



PREVALENCE, ANTIMICROBIAL RESISTANCE AND RISK FACTORS FOR *CAMPYLOBACTER* COLONISING DOGS AND CATS IN GREECE

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Summary

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The study was conducted to determine the prevalence, antimicrobial resistance and risk factors for *Campylobacter* colonising dogs and cats in Greece. Faecal specimens were collected from 181 dogs and 132 cats. Culture methods were applied to detect *Campylobacter* spp. and a multiplex PCR assay to identify the isolates. The prevalence of *Campylobacter* spp. was 3.8% in dogs and 12.1% in cats. The most frequently identified *Campylobacter* species in dogs was *C. jejuni* (57.1%) followed by *C. coli* (42.9%). All feline isolates were identified as *C. jejuni* apart from one isolate that was characterised as *Campylobacter*-like organism. Gender, age, breed, life style, diarrhoea and type of diet of dogs and cats did not significantly correlate ($P>0.05$) with *Campylobacter* isolation. Possible predictors regarding *Campylobacter* presence in dogs and cats were assessed by binary logistic regression. A tendency towards higher risk for *Campylobacter* contamination was observed in dogs consuming a homemade diet and in outdoor cats. Disk diffusion method revealed that all *Campylobacter* isolates exhibited susceptibility to erythromycin, gentamicin and streptomycin. Contrariwise, 66.7% of canine isolates were resistant concurrently to tetracycline and quinolones and 59.0%, 13.6% and 4.5% of feline isolates were resistant to quinolones, quinolones along with tetracycline and tetracycline alone, respectively.

Key words: antibiotic resistance, *Campylobacter*, *C. jejuni*, *C. coli*, cats, dogs

INTRODUCTION

Campylobacteriosis is the most frequently reported zoonosis of bacterial origin in the EU (EFSA & ECDC, 2015a). *Campylobacter* species are widely distributed in the gastrointestinal tract of most warm-

blooded animals such as farm animals and pets, including cats and dogs, which are usually asymptomatic carriers (WHO, 2011; EFSA & ECDC, 2015a). The vast majority of human campylobacteriosis is

due to foodborne contamination with thermophilic *Campylobacter jejuni* (*C. jejuni*) and *C. coli*, but direct contact with carrier pet animals (faecal-oral route) has been also recognised as a risk factor (Rossi *et al.*, 2008; Kittl *et al.*, 2013; Mughini Gras *et al.*, 2013; EFSA & ECDC, 2015a). In particular, epidemiological data indicate that up to 9.0% of human campylobacteriosis incidents are attributed to *Campylobacter* strains acquired from pet animals (Rossi *et al.*, 2008, Kittl *et al.*, 2013).

Healthy dogs and cats can be potential carriers of *C. jejuni*, *C. coli*, *C. helveticus*, *C. hyointestinalis*, *C. upsaliensis*, *C. lari*, *C. fetus*, *C. gracilis*, *C. curvus*, *C. mucosalis*, *C. rectus*, *C. showae* and *C. sputorum* (Sandberg *et al.*, 2002; Hald *et al.*, 2004; Wieland *et al.*, 2005; Chaban *et al.*, 2010). Isolation of *Campylobacter* spp. from healthy dogs and cats varies considerably among studies from different countries (dogs 5–76%, cats 5–41.9%) and relates to the characteristics of the sample population (e.g. age, life style) and to the applied diagnostic methods (Sandberg *et al.*, 2002; Wieland *et al.*, 2005; Gargiulo *et al.*, 2008; Rossi *et al.*, 2008; Carbonero *et al.*, 2012). *Campylobacter* contamination and the presence of diarrhoea in dogs and cats do not always co-exist since similar patterns of shedding between healthy and diarrhoeic animals have been observed (Sandberg *et al.*, 2002; Rossi *et al.*, 2008; Chaban *et al.*, 2010). It has been reported that animals less than one year old carry campylobacters more often than older animals (Acke *et al.*, 2010; Parsons *et al.*, 2010; Salihu *et al.*, 2010; Carbonero *et al.*, 2012; Amar *et al.*, 2014) and that *Campylobacter* is transmitted between animals of the same or different species when they come in contact (Wieland *et al.*, 2005).

Available data suggest that isolation of *C. upsaliensis* and *C. helveticus* from dogs and cats can exceed that of *C. jejuni* (Hald *et al.*, 2004; Sandberg *et al.*, 2002; Rossi *et al.*, 2008; Chaban *et al.*, 2010; Carbonero *et al.*, 2012) and that there is an epidemiological correlation between these animals and human campylobacteriosis caused by *C. upsaliensis* and *C. jejuni*, especially regarding children (Damborg *et al.*, 2004; Ramonaite *et al.*, 2014). Moreover, Damborg *et al.* (2004) have reported direct transmission of quinolone-resistant *Campylobacter* from dogs and cats to humans, highlighting the importance of these pet animals to the inter-species spreading of antibiotic-resistant campylobacters.

In Greece, there are no available data regarding the prevalence of *Campylobacter* spp. in dogs and cats. Thus, the objective of the present study was twofold: first, to investigate the role of dogs and cats as reservoirs of campylobacters along with the significance of possible predictors associated with their contamination in the region of Thessaloniki, Greece, and second – to determine the potential antimicrobial resistance of the identified *Campylobacter* isolates.

MATERIALS AND METHODS

Study population and sample collection

A total of 181 dogs and 132 cats presented at the Companion Animal Clinic of the Veterinary School, Aristotle University of Thessaloniki, with various clinical signs, were randomly selected for the study. One faecal specimen using a sterile cotton-tipped swab was taken directly from the rectum of each individual animal. Owners were asked to complete a questionnaire with information relevant to the history of the animal (gender, age, breed,

life style, diarrhoea and type of diet) and to give their consent for sampling their animals. Regarding life style, dogs and cats that lived indoors but were allowed to spend time outdoors, even occasionally, were regarded as outdoor animals. Complete history data regarding diarrhoea were not available for stray animals (5 dogs and 69 cats) that were about to be sheltered in animal welfare unions and visited the clinic for veterinary care immediately after collection, whereas, consent for sampling these animals was provided by the animal shelter representative. Missing data for these animals were addressed as 'unknown'.

Microbiological analysis

Samples were transported in a temporary culture media (Transwab, Medical Wire & Equipment Co. Ltd., Corsham, England) under refrigeration (<4 °C) to the Laboratory of Hygiene of Food of Animal Origin, School of Veterinary Medicine, Aristotle University of Thessaloniki, where they were analysed within four hours from the time of collection. The rectal swabs were directly inoculated onto a modified Charcoal Cefoperazone Deoxycholate agar (*Campylobacter* blood-free selective agar, mCCDA, Merck, Germany) and a Karmali agar (Oxoid Ltd., Basingstoke, Hampshire, UK) plate that were subsequently incubated at 41.5 °C for 44±4 h under microaerophilic conditions in a jar (Genbox jar, Genbox Microaer Generator, Biomérieux, Lyon, France). After incubation, one typical or suspected colony of *Campylobacter* species was selected from each selective medium and, thus, two were the maximum number of isolates analysed per animal. Each selected colony was subcultured onto a Columbia blood agar (CBA) plate (Biomérieux) and incubated at 41.5 °C for 44±4 h under mic-

roaerophilic conditions. Pure cultures were examined for morphology, motility, catalase and oxidase activity and aerobic growth at 25 °C. Isolates were stored at -80 °C in Nutrient Broth No. 2 (Oxoid) supplemented with 5% lysed horse blood (Oxoid) and 20% glycerol (BDH Laboratory Suppliers, Poole, England) for forthcoming analysis.

Identification of Campylobacter species

DNA was extracted using an in-house developed protocol as previously described (Lazou *et al.*, 2014). The multiplex PCR (m-PCR) assay developed by Wang *et al.* (2002) was used to identify *Campylobacter* at species level. The assay simultaneously detects the *hipO* gene for *C. jejuni*; the *glyA* gene for *C. coli*, *C. lari*, and *C. upsaliensis*; and the *sapB2* gene for *C. fetus* subsp. *fetus*. Moreover, the assay includes a pair of primers as an internal control for *Campylobacter* 23S rRNA and the corresponding amplicon is detected in *Campylobacter*, *Arcobacter*, and *Helicobacter* isolates. Specific identification was achieved by applying Restriction Fragment Length Polymorphism (RFLP) digestion using the restriction endonucleases *BsrDI*, *AluI*, *ApoI*, *DdeI*, *BcII* and *HhaI*, resulting in specific restriction fragments for each *Campylobacter* species (Wang *et al.*, 2002). The strains *C. jejuni* ATCC 33291, *C. coli* ATCC 43478, *C. lari* ATCC 35221, *C. upsaliensis* ATCC 43954, and *C. fetus* subsp. *fetus* ATCC 25936 were included as positive controls. The strain *E. coli* ATCC 11303 and a blank were included as negative controls.

Antimicrobial susceptibility testing

The disk diffusion method according to Bauer *et al.* (1966) was applied in order to screen the antibiotic susceptibility profile

of the *Campylobacter* isolates towards six critically important antimicrobials as recommended by the World's Health Organization (WHO, 2009), including ciprofloxacin, erythromycin, gentamicin, nalidixic acid, streptomycin, and tetracycline (BBL-DIFCO Microbiology, Becton, Dickinson and Company, USA). The observed inhibition zones were interpreted according to a) the European Committee on Antimicrobial Susceptibility Testing (EUCAST) guidelines (EUCAST, 2013) for *Campylobacter* as regards ciprofloxacin, erythromycin and tetracycline, b) the Clinical and Laboratory Standards Institute (CLSI) guidelines for *Enterobacteriaceae* (CLSI, 2010) as regards gentamicin and streptomycin (Maćkiw *et al.*, 2012), and c) the British Society for Antimicrobial Chemotherapy (BSAC) guidelines for *Campylobacter* (BSAC, 2012) regarding nalidixic acid. The strains *C. jejuni* ATCC 33560 and *C. coli* ATCC 33559 were included as quality controls.

Statistical Analysis

Chi-square analysis was used to compare categorical traits between positive and *Campylobacter*-free dogs and cats. Moreover, two binary logistic regression models were built in order to assess the effects of possible predictors regarding the infection with *Campylobacter* spp. in dogs and cats. Variables used for the models were selected after a stepwise regression

analysis as being the most significant ones. Gender (2 levels, male and female), age (2 levels, <1 year and >1 year) and life style (2 levels, outdoors and indoors) were forced as possible predictors in both models. Especially in dogs, diet (2 levels, homemade and commercial diet) was also used as a predictor variable. All statistical analyses were performed using the statistical package IBM SPSS Statistics 21.

RESULTS

In total, 23 out of 313 dogs and cats (7.3%) were *Campylobacter*-positive. The identification of species by m-PCR and RFLP is presented in Table 1 and indicative PCR amplification products are illustrated on Fig. 1.

The most frequently identified *Campylobacter* species in dogs was *C. jejuni* (57.1%) followed by *C. coli* (42.9%) whereas coinfection with both *C. jejuni* and *C. coli* was not detected in any dog. In cats, the vast majority of isolates were identified as *C. jejuni* (94.7%). The single isolate from a stray cat, although it yielded the 23S rRNA amplicon, could not be identified at species level by m-PCR despite repeated attempts and was characterised as *Campylobacter*-like organism (CLO) (Fig. 1).

Chi-square test revealed no statistically significant association ($P > 0.05$) between the presence/absence of *Campylo-*

Table 1. Isolation rates of *Campylobacter* species in dogs (n=181) and cats (n=132)

Species	Absence of diarrhoea	Diarrhoeic	No data regarding diarrhoea (stray animals)	Total
Dogs	n=154	n=22	n=5	n=181
<i>C. jejuni</i>	2 (1.3%)	2 (9.1%)	–	4 (2.2%)
<i>C. coli</i>	3 (1.9%)	–	–	3 (1.6%)
				7 (3.8%)
Cats	n=57	n=6	n=69	n=132
<i>C. jejuni</i>	4 (7.0%)	1 (1.7%)	11 (15.9%)	16 (12.1%)

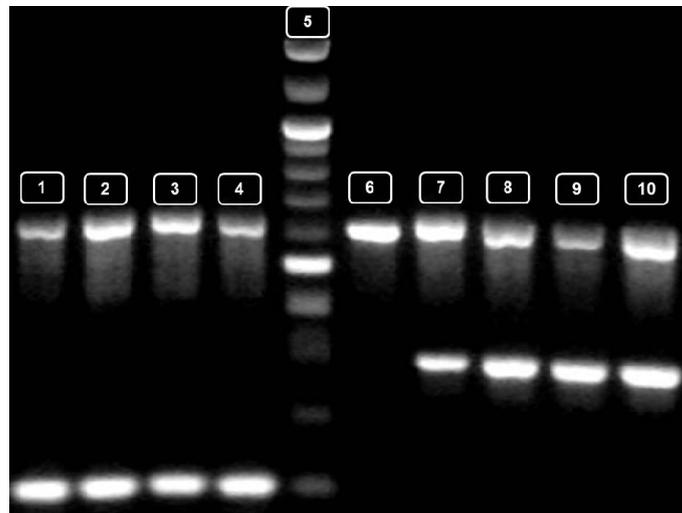


Fig. 1. Indicative amplification products of the multiplex PCR assay used in this study for the identification of *Campylobacter* isolates from dogs and cats. Lanes 1 to 3: *C. coli* isolates from dogs, lane 4: *C. coli* ATCC 43478, lane 5: 100-bp DNA Ladder (New England Biolabs, Ipswich, MA, USA), lane 6: *Campylobacter*-like organism (CLO) isolate from a stray cat, lane 7: *C. jejuni* ATCC 33291, lanes 8 to 10: *C. jejuni* isolates from cats.

bacter and gender, age, breed, life style, diarrhoea and type of diet of the dogs and cats.

When the full regression models were tested against constant only models, the result was not statistically significant for both of the models (chi square= 6.789, $P=0.147$ with $df=4$ and chi square= 6.966, $P=0.073$ with $df=3$, for dog and cats, respectively), indicating their low predictive power. Moreover, the two models failed to produce a strong relationship between the predictors used and the infection with *Campylobacter* (Nagelkerke $R^2= 11.4\%$ and 9.7% for dogs' and cats' model, respectively). The effects of the predictors for the two models are summarised in Table 2. However, among the predictors, diet tended to have a significant effect on *Campylobacter* infection in dogs; dogs eating a homemade diet were about 6.2 times more likely to be infected with *Campylobacter* when compared to dogs

consuming strictly a commercial diet ($P=0.09$, CI 95% 0.75 to 52.06). None of the other predictors was found to have a significant effect on the presence of *Campylobacter*. In cats, life style displayed a tendency to affect infection with *Campylobacter* since indoor cats were 3.4 times less likely to be infected ($P=0.07$, CI 95% 0.92 to 12.91) compared to outdoor cats. The effects of the rest predictors, in both models, were also not statistically significant.

Isolates originating from the same animal displayed a common antimicrobial profile and the exhibited antimicrobial resistance profile of the examined *Campylobacter* isolates is presented in Table 3. Both canine and feline isolates exhibited susceptibility to erythromycin, gentamicin and streptomycin. On the other hand, 66.7% of *Campylobacter* isolates from dogs were resistant concurrently to tetracycline and quinolones (ciprofloxacin and

Table 2. The effects of the predictors used in the two binary logistic regression models for *Campylobacter* infection in dogs (n=181) and cats (n=132)

Animal species	Predictor							95% CI for Odds Ratio	
			B	S.E.	P	Odds ratio	Lower	Upper	
Dogs	Gender	Male (n=84)	-1.14	0.83	0.17	0.32	0.06	1.63	
		Female (n=97)	Ref.*	-	-	-	-	-	
	Age	< 1 year (n=44)	0.18	0.86	0.84	1.19	0.22	6.38	
		> 1 year (n=137)	Ref.*	-	-	-	-	-	
	Diet	Homemade (n=114)	1.83	1.08	0.09	6.23	0.75	52.06	
		Commercial (n=67)	Ref.*	-	-	-	-	-	
	Life style	Outdoors (n=84)	-0.69	0.75	0.36	0.50	0.12	2.21	
Indoors (n=97)		Ref.*	-	-	-	-	-		
Constant			-3.70	1.04	0.00	0.03	-	-	
Cats	Gender	Male (n=48)	0.59	0.53	0.26	1.81	0.64	5.09	
		Female (n=84)	Ref.*	-	-	-	-	-	
	Age	< 1 year (n=20)	-1.41	1.07	0.19	0.24	0.03	1.99	
		> 1 year (n=112)	Ref.*	-	-	-	-	-	
	Life style	Outdoors (n=91)	1.24	0.68	0.07	3.44	0.92	12.91	
		Indoors (n=41)	Ref.*	-	-	-	-	-	
	Constant			-2.80	0.68	0.00	0.06	-	-

Table 3. Antimicrobial resistance profile of the investigated *Campylobacter* isolates from dogs and cats.

Antimicrobial resistance profile*	<i>Campylobacter</i> spp. (n = 34)		
	Dog isolates		Cat isolates
	<i>C. jejuni</i> (n=8)	<i>C. coli</i> (n=4)	<i>C. jejuni</i> (n=22)
TE	0 (0%)	0 (0%)	1 (4.5%)
CIP + NA	0 (0%)	0 (0%)	13 (59.0%)
CIP + NA + TE	4 (33.3%)	4 (33.3%)	3 (13.6%)
Total	33.3%	33.3%	17 (77.3%)

* Key to antimicrobial agents: TE: tetracycline; NA: nalidixic acid; CIP: ciprofloxacin.

nalidixic acid). In cats, approximately 60.0% of *Campylobacter* isolates were resistant to quinolones, 14.0% to quinolones along with tetracycline, and 5.0% only to tetracycline.

DISCUSSION

In this study, *Campylobacter* was isolated from approximately 4% of dogs and 12%

of cats. These proportions, although similar to previous studies (Lee *et al.*, 2004; Gargiulo *et al.*, 2008; Ramonaite *et al.*, 2014), could be considered rather low compared to other reported results (Wieland *et al.*, 2005; Parsons *et al.*, 2010; Carbonero *et al.*, 2012). It should be noted that each animal was sampled only once according to the study design, whereas, higher frequencies have been recorded in longitudinal studies where

serial sampling was performed from each animal (Hald *et al.*, 2004; Sandberg *et al.*, 2002). Nevertheless, the proportion of *Campylobacter*-positive cats was higher than that of dogs and this difference proved to be statistically significant ($P=0.012$). Both higher and lower rates of *Campylobacter* infection in cats than in dogs or even similar infection rates between these two animal species have been previously reported (Sandberg *et al.*, 2002; Wieland *et al.*, 2005; Mohan, 2015). The most likely explanation of the significant divergence between the *Campylobacter* contamination rates of cats and dogs observed in this study could be the fact that approximately 52.3% of the cats whereas only 2.8% of the dogs examined were stray animals. Indeed, stray animals have been found more commonly infected by campylobacters than household animals that are kept mostly indoors (Tsai *et al.*, 2007). Stray animals that are not sheltered may easily come in direct or indirect contact with wild birds that are regarded as reservoirs for *Campylobacter* (Mohan, 2015). Moreover, crowded animal housing with recurrent turnover in shelters and financial limitations for pathogen surveillance in shelters for stray animals have been indicated as predisposing factors for pathogen transmission between them (Tsai *et al.*, 2007).

In dogs, *C. jejuni* was isolated more frequently (57.1%) followed by *C. coli* (42.9%) and the vast majority of isolates from cats were identified as *C. jejuni* (94.7%). Comparable results regarding the predominance of *C. jejuni* in samples from dogs and cats have been reported in previous studies (Gargiulo *et al.*, 2008; Badlík *et al.*, 2014; Giacomelli *et al.*, 2015). None of the *Campylobacter* isolates in the present study has been identified as *C. upsaliensis*, though the pre-

dominance of this species over *C. jejuni* in faecal samples from both diarrhoeic and healthy dogs and cats is a common finding (Hald *et al.*, 2004; Wieland *et al.*, 2005; Chaban *et al.*, 2010; Parsons *et al.*, 2010; Salihu *et al.*, 2010; Carbonero *et al.*, 2012). Available data suggest that the recovery of *C. upsaliensis* presupposes a series of crucial parameters such as either a filtration method or the use of up to four agar plates containing cefoperazone, teicoplanin, and amphotericin B (CAT medium) combined with extension of the incubation period for at least four days or even combination of different culture methods (Goossens *et al.*, 1991; Moreno *et al.*, 1993; Hald *et al.*, 2004; Acke *et al.*, 2009). Moreover, the higher cefoperazone content of mCCDA compared to CAT has been reported to impose an inhibitory effect to the growth of *C. upsaliensis* (Hald *et al.*, 2004). Therefore, the applied methodology in the present study (direct inoculation of mCCDA and Karmali agar plates, incubation for 48 hours) could not be regarded as ideal for the isolation of *C. upsaliensis*.

With only 7 and 16 *Campylobacter*-positive dogs and cats, respectively, the power to detect significant risk factors by the applied statistical models was limited. Nevertheless, an overall tendency of higher odds for *Campylobacter* contamination in dogs consuming a homemade instead of a commercial diet was observed though not significant in the statistical model. This tendency could be enlightened by the fact that the hygiene status of homemade diets may vary considerably and include leftovers of raw meat, such as poultry, that serve as vehicles for *Campylobacter* transmission to dogs and cats. The indication of a higher risk of *Campylobacter* contamination for outdoor cats was also weak but may reflect the contact

with other animals, consumption of raw meat and exposure to environments harbouring campylobacters. Conversely, indoor cats use their litter pan and have limited or even no contact with other animals. Similar findings have been reported previously and were related to the stray behaviour of cats (e.g. ground digging) (Sandberg *et al.*, 2002; Wieland *et al.*, 2005).

In the present study, no statistically significant ($P>0.05$) association between *Campylobacter* spp. isolation and gender, age and presence of diarrhoea was observed in the dogs and cats. No significant difference in the prevalence of *Campylobacter* relevant to the gender of dogs and cats has been reported previously (Gargiulo *et al.*, 2008; Salihu *et al.*, 2010). It has been found that the age of dogs is not a risk factor for *C. jejuni* and *C. coli* infection (Wieland *et al.*, 2005; Selwet *et al.*, 2015) but dogs and cats younger than 36 months old have significantly higher odds of carrying *C. upsaliensis* and *C. helveticus* (Wieland *et al.*, 2005). In a longitudinal study by Hald *et al.* (2004), three month-old dogs were 60% *Campylobacter*-positive, with the prevalence of contamination reaching almost 100% at 12 months of age and then decreasing to 67% when the dogs were 24 months old. Other studies have not detected any association between *Campylobacter* carriage and intestinal disease in dogs (Acke *et al.*, 2006; Rossi *et al.*, 2008; Parsons *et al.*, 2010). In accordance to the results of this study, the prevalence of *Campylobacter* in dogs and cats with diarrhoea and healthy animals in Norway was not significantly different (Sandberg *et al.*, 2002) and recent diarrhoeic episodes in dogs in Switzerland did not have a significant impact on *C. jejuni* isolation (Wieland *et al.*, 2005). On the other hand, intestinal-related signs have been recognised as a

significant factor for the isolation of *Campylobacter* spp., *C. jejuni* and *C. upsaliensis* in dogs in Spain (Carbonero *et al.*, 2012). The contribution of *Campylobacter* to the aetiopathogenesis of diarrhoea of the dogs and cats in the present study could not be excluded, although disorders in the gastrointestinal tract other than the presence of campylobacters constituted the definitive diagnoses.

Antimicrobial resistance of pathogenic bacteria towards critically important antimicrobials (WHO, 2009), as the ones applied in this study, is a topic of public health interest. All of the tested *Campylobacter* isolates from dogs and cats exhibited susceptibility to erythromycin, gentamicin and streptomycin, which are administered for campylobacteriosis in humans (Blaser & Engberg, 2008). This finding is comforting from a public health point of view. High susceptibility rates of *Campylobacter* isolates from dogs and cats to these antimicrobials have been reported in other studies (Sandberg *et al.*, 2002; Lee *et al.*, 2004; Carbonero *et al.*, 2012). However, resistance to quinolones was exhibited by the majority of canine and feline *Campylobacter* isolates in this study, while cross-resistance to nalidixic acid and ciprofloxacin was always observed. These results can be attributed to the fact that enrofloxacin is widely used in veterinary practice in Greece and this drug has been associated to the increase in fluoroquinolone-resistant *C. jejuni* strains (van Looveren *et al.*, 2001). Tetracycline is an alternative drug for campylobacteriosis treatment (Blaser & Engberg, 2008) and resistance of *Campylobacter* isolated from dogs and cats towards tetracycline varies among different studies (0–77.5%) (Sandberg *et al.*, 2002; Lee *et al.*, 2004; Carbonero *et al.*, 2012). In the present study, approximately 67% of canine and

18% of feline *Campylobacter* isolates displayed resistance to this antimicrobial agent. Since tetracycline is not commercially available for veterinary use in dogs and cats in Greece in contrast to doxycycline, the observed resistance could be attributed to cross-resistance to the latter (Karmali *et al.*, 1981) or to the primary infection of the dogs and cats with tetracycline-resistant isolates commonly found in other animal species and food (EFSA & ECDC, 2015b).

CONCLUSION

To our knowledge, this is the first study on the prevalence and antibiotic resistance of *Campylobacter* in dogs and cats in Greece. The data presented in the current study support previously reported results that not only diarrhoeic but also healthy dogs and cats can shed campylobacters. *Campylobacter* infection of humans from dogs and cats via accidental exposure to this pathogen is possible. The awareness that even healthy dogs and cats can pose a zoonotic risk for humans may itself be a first step towards reducing its transmission by adopting good hygiene practices. Another finding of public health interest is the fact that erythromycin, gentamicin and streptomycin displayed the highest *in vitro* efficiency against the tested *Campylobacter* isolates of canine and feline origin. Further research is deemed necessary in order to clarify the actual role of dogs and cats as sources of human campylobacteriosis in Greece.

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