M, Schlegel S, Truyen U, Speck S. Impact of UVC-sustained recirculating air filtration on airborne bacteria and dust in a pig facility. *PLoS One*. (2019) 14:e0225047. doi: 10.1371/journal.pone.0225047

24. Tenzin S, Ogunniyi AD, Khazandi M, Ferro S, Bartsch J, Crabb S, et al. Decontamination of aerosolised bacteria from a pig farm environment using a pH neutral electrochemically activated solution (Ecas4 anolyte). *PLoS One*. (2019) 14:e0222765. doi: 10.1371/journal.pone.0222765

25. Wei M, Liu H, Chen J, Xu C, Li J, Xu P, et al. Effects of aerosol pollution on PM2.5-associated bacteria in typical inland and coastal cities of northern China during the winter heating season. *Environ Pollut*. (2020) 262:114188. doi: 10.1016/j.envpol.2020.114188

26. Dakić I, Morrison D, Vuković D, Savić B, Shittu A, Jezek P, et al. Isolation and molecular characterization of *Staphylococcus sciuri* in the hospital environment. *J Clin Microbiol*. (2005) 43:2782–5. doi: 10.1128/JCM.43.6.2782-2785.2005

27. Shittu A, Lin J, Morrison D, Kolawole D. Isolation and molecular characterization of multiresistant Staphylococcus sciuri and *Staphylococcus haemolyticus* associated with skin and soft-tissue infections. *J Med Microbiol*. (2004) 53:51–5. doi: 10.1099/jmm.0.05294-0

28. Kolawole DO, Shittu AO. Unusual recovery of animal staphylococci from septic wounds of hospital patients in Ile-Ife, Nigeria. *Lett Appl Microbiol*. (1997) 24:87–90. doi: 10.1046/j.1472-765x.1997.00337.x

29. Kloos WE. Natural populations of the genus Staphylococcus. *Annu Rev Microbiol*. (1980) 34:559–92. doi: 10.1146/annurev.mi.34.100180.003015

30. Frana TS. Staphylococcosis In: JJ Zimmerman, LA Karriker, A Ramirez, KJ Schwartz and GW Stevenson, editors. *Diseases of swine*. Oxford: Wiley-Blackwell (2012). 834–40.

31. Sasaki Y, Yamanaka M, Nara K, Tanaka S, Uema M, Asai T, et al. Isolation of ST398 methicillin-resistant *Staphylococcus aureus* from pigs at abattoirs in Tohoku region, Japan. *J Vet Med Sci*. (2020) 82:1400–3. doi: 10.1292/jvms.20-0184

32. Wendt C, Dietze B, Dietz E, Rüden H. Survival of *Acinetobacter baumannii* on dry surfaces. *J Clin Microbiol*. (1997) 35:1394–7. doi: 10.1128/jcm.35.6.1394-1397.1997

33. Neely AN, Maley MP. Survival of enterococci and staphylococci on hospital fabrics and plastic. *J Clin Microbiol*. (2000) 38:724–6. doi: 10.1128/JCM.38.2.724-726.2000

34. Hanczvikkel A, Tóth Á. Quantitative study about the role of environmental conditions in the survival capability of multidrug-resistant bacteria. *J Infect Public Health*. (2018) 11:801–6. doi: 10.1016/j.jiph.2018.05.001

35. Katzenberger RH, Rösel A, Vonberg RP. Bacterial survival on inanimate surfaces: a field study. *BMC Res Notes*. (2021) 14:97. doi: 10.1186/s13104-021-05492-0

36. Makita K, Goto M, Ozawa M, Kawanishi M, Koike R, Asai T, et al. Multivariable analysis of the association between antimicrobial use and antimicrobial resistance in *Escherichia coli* isolated from apparently healthy pigs in Japan. *Microb Drug Resist*. (2016) 22:28–39. doi: 10.1089/mdr.2014.0311

37. Dohoo I, Martin W, Stryhn H. Ecological and group-level studies In: I Dohoo, W Martin, H Stryhn and SM McPike, editors. *Veterinary epidemiologic research*. 2nd ed. Prince Edward Island, Canada: VER Inc (2014). 773–88.

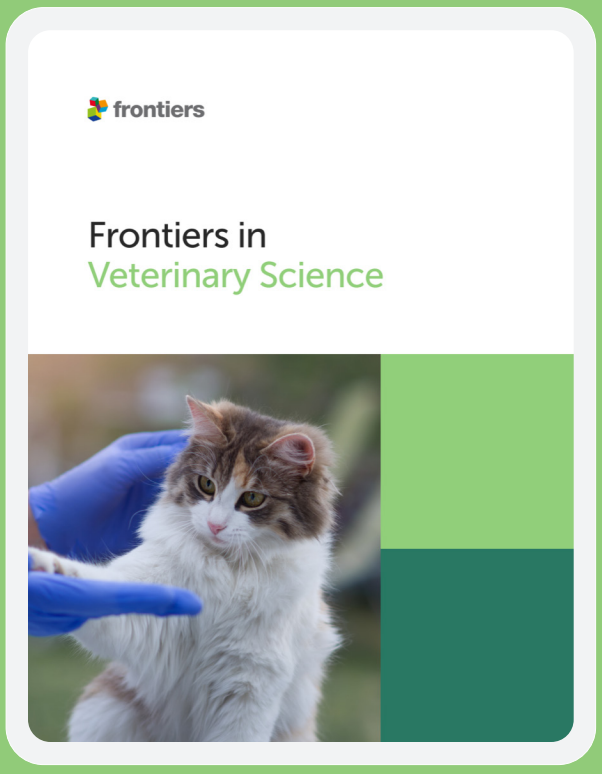
Frontiers in Veterinary Science

Transforms how we investigate and improve animal health

The third most-cited veterinary science journal, bridging animal and human health with a comparative approach to medical challenges. It explores innovative biotechnology and therapy for improved health outcomes.

Discover the latest Research Topics

See more →



Frontiers

Avenue du Tribunal-Fédéral 34
1005 Lausanne, Switzerland
frontiersin.org

Contact us

+41 (0)21 510 17 00
frontiersin.org/about/contact