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Identification and antimicrobial resistance of *Campylobacter* species isolated from animal sources

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Background: *Campylobacter* spp. are together with *Salmonella* spp. the leading causes of human bacterial gastroenteritis worldwide. The most commonly isolated species in humans are *Campylobacter jejuni* and *C. coli*. The isolation, identification, and antimicrobial resistance of *Campylobacter* spp. from poultry and raw meat from slaughterhouses, has been investigated for the first time in Greece. During the period from August 2005 to November 2008 a total of 1080 samples were collected: (a) 830 fecal samples from five poultry farms, (b) 150 cecal samples from chicken carcasses in a slaughterhouse, and (c) 100 fecal samples from one pig farm near the region of Attica. The identification of the isolates was performed with conventional (sodium hippurate hydrolysis and commercial identification system (Api CAMPY system, bioMerieux, France), as well as with and molecular methods based on 16S rRNA species specific gene amplification by PCR and subsequent sequence analysis of the PCR products. **Results:** Sixteen *Campylobacter* strains were isolated, all collected from the poultry farms. None of the strains was identified as *C. jejuni*. Antimicrobial susceptibility to six antimicrobials was performed and all the strains were susceptible to ciprofloxacin, amoxicillin–clavulanic acid, and gentamicin. Thirteen out of 14 *C. coli* were resistant to erythromycin and all *C. coli* strains were resistant to ampicillin. **Conclusion:** Our results emphasize the need for a surveillance and monitoring system with respect to the prevalence and antimicrobial resistance of *Campylobacter* in poultry, as well as for the use of antimicrobials in veterinary medicine in Greece.

Keywords: *Campylobacter*, animals, sequencing identification, antimicrobial resistance

INTRODUCTION

Campylobacter spp. are together with *Salmonella* spp. the leading causes of human bacterial gastroenteritis worldwide. The most commonly isolated species in humans are *Campylobacter jejuni*, *C. coli*, and *C. lari*, but other species like *C. fetus*, seem to be also involved in human disease (Lastovica and Skirrow, 2000). Sporadic human cases have been associated with consumption of undercooked poultry meat, while larger outbreaks are associated with raw milk (Karagiannis et al., 2010; Denis et al., 2011). Contaminated drinking water has been the cause of sporadic cases, as well as of larger outbreaks (Peterson, 2003). Antimicrobial resistance of *Campylobacter* spp. to fluoroquinolones, which are generally used for the empiric treatment of bacterial gastroenteritis, has increased during the past two decades, mainly as a result of the approval of this group of antimicrobials for the use in food producing animals (Nelson and Harris, 2006; Han et al., 2009). Macrolide-resistant *Campylobacter* spp. isolated from food, animals, and humans have also been reported (Kang et al., 2000; Belanger and Shryock, 2007; Gallay et al., 2007). In Greece epidemiological data about the prevalence and antimicrobial susceptibility of *Campylobacter* spp. are restricted to strains from clinical samples belonging mainly to *C. jejuni*, isolated from children with gastroenteritis (Kafetzis et al., 2001; Chatzipanagiotou et al., 2002, 2003a,b; Maraki et al., 2003; Ioannidis et al., 2006; Papavasileiou et al., 2007).

There is, neither an official surveillance and monitoring system for the presence of *Campylobacter* in animals, nor for the use of antimicrobials in veterinary medicine. The aim of this study was (1) to evaluate the prevalence of *Campylobacter* spp. in positive samples in poultry meat, farms, and slaughterhouses; (2) to report for the first time the isolation, identification, and antimicrobial resistance of *Campylobacter* spp. from poultry in Greece; and (3) to investigate any possible epidemiological association with previous reports referring to isolates from clinical cases.

MATERIALS AND METHODS

SAMPLE COLLECTION

In the period from August 2005 to November 2008 a total of 1080 samples were collected from poultry farms, pig farms, and slaughterhouses as follows: (a) 830 fecal samples from five poultry farms near the region of Attica, (b) 150 cecal samples from chicken carcasses in a slaughterhouse, and (c) 100 fecal samples from one pig farm near the region of Attica.

Table 1 | Prevalence rates of *Campylobacter* spp. isolated from poultry and other animal sources.

Clusters	Total clusters' units	Number of units sampled	*Campylobacter* spp. positive strains found	Cluster's percentage rate	Cluster's estimated number of *Campylobacter* spp. positive strains
1 (poultry farm)	2000	85	0	0.00	0
2 (poultry farm)	5000	80	0	0.00	0
3 (poultry farm)	2000	80	4	5.00	100
4 (poultry farm)	2000	270	8	2.96	59
5 (poultry farm)	10000	315	4	1.27	126
6 (slaughterhouse)	15000	150	0	0.00	0
7 (pig farm)	10000	100	0	0.00	0
Total	46000	1080	16		285

All samples were collected with a sterile swab from fresh feces immediately dropped to the ground. The sampling places covered geographically the location of Sterea Ellada, which is situated in Central Greece and includes the Department of Attica, the most populous Department in Greece, and also the Departments of Viotia, Evia, Evritania, Fthiotida, and Fokida. Thirteen percent of the total Greek meat production is concentrated in this location and five per cent in Attica.

At these locations, 61 poultry farms, 52 pig farms, and approximately 9 slaughterhouses are situated, most of them located in the Department of Attica and Viotia (40 poultry farms, 29 pig farms, and 4 slaughterhouses).

In this study, the method of two-stage cluster sampling was used. Under the well known cluster sampling scheme the population is divided in representative clusters, then a sample of these clusters is randomly chosen and all the units contained in the chosen clusters are deemed as the sample. The units within a cluster should ideally be as heterogeneous as possible, but there should be homogeneity between cluster means. Here we assume that there is homogeneity between all the farms as well as all the slaughterhouses since there are EU laws that impose a minimum level of neatness.

Nevertheless, in our case the cluster sampling is not adequate, since the units that are contained in each cluster are thousands, thus we choose the method of two-stage cluster sampling. There are many ways to modify cluster sampling for more complex sampling situations. However, the most common modification is to take a sample of secondary units from within sampled primary units (clusters) instead of inspecting every secondary unit within each primary unit (cluster).

Specifically, let us suppose that a simple random sample of n primary units is selected from a population of N primary units. Then a simple random sample of secondary units of sizes m_1, m_2, . . ., m_n are selected from within these n primary units. The total sample of $m = \sum_{i=1}^{n} m_i$ secondary units is called a two-stage cluster sample. In two-stage cluster sampling, we select m_i units from the M_i secondary units ($m_i \leq M_i$) in primary unit i.

Specifically in our case, at the first stage, we selected seven clusters (each farm and slaughterhouse is deemed as a cluster), which represent about the 5.75% of the total number of the farms and slaughterhouses in the area under study (122 units in total population). The seven clusters (five poultry farms, one pig farm, and chicken carcasses) have a total animal population equal to 46000 animals (**Table 1**). At the second stage, from each one of the seven clusters, random samples were selected. The distribution of the samples taken from each cluster as well as the each cluster's estimated number of *Campylobacter* spp. positive strains are given in **Table 1** (an unbiased estimator of the unit total is $\hat{y}_i = M_i \cdot \bar{y}_i$, where $\bar{y}_i$ is cluster's percentage rate).

CULTURE, ISOLATION, AND IDENTIFICATION OF BACTERIA

Campylobacter isolation and bacteriological identification

The swabs were immediately inoculated into 10 mL transport *Campylobacter* selective enrichment broth (Preston broth, Oxoid, CM0067 + SR0084 + SR117E, Basingstoke, UK) and refrigerated at 5˚C for a maximum of 2 h after sampling and before dispatch to the laboratory.

The samples in Preston broth were incubated for 48 h at 42˚C and were then subcultured onto *Campylobacter* selective Skirrow agar (manufacturer Oxoid, CM169 + SR0069, Basingstoke UK). The agar plates were further incubated at 42˚C under microaerophilic conditions (GENbox microaer, bioMerieux, France).

All isolates were stored in brain heart infusion broth with 50% glycerol at −80˚C for any further investigation.

The suspected colonies, showing typical morphology and positive oxidase and catalase reaction were microscopically examined after Gram-stain and typical isolates were primarily identified through sodium hippurate hydrolysis as well as by means of a commercial identification system (Api CAMPY system, bioMerieux, France).

Campylobacter sequence identification

All isolates were further identified by a molecular method based on 16S rRNA species specific gene amplification by PCR and subsequent sequence analysis of the PCR products.

DNA extraction and PCR. Bacterial DNA was extracted using the commercially available InstaGene matrix reagent (6% Polystyrene divinylbenzene iminodiacetate in Aqua bides; BioRad Laboratories, CA, USA). For the PCR reaction JumpStart REDTaq ReadyMix solution (Sigma Laboratories, St. Louis, USA) was used. The following oligonucleotide primer pairs were applied corresponding to three 16S

rRNA gene fragments, run in separate reactions (Gorkiewicz et al., 2003): (1) Ps5/1 (5′-TATGGAGAGTTTGATCCTGG-3′) and Ps3/1 (5′ GTTAAGCTGTTAGATTTCAC-3′), (2) Ps5/2 (5′-AGCGTTACTCGGAATCACTG-3′) and Ps3/2 (5′-ACAGCCGTGCAGCACCTGTC-3′), (3) Ps5/3 (5′-AACCTTACC TGGGCTTGATA-3′) and Ps3/3 (5′-AAGGAGGTGATCC AGCCGCA-3′).

Additional oligonucleotide primers were applied to amplify the variable 16S rRNA regions (Vc regions) in order to facilitate detection of sequence variation: The primer pair Vc5/6-F (5′-AAAGCGTGGGGAGCAAACAG-3′) and Vc5/6-R (5′-ACTTAACCCAACATCTCACG-3′) was used for a 334-bp DNA fragment and the primer pair Vc1/2-F (5′-AGAGTTTGATCCTGGCTCAG-3′) andVc1/2R (5′-TGATCATCC TCTCAGACCAG-3′) was used to amplify a 330-bp DNA fragment (Gorkiewicz et al., 2003).

Campylobacter. *16S rRNA sequencing identification* The 16S rRNA sequencing of the PCR products was performed by Macrogen Incorporation (908 World Meridian Center #60-24 Gasan-dong, Geumcheon-Gu Seoul, Republic of Korea) and the analyzed 16S rRNA gene fragments were submitted to the GenBank (using nucleotide BLAST) and compared with sequences already accessed from other studies.

For the quality assessment of the method the following reference strains were used: *C. jejuni* 0:37 NCO 12539, *C. jejuni* 0:38 NCO 12540, *C. jejuni* 0:41 NCO 12542, *C. coli* NCO 11366, *C. lari* NCO 11352.

CAMPYLOBACTER ANTIMICROBIAL SUSCEPTIBILITY TESTING

Antimicrobial susceptibility testing was performed by using the standard E-test method (AB Biodisk, Sweden). The antibiotics included were nalidixic acid, ciprofloxacin, ampicillin, amoxicillin–clavulanic acid, gentamicin, and erythromycin. The bacterial inoculum was adjusted to 0.5 McFarland standard turbidity and Mueller–Hinton agar supplemented with 5% lysed sheep blood were used. Plates were incubated at 37°C for 48 h under microaerophilic conditions. Results were interpreted according to the Clinical Laboratory Standards Institute (CLSI) guidelines (CLSI document M45-A and M100-S17; Clinical and Laboratory Standards Institute, 2006, 2007) using the following resistance breakpoints of *C. jejuni*/*coli*: ampicillin >16 mg/L, ciprofloxacin >4 mg/L, gentamicin >16 mg/L, erythromycin >32 mg/L, nalidixic acid >32 mg/L, and amoxicillin–clavulanic acid >16 mg/L).

The following reference strains were used as controls: *C. jejuni* 0:37 NCO 12539, *C. jejuni* 0:38 NCO 12540, *C. jejuni* 0:41 NCO 12542, *C. coli* NCO 11366, *C. lari* NCO 11352.

STATISTICAL ANALYSIS

For the analysis the statistical package SPSS (version 18) was used. The chi-square test was the statistical method used (as a distribution goodness of fit test).

The chi-square test is used when we are interested as to how well a model actually reflects empirical data. Specifically, when we are interested in how "close" the observed values are to those which would be expected under a theoretical model. In our case, we used it in order to analyze multi-category percentages (e.g., test the null hypothesis that the percentage of *Campylobacte*r strains isolated in poultry farms was equal to the percentages of *Campylobacter* strains isolated in farm pigs and chicken carcasses).

Details about the chi-square test (as a distribution goodness of fit test) are given in Armitage et al. (2002). Finally, the significance of the test was set equal to 0.05.

Table 2 | 16S rRNA gene sequencing identification of *Campylobacter* spp. isolated from poultry and other animal sources.

Sample origin	Number of samples	*C. coli*	*C. jejuni* subsp. *doylei*	Total
Poultry farms	830	14 (1.68%)	2 (0.24%)	16 (1.93%)
Farm pigs	100	–	–	–
Chicken carcasses	150	–	–	–
Total	1080	14 (1.3%)	2 (0.18%)	16 (1.48%)

RESULTS

CAMPYLOBACTER ISOLATION

From the total of 1080 collected samples 16 *Campylobacter* strains were isolated: (a) 16 out of 830 fecal samples (1.93%) from the five poultry farms, (b) none out of the 150 cecal samples from chicken carcasses, and (c) none out of the 100 fecal samples from the pig farm (**Table 2**). According to the statistical analysis the percentage of *Campylobacter* strains isolated in poultry farms was significantly different (p-value <0.05%) from the percentage of *Campylobacter* strains isolated from the farm pigs and chicken carcasses.

CAMPYLOBACTER BACTERIOLOGICAL IDENTIFICATION

All isolated strains were negative for sodium hippurate hydrolysis. Through the commercial identification system (Api CAMPY) only 7 out of the 16 isolated strains were identified: four as *C. coli*, two as *C. lari*, and one as *Arcobacter cryaerophilus*.

CAMPYLOBACTER SEQUENCING IDENTIFICATION

The 16S rRNA sequencing analysis identified all the strains as follows: 14 *C. coli* and 2 *C. jejuni* subsp. *doylei* (**Table 2**). The accession numbers of the nucleotide sequences admitted to GenBank are shown in **Table 3**. In addition, the statistical analysis revealed that the percentage of *C. coli* strains isolated in total was significantly different (p-value <0.05%) from the percentage of the isolated *C. jejuni* subsp. *doylei*.

CAMPYLOBACTER ANTIMICROBIAL SUSCEPTIBILITY TESTING

The results for antimicrobial susceptibility in relation to species and origin are shown in **Table 4**. All the strains were susceptible to ciprofloxacin and amoxicillin–clavulanic acid, while 13 were resistant to erythromycin, 14 to ampicillin, 2 to nalidixic acid, and 2 to gentamicin.

DISCUSSION

Our study is the first report ever published from Greece, referring to the isolation, identification, and antimicrobial resistance of

Campylobacter spp. from poultry farms. To our knowledge there are only a few published data from Greece upon the infection rate of *Campylobacter* spp. in human sporadic cases. Kafetzis et al. (2001) isolated *Campylobacter* spp. in 9% of 294 stool samples of hospitalized children, Maraki et al. (2003) isolated *Campylobacter* spp. in 4.2% from 7090 human stool samples, and Gousia et al. (2011) reported the prevalence of *Campylobacter* spp. of raw and processed meat products from retail shops.

In the present study, the isolation rate of positive samples of *Campylobacter* spp. in the examined poultry farms and in the total of samples was 1.93 and 1.48% respectively, which is low compared to those reported from other countries. In 2009, a total of 24 countries reported data about the presence of *Campylobacter* in broiler flocks (Scientific Report of European Food Safety Authority, 2011) and the recorded rates of positive samples were variable, ranging from 0 to 100.0%. Low and moderate levels were only observed in Estonia, Finland, Sweden, and Norway and high levels (>50%) were observed in Austria, France, Romania, and Spain. In the present study, we did not isolate any strain of *Campylobacter* spp. from chicken carcasses and farm pigs and this might be due to good hygiene practices in the slaughterhouse we visited, including daily cleaning and disinfection of the equipment used, existence of sufficient air system ventilation, and use of appropriate clothes (Young et al., 2010). Likewise, we did not isolate any strain of *C. jejuni* and this could explain the fact that so far no outbreaks due to *Campylobacter* spp. have been notified in Greece, as all the hitherto reported cases are sporadic and community acquired. The lack of outbreaks can also be explained by the fact that according to the Greek alimentary customs, meat is always well-cooked.

As shown by the results in **Table 1**, there are 285 estimated positive samples in the 7 primary sampling units (40.71 in each unit on average). Thus, the estimated prevalence rate of *Campylobacter* spp. positive animals was 0.622%. An upper bound for prevalence rate, with 95% confidence, is approximately 1.226%, while a lower bound is zero. For estimating prevalence rate and confidence intervals, appropriate estimators were used, corresponding to the sampling scheme that was applied (Cochran, 1977; Kish, 1995). In the total of 122 poultry/pig farms and slaughterhouses (which are situated in the examined location of Greece), 4967.2 animals are expected to be found as positive for *Campylobacter* spp. in the total estimated population of 801715 animals. These figures were estimated using appropriate estimators corresponding to the multistage sampling scheme that was applied (combining and weighting the n independent unbiased estimates $\hat{y}_i$ provides us with an unbiased estimate of the population total $t = \frac{N}{n}\sum_{i-1}^{n}\hat{y}_i = 122 \cdot 40.71 \approx 4967.2$ while the prevalence ratio is calculated by the dividing 4967.2 by the estimated total number of units in all the clusters which is 801715).

In the poultry samples, *C. coli* was prevailing with 14 out of 16 strains, a relatively high rate compared to other studies from European countries and the USA (Atanassova et al., 2007; Han et al., 2009). This predominance could be specific for the geographical region we visited and might be different in other Greece regions. Besides, the present survey was performed in a specific region near Athens and that could explain a probable clonality of the strains, which might be not representative of the general animal population. In our case another reason could be the protein-based broilers feed, which influences the digestive bacterial flora equilibrium of chickens. Previous reports showed that the ceca of birds that receive plant protein-based feed are less likely to be colonized with *C. jejuni,* than the ceca of birds that receive other types of feed (Udayamputhoor et al., 2003).

There was a remarkable discrepancy in the identification of *Campylobacter* strains between biotyping by the commercial Api CAMPY system and the molecular methods. In our study as well as in previous reports, the 16S rRNA gene sequencing was shown to be the most suitable test for the identification of the less common *Campylobacter* spp. (Al Amri et al., 2007; Caner et al., 2008).

Table 3 | The accession numbers of the nucleotide sequences admitted to GenBank.

Strain	Source	Accession number to GenBank	Result from sequencing
9/05-85	Poultry farm (3)	AF 550623	*C. coli*
9/05-90	Poultry farm (3)	AF 550622	*C. coli*
8/05-3	Poultry farm (3)	P 6007681	*C. jejuni* subsp. *doylei*
8/05-36	Poultry farm (3)	P 0007681	*C. jejuni* subsp. *doylei*
6/06-36	Poultry farm (4)	AF 550623	*C. coli*
12/06-52	Poultry farm (4)	AF550623	*C. coli*
12/06-33	Poultry farm (4)	AF550624	*C. coli*
6/06-78	Poultry farm (4)	AF 550625.1	*C. coli*
6/06-77	Poultry farm (4)	AF 550623	*C. coli*
6/06-35	Poultry farm (4)	AF 550625	*C. coli*
6/06-75	Poultry farm (4)	AF 550622	*C. coli*
6/06-15	Poultry farm (4)	AF 372092	*C. coli*
7/08-4	Poultry farm (5)	EU 127530.1	*C. coli*
7/08-1	Poultry farm (5)	AF 372096.1	*C. coli*
7/08-2	Poultry farm (5)	AF 372092.1	*C. coli*
7/08-3	Poultry farm (5)	AF 550622.1	*C. coli*

Table 4 | Antimicrobial resistance rates of *Campylobacter* spp. isolated from poultry and other animal sources in relation to species and sample origin.

Species/origin	Total	Nalidixic acid	Ciprofloxacin	Erythromycin	Ampicillin	Amoxicillin–clavulanic acid	Gentamicin
C. coli (poultry farm)	14	2 (14.28%)	–	13 (92.8%)	13 (92.8%)	–	2 (14.3%)
C. jejuni subsp. *doylei* (poultry farm)	2	–	–	–	1 (50%)	–	–

Regarding the antimicrobial susceptibility, there was a remarkably high resistance rate to erythromycin and ampicillin of *C. coli* isolates from poultry farms (92.85 and 100% respectively). Unfortunately, there is no surveillance and monitoring system for the notification of antibiotic use in veterinary medicine and together with the possibility that antibiotics were administered to animals for growth, the possible use of erythromycin, and ampicillin in poultry can only be an assumption. The potential use of ampicillin and erythromycin in veterinary medicine or for animal growth could increase the prevalence of high-resistant *C. coli* strains in poultry farms. On the other hand, *C. coli* isolates are generally more resistant than *C. jejuni* strains (Gallay et al., 2007). According to a pan–European survey upon the antimicrobial susceptibility of *Campylobacter* spp. isolated from chickens (de Jong et al., 2009), 60.2% of the isolated *C. coli* isolates were resistant to ciprofloxacin, 61.4% to nalidixic acid, 12.0% to erythromycin, and 1.2% to gentamicin. Macrolide resistance is based on mechanisms including target modification by point mutation or methylation of 23S rRNA gene, hydrolysis of the drug, and efflux pumps (Belanger and Shryock, 2007). Since no transmission of erythromycin resistance genes through plasmid mechanism has been described so far, either among strains of the same *Campylobacter* species, or of different *Campylobacter* species, it seems that no clear association could be assumed, between the use of macrolides in veterinary medicine and the emergence of macrolide-resistant *Campylobacter* strains in humans.

None of the strains was resistant to ciprofloxacin and amoxicillin–clavulanic acid. Two *C. coli* isolates from the poultry farms were resistant to nalidixic acid and susceptible to ciprofloxacin, a phenotype which could be considered as a type 2 mutant being previously described (Reina et al., 1994; Ioannidis et al., 2009).

CONCLUSION

The isolation, identification, and antimicrobial resistance of *Campylobacter* spp. from poultry farms and slaughterhouses has been investigated and reported for the first time in Greece. The results showed a low prevalence of *Campylobacter* spp. in a geographical region around Athens, with the predominance of *C. coli*. There was a remarkably high resistance rate of *C. coli* isolates from poultry farms to ampicillin and erythromycin, indicating the possible use of erythromycin and ampicillin in poultry. Our results also emphasize the need for a surveillance and monitoring system for the prevalence and antimicrobial resistance of *Campylobacter* in poultry and other food animals.

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