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RESEARCH ARTICLE

Recovery of Thermophilic *Campylobacter* spp. in Healthy and Diarrhoeic Pets by Three Culture Methods and Identification of the Isolates by Multiplex Polymerase Chain Reaction (mPCR) [1]

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Summary

In this study, the determination of prevalence of thermophilic *Campylobacter* species in dogs and cats with and without diarrhoea using 3 different cultural methods was aimed. For this purpose, rectal swabs were collected from 120 dogs and 15 cats and 14 of them (12 dogs and 2 cats) were taken from diarrhoeic animals. The isolation of thermophilic *Campylobacter* spp. was conducted by direct plating onto modified charcoal cefoperazone deoxycholate agar (mCCDA) supplemented with CCDA (cefoperazone, amphotericin B) or CAT (cefoperazone, amphotericin and teicoplanin) for all samples and membrane filtration method onto Mueller-Hinton Agar supplemented with 5% defibrinated sheep blood for samples from diarrhoeic pets and identification of isolates was performed using multiplex polymerase chain reaction (mPCR). The overall prevalence of *Campylobacter* species was found to be 40.0% and 26.7% in dogs and cats, respectively. *Campylobacter jejuni* was the most frequent bacterium isolated from 36 dogs and 4 cats. *C. upsaliensis, C. coli* and *C. lari* were isolated from 10 dogs and 1 cat, 5 dogs and 2 healthy dogs respectively. For the isolation of thermophilic *Campylobacter* spp., whilst the method using CAT as selective supplement being more sensitive in dogs, the membrane filtration appeared as the most suitable method in diarrhoeic dogs. These results showed the occurrence of a relatively high carriage of *Campylobacter* spp., particularly in healthy dogs that may constitute a non negligible risk for public health.

Keywords: *Campylobacter spp., Membrane filtration method, mPCR, Pet animals, Rectal swab*

Sağlıklı ve İshalli Pet Hayvanlarda Termofilik *Campylobacter* spp.'nin Üç Kültür Metodu ile İzolasyonu ve İzolatların Multipleks Polimeraz Zincir Reaksiyonu m(PZR) İle İdentifikasyonu

Özet

Bu çalışmada, sağlıklı ve ishalli kedi ve köpeklerde 3 farklı kültür metodu kullanılarak termofilik *Campylobacter* türlerinin prevalansının belirlenmesi amaçlandı. Bu amaçla, 120 köpek, 15 kediden rektal svap örneği toplandı ve bunların 14'ü (12 köpek ve 2 kedi) ishalli hayvanlardan alındı. Termofilik *Campylobacter* spp.'nin izolasyonunda, tüm örnekler için CCDA (cefoperazone, amphotericin B) ya da CAT suplement (cefoperazone, amphotericin and teicoplanin) ilave edilmiş modified charcoal cefoperazone deoxycholate agar (mCCDA)'a (sefoperazon, amfoterisin B) direkt ekim, ishalli hayvanlardan alınan örnekler için de %5 defibrine koyun kanı ilave edilmiş Mueller- Hinton Agar üzerine membran filtrasyon yöntemi kullanıldı. İzolatların identifikasyonu multipleks polimeraz zincir reaksiyonu (mPZR) ile gerçekleştirildi. *Campylobacter* türlerinin köpek ve kedilerde genel prevalansı sırasıyla %40.0 ve %26.7 olarak bulundu. *Campylobacter jejuni* en sık rastlanan tür olup 36 köpek ve 4 kediden izole edildi. Ayrıca, 10 köpek ve 1 kediden *C. upsaliensis,* 5 köpekten *C. coli* ve 2 sağlıklı köpekten *C. lari* izole edildi. Termofilik *Campylobacter* spp. izolasyonu için CAT selektif suplementin kullanıldığı metot köpeklerde daha duyarlı iken ishalli köpeklerde membran filtrasyonun en uygun metot olduğu görüldü. Bu sonuçlar, özellikle sağlıklı köpeklerde oldukça yüksek oranlarda bulunan *Campylobacter* spp. taşıyıcılığının halk sağlığı için göz ardı edilemez risk oluşturduğunu göstermektedir.

Anahtar sözcükler: *Campylobacter spp., Membran filtrasyon metodu, mPZR, Pet hayvanları, Rektal svap*

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INTRODUCTION

The genus *Campylobacter* currently contains 18 species with six sub-species and two biovars [1-3]. *Campylobacter* bacteria are the most commonly reported agents causing gastroenteritis in humans in the industrialized countries [4-6]. Campylobacteriosis in humans is a zoonotic disease and the bacteria are frequently found as commensals in the gastrointestinal tract of many domestic and wild animals, especially birds [7-12]. *C. jejuni* is by far the most frequently isolated species from human cases, but other thermophilic *Campylobacter* spp. such as *C. upsaliensis, C. coli and C. lari* have also been associated with diseases in humans [5,6] Consumption of undercooked chickens and handling raw chicken carcasses has been identified as significant risk factors for human infections [2,10,13]. Other known risk factors are consumption of unpasteurised milk or water, travelling abroad and living or working on a farm [2,13-15]. Cat and dogs can harbour *Campylobacter* spp. in their gastrointestinal systems [16-18] and daily contact with pet dogs and cats have been identified as another risk factor for human campylobacteriosis. There are many reports describing presumed or proven associations between *Campylobacter* infections and pet exposure [19,20].

However, there is no detailed report by using different isolation methods and molecular method for *Campylobacter* species from dogs and cats in Turkey. The objective of this study was to evaluate the prevalence of Thermophilic *Campylobacter* spp. in dogs and cats with and without diarrhoea using three different isolation methods and to identify isolates using mPCR (multiplex polymerase chain reaction).

MATERIAL and METHODS

Samples

A total of 135 rectal swabs taken from 120 dogs and 15 cats submitted to Erciyes University, Faculty of Veterinary Medicine, Turkey, was analysed between November 2008 and April 2009. The animals were at different ages and breeds. Fourteen (12 dogs and 2 cats) of the 135 rectal swabs examined were taken from diarrhoeic animals whereas 108 dogs and 13 cats were healthy (being presented for a health check, vaccination or neutering). The majority of the animals was from Kayseri and kept as indoor pets. The samples were immediately transported to the laboratory in a cool box and examined within 15 min after sampling.

Isolation Procedures

Each swab sample taken from animals was homogenized with 500 µL distilled water and 100 µL of this inoculum was plated directly onto mCCDA (modified charcoal cefoperazone deoxycholate agar, Oxoid, CM0739) with CCDA selective supplement (cefoperazone, amphotericin B, Oxoid, SR0155E) (medium 1), and mCCDA with CAT (cefoperazone, amphotericin and teicoplanin, Oxoid, SR174E) selective supplement (medium 2), respectively. Membrane filtration method was used as a third method. In the filtration method, 300 µL of faecal suspension were placed on a 47-mm diameter, 0.45µm-pore-size cellulose acetate membrane filter (Sartorius AG, Goettingen, Germany) placed on Mueller-Hinton Agar supplemented with 5% defibrinated sheep blood without any selective supplement. After incubation at 37°C for 30 min under aerobic conditions, the filter was removed [21]. The plates were then incubated microaerobically for 48-96 h at 42°C. The first and second methods were used both in diarrhoeic and non diarrhoeic animals, but the third method was used in diarrhoeic animals only. After the incubation period, *Campylobacter* spp. were initially identified by observing characteristic morphology and motility using phase contrast microscopy and using morphological features of the colonies (1-3 mm in diameter, white to cream to silver in colour and round in outline), Gram staining, oxidase reaction and catalase production [8,9,22]. *C. jejuni* NCTC 11168 was used as the reference strain. Presumed *Campylobacter* spp. colonies were sub-cultured on mCCDA supplemented with 5% defibrinated sheep blood under the same conditions as described above for purification and the isolates were stored at -80°C until further analysis.

Differentiation of Campylobacter Isolates by Colony mPCR

The primers and PCR assay conditions were used for the simultaneous identification and differentiation of the *Campylobacter* isolates as previously described by Wang et al.[23]. This method was slightly modified and *Campylobacter fetus* primers were not used in the current mPCR. Only 5 pairs of primers were used to identify the genes *hipO* from *C. jejuni*, *glyA* from *C. coli*, *C. lari*, and *C. upsaliensis*; and the internal control 23S rRNA [23]. Chromosomal DNA was prepared by suspending again the cell pellets in 100 µL of sterile distilled water and boiling the suspensions for 10 min. After centrifugation (in 10.000xg, for 10 min, at +4°C), the supernatants were used as DNA templates in mPCR. The primers and expected PCR amplicons are shown in *Table 1*.

The mPCR consisted of 30 cycles (Touchgene Gradient,

Table 1. *Predicted sizes of amplified products of mPCR and primer pairs for thermophilic Campylobacter species used*
Tablo 1. *Termofilik Campylobacter türleri için mPCR amplifikasyon ürünlerinin beklenen band büyüklükleri ve kullanılan primer çiftleri*

Gene	Primer	PCR Amplicon Size (in bp)
C. jejuni hipO	CJF, CJR	323
C. coli glyA	CCF, CCR	126
C. lari glyA	CLF, CLR	251
C. upsaliensis glyA	CUF, CUR	204
C. jejuni 23S rRNA	23SF, 23SR	650

Techne, UK). Amplified products were detected by electrophoresis (EC340 Maxicell, Thermo, USA) on a 1.5% agarose at 100 V for 40 min (EC250-90, Thermo, USA). The gels were stained with ethidium bromide and inspected visually under a UV transilluminator (Vilber Lourmat, Marne La Vallée, France).

RESULTS

All *Campylobacter* spp. isolates were identified at the species level by mPCR *(Fig. 1)*. A total of 48 samples in dogs and 4 samples in cats were positive for *Campylobacter* spp. with at least one of the 3 isolation methods leading to overall prevalences of 40.0% and 26.7%, respectively. As shown in *Table 2* and *Table 3*, *C. jejuni* was the most predominant species identified in sampled dogs (in 32 non diarrhoeic dogs and in 4 diarrhoeic dogs) and cats (in 3 healthy animals and in one with diarrhoea) with at least one isolation method and sometimes found in association with other species such as *C. upsaliensis* (3 times in dogs and once in cats) and *C. coli* (Once in healthy dogs). Three other *Campylobacter* species, *C. upsaliensis*, *C. coli* and *C. lari*, were also isolated in 20.8%, 10.4% and 4.2% respectively dog rectal swab samples.

The 2 isolation methods based on isolation on mCCDA medium with CCDA (method 1) or CAT (method 2) as selective supplements showed different efficiencies. Firstly, 24.1% and 32.4% of the healthy dogs and 25.0% and 41.7% of the diarrhoeic dogs were positive using methods 1 and 2, respectively *(Table 2)* and in healthy cats, the isolation rates were 23.1% and 15.4% for methods 1 and 2, respectively. Contrary to the method 1, no rectal sample from diarrhoeic cats gave positive isolation with the medium 2 *(Table 3)*. As reported in *Table 4*, the overall agreement score (number of identical scores (positive or negative isolation for *Campylobacter* spp.) in dogs and cats) was 81.5% and the agreement score was slightly higher in cats (86.7%) than in dogs (80.8%). Among the 50 samples (46 from dogs and 4 from cats) positive for *Campylobacter* spp. isolation, 25 (23 from dogs and 2 from cats) were positive for the 2 methods (positive agreement score: 50.0%) and these positive scores were similar and have remained as moderate in healthy animals than in diarrhoeic ones (approximately 50.0%). The second method was found to be more appropriate and sensitive in detecting various species of *Campylobacter* spp. in the dog rectal samples (40 positive samples versus 29 with the first method) whereas in cats 4 samples were positive with the first method and only 2 with the second method. In diarrhoeic cases, the membrane filtration method (third method) appeared as the most suitable in dogs *(Table 2)* evidencing *Campylobacter* spp. in 7 samples (versus 5 with the method 2) whereas it failed to detect bacteria in diarrhoeic cats *(Table 3)*.

DISCUSSION

This is the first report using different isolation techniques and mPCR for detecting of thermophilic campylobacters from dogs and cats in Turkey. Several isolation media have been developed and evaluated for the isolation of *Campylobacter* spp. from clinical, food, environmental and animal samples. The most widely used method for the

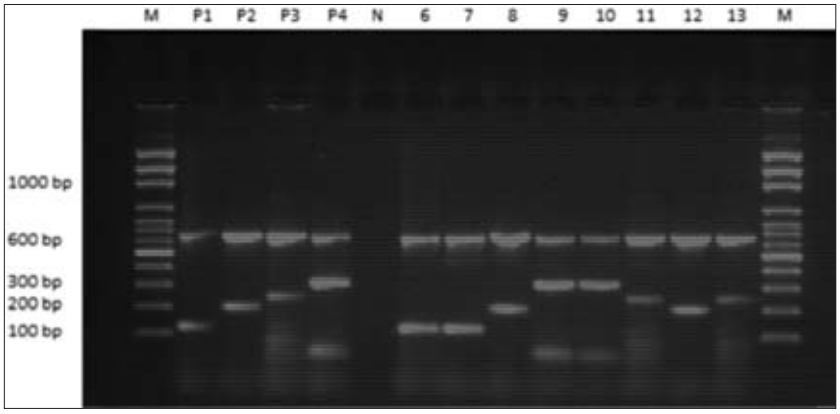

Fig 1. mPCR products from *Campylobacter* isolates by 1.5% agarose gel electrophoresis
M: Marker; **P1:** positive control for *C. coli* DCC2 (126 bp); **P2:** positive control for *C. upsaliensis* DCC3 (204 bp); **P3:** positive control for *C. lari* DCC4 (251 bp); **P4:** positive control for *C. jejuni* NCTC 11168 (323 bp); **N:** negative control; **lanes 6-13:** dog rectal swap isolates (*C. coli* in lanes 6 and 7, *C. upsaliensis* in lanes 8 and 12, *C. jejuni* in lanes 9 and 10, *C. lari* in lane 11and 13); **650 bp:** fragment of 23S rRNA (which occurred from all *Campylobacter* spp.)

Şekil 1. *Campylobacter* türlerine ait mPZR ürünlerinin %1.5 agaroz jel görüntüsü
M: Moleküler marker; **P1:** *C. coli* DCC2 pozitif kontrol (126 bp); **P2:** *C. upsaliensis* DCC3 pozitif control (204 bp); **P3:** *C. lari* DCC4 pozitif control (251 bp); **P4:** *C. jejuni* NCTC 11168 pozitif control (323 bp); **N:** negatif kontrol; **sıra 6-13:** köpek rektal svap izolatları (6,7: *C. coli*, 8,12: *C. upsaliensis,* 9,10: *C. jejuni*, 11,13: *C. lari*); **650 bp:** 23S rRNA (*Campylobacter* spp. için genus pozitif bandlar)

Table 2. *Isolation rates of thermophilic Campylobacter spp. by different methods in diarrhoeic (n = 12) and in healthy (n = 108) dogs*
Tablo 2. *İshalli ve sağlıklı köpeklerden termofilik Campylobacter türlerinin farklı metodlar ile izolasyon oranları*

Media Used	Healthy Dogs (n = 108)	Diarrhoeic Dogs (n = 12)	Total (n = 120)
Positive samples			
Medium 1	26 (24.1%)	3 (25.0%)	29 (24.2%)
Medium 2	35 (32.4%)	5 (41.7%)	40 (33.3%)
Medium 3	ND	7 (58.3%)	ND
Total[1]	**41 (38.0%)**	**7 (58.3%)**	**48 (40.0%)**
***Campylobacter* spp**			
C. jejuni			
Medium 1	22 (84.6%)	3 (100.0%)	25 (86.2%)
Medium 2	28 (80.0%)	4 (80.0%)	32 (80.0%)
Medium 3	ND	4 (57.1%)	ND
Total[1]	**32**	**4**	**36 (75%)**
C. coli			
Medium 1	3 (11.5%)	0 (0.0%)	3 (10.3%)
Medium 2	1 (2.9%)	0 (0.0%)	1 (2.5%)
Medium 3	ND	1 (14.3%)	ND
Total[1]	**4**	**1**	**5 (10.4%)**
C. lari			
Medium 1	1 (3.8%)	0 (0.0%)	1 (3.4%)
Medium 2	1 (2.9%)	0 (0.0%)	1 (2.5%)
Medium 3	ND	0 (0.0%)	ND
Total[1]	**2**	**0**	**2 (4.2%)**
C. upsaliensis			
Medium 1	1 (3.8%)	0 (0.0%)	1 (3.4%)
Medium 2	8 (22.9%)	1 (20.0%)	9 (22.5%)
Medium 3	ND	2 (28.6%)	ND
Total[1]	**8**	**2**	**10 (20.8%)**

Medium 1: *mCCDA (modified charcoal cefoperazone deoxycholate agar) with CCDA (cefoperazone, amphotericin B) selective supplement;* ***Medium 2:*** *mCCDA (modified charcoal cefoperazone deoxycholate agar) with CAT (cefoperazone, amphotericin and teicoplanin) selective supplement;* ***Medium 3:*** *Mueller-Hinton Agar supplemented with 5% defibrinated sheep blood (membrane filtration method);* ***ND:*** *not detected;* [1] *number of positive samples with at least one isolation method*

detection of *Campylobacter* spp. in animals is direct plating of a faecal swab sample onto selective media containing various combinations of antibacterial agents (such as, Preston agar, CAT agar, mCCDA medium, and Karmali medium) [24,25]. In addition, enrichment of campylobacters in a broth medium is used for the isolation of campylobacters when the numbers of bacteria are presumed to be low [26,27]. Membrane filtration method has been extensively used for the isolation of *Campylobacter* spp. (non selective agar base e.g., blood agar base, Mueller Hinton agar, Brucella agar supplemented with 5-7% defibrinated sheep blood) [26-28]. Although faecal-based methods are still the most widely used and considered to be reliable detection methods for *Campylobacter* in animals, their detection ranges are variable with each procedure [9,26-29]. Thermophilic *Campylobacter* spp., *C. jejuni*, *C. coli*, *C. lari* and *C. upsaliensis* are commensally present in the intestinal flora of dogs and cats [30,31]. Hence, dogs and cats also present a risk factor for human campylobacteriosis [19,20]. Isolation rate of *Campylobacter* spp. from these animals have been shown to vary in different studies. Acke et al.[26] isolated *Campylobacter* spp. from both healthy and diarrhoeic animals at a rate of 45.2% in dogs and cats. In another study performed by the same researchers [32], the isolation rate of *Campylobacter* spp. was determined to be 42.9% and 41.5% in cats and dogs, respectively. Sandberg et al.[22] found campylobacters in 18% and 23% of healthy cats and dogs respectively. In the present study, the isolation rates of *Campylobacter* spp. were found to be 25.0%, 41.7% and 58.3% in diarrhoeic dogs by using the method 1, method 2 and method 3, respectively and healthy animals had 24.1% and 32.4% *Campylobacter* isolation rates with methods 1 and 2, respectively. Similar to other studies [22,32], it was found that dogs with diarrhoea were more likely to be carriers of campylobacters than healthy animals *(Table 2)*. In healthy cats, the isolation rates were 23.1% and 15.4% for method 1 and method 2, respectively. The isolation rates of campylobacters in this study were found to be different from the earlier studies, which can be attributed to several factors, such as isolation media and procedures employed, sample size, sampling time.

However, the detection rate of *Campylobacter* spp. in the 135 pets sampled was significantly increased using a combination of the 3 isolation methods in the current study leading to bacteria prevalence of 40.0% (48 positive cases) in dogs and of 26.7% (4 positive cases) in cats (*Table 2* and *3*). As the majority of *Campylobacter* spp. was

Table 3. *Isolation rates of thermophilic Campylobacter spp. by different methods in diarrhoeic (n = 2) or in healthy (n = 13) cats*
Tablo 3. *İshalli ve sağlıklı kedilerden termofilik Campylobacter türlerinin farklı metodlar ile izolasyon oranları*

Media Used	Healthy Cats (n = 13)	Diarrhoeic Cats (n = 2)	Total (n = 15)
Positive samples			
Medium 1	3 (23.1%)	1	4 (26.7%)
Medium 2	2 (15.4%)	0	2 (13.3%)
Medium 3	ND	0	ND
Total[1]	**3 (23.1%)**	**1**	**4 (26.7%)**
***Campylobacter* spp**			
C. jejuni			
Medium 1	3 (100%)	1	4
Medium 2	2 (100%)	0	2
Medium 3	ND	0	ND
Total[1]	**3**	**1**	**4**
C. coli			
Medium 1	0 (0%)	0	0
Medium 2	0 (0%)	0	0
Medium 3	ND	0	ND
Total[1]	**0**	**0**	**0**
C. lari			
Medium 1	0 (0%)	0	0
Medium 2	0 (0%)	0	0
Medium 3	ND	0	ND
Total[1]	**0**	**0**	**0**
C. upsaliensis			
Medium 1	0 (0%)	0	0
Medium 2	1 (50%)	0	1
Medium 3	ND	0	ND
Total[1]	**1**	**0**	**1**

Medium 1: *mCCDA (modified charcoal cefoperazone deoxycholate agar) with CCDA (cefoperazone, amphotericin B) selective supplement;* ***Medium 2:*** *mCCDA (modified charcoal cefoperazone deoxycholate agar) with CAT (cefoperazone, amphotericin and teicoplanin) selective supplement;* ***Medium 3:*** *Mueller-Hinton Agar supplemented with 5% defibrinated sheep blood (membrane filtration method);* ***ND:*** *not detected; [1] number of positive samples with at least one isolation method*

Table 4*. Agreement scores between the methods used for the isolation of Campylobacter spp. from rectal swab samples in healthy (108 dogs and 13 cats) and diarrhoeic (12 dogs and 2 cats) animals*
Tablo 4. *Sağlıklı ve İshalli hayvanların (12 köpek ve 2 kedi) rektal svap örneklerinden Campylobacter spp. izolasyonunda kullanılan metodlar arasındaki uyum değerleri*

Medium 2 and Aggrement Score	Medium 1																	
	Positive (n = 33)									Negative (n = 102)								
	Healthy			Diarrhoeic			Total			Healthy			Diarrhoeic			Total		
	Dogs	Cats	Dogs + Cats	Dogs	Cats	Dogs + Cats	Dogs	Cats	Dogs + Cats	Dogs	Cats	Dogs + Cats	Dogs	Cats	Dogs + Cats	Dogs	Cats	Dogs + Cats
Medium 2																		
Positive (n = 42)	20	2	22	3	0	3	23	2	25	15	0	15	2	0	2	17	0	17
Negative (n=93)	6	1	7	0	1	1	6	2	8	67	10	77	7	1	8	74	11	85
Agreement score																		
in dogs	20			3			23			67			7			74		
in cats		2			0			2			10			1			11	
Total			22			3			25			77			8			85

Medium 1: *mCCDA (modified charcoal cefoperazone deoxycholate agar) with CCDA (cefoperazone, amphotericin B) selective supplement;* ***Medium 2:*** *mCCDA (modified charcoal cefoperazone deoxycholate agar) with CAT (cefoperazone, amphotericin and teicoplanin) selective supplement; Agreement score: number of samples given a same result with the 2 methods*

recovered by direct plating onto mCCD agar medium with CAT supplement (method 2), this would be the method of choice if only a method was selected for detection of the most common *Campylobacter* spp. in pets. The findings of

this method used in this study are in agreement with those of previous studies for the isolation of *Campylobacter* [26,28,32]. Indeed, the overall agreement score between method 1 (mCCD agar medium with CCDA supplement) and method 2 (mCCD agar medium with CAT supplement) was relatively moderate in pets (81.5%) and the positive agreement score (number of samples given positive by the 2 methods) was quite low (50.0%), showing great variations in sensitivity between the 2 methods. As the membrane filtration method was used only in diarrhoeic animals, the number of cases was quite insufficient for determining agreement scores with the 2 other isolation methods.

C. jejuni was the most commonly isolated species from dogs and cats, and *C. upsaliensis* was the second most commonly isolated species in pets with all three method used in the current study. In contrary, it has been reported that *C. upsaliensis* was the predominant species in the recent studies [17,18,33].

Campylobacter spp. can be found as an opportunistic infectious agent in dogs and cats with gastrointestinal signs caused from endoparasites or parvovirus infection and they may act as a primary or secondary pathogen [32]. The link between the gastrointestinal symptoms and the presence of campylobacters in the gastrointestinal system has been studied but it remains obscure [29,32] and in the present study, the sampled animals were found to be negative for endoparasites or parvovirus infections.

Concurrent association between several *Campylobacter* species in dogs and cats has also been reported by other researchers [25,29,34-36]. Koene et al.[25] detected more than one *Campylobacter* species in six samples taken from healthy dogs. Similarly Hald et al.[37] reported that ten dogs were positive for concurrent infection with *Campylobacter* species. Similar findings have been reported by Workman et al.[29] for cat rectal samples. Such *Campylobacter* spp. associations were detected in five samples from pets in the present study, indicating that infections may be simultaneously caused by several bacterial species. In addition, different colony type of *Campylobacter* bacterium should be evaluated in one examined samples. Although *C. lari* was generally found in poultry intestinal system [38] some researchers reported that this bacterium was also encountered in dog intestines [25,32]. Similarly, *C. lari* was found in 2 healthy dog rectal swabs.

As a conclusion, the overall prevalence of *Campylobacter* spp. was 40.0% (48 cases) and 26.7% (4 cases) in dogs and cats, respectively. This study illustrates that dogs and cats carry a potential risk as possible reservoirs for human infections by these bacteria. The mCCD basal agar with CAT supplement (method 2) was found to be more appropriate and sensitive in detecting various species of *Campylobacter* in healthy dog rectal swab samples while in diarrhoeic animals, the membrane filtration was the most effective method for cultural detection.

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