JOURNAL OF CLINICAL MICROBIOLOGY, Feb. 2004, p. 819–821 Vol. 42, No. 2
0095-1137/04/$08.00+0 DOI: 10.1128/JCM.42.2.819–821.2004

Simultaneous Presence of Multiple *Campylobacter* Species in Dogs

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Received 9 June 2003/Returned for modification 18 August 2003/Accepted 11 November 2003

The prevalence of coinfection of *Campylobacter* species in dogs was determined using four isolation methods. In 26% of the positive-testing stools, multiple *Campylobacter* species were identified. The use of multiple isolation methods as well as the time lapse between sampling and processing are important for detection of coinfection.

In humans, *Campylobacter* is considered the most frequent bacterial cause of enteritis. The species *Campylobacter jejuni* is the prime etiologic agent, with minor contributions of *Campylobacter coli* and *Campylobacter lari* (1, 19). The role of *Campylobacter* as an enteric pathogen in dogs is much less evident. It is frequently isolated both from animals with symptoms of enteritis and from healthy animals (3, 9, 12, 16). The poor identification of *Campylobacter* species as animal pathogens may result from the simultaneous presence of multiple strains or species with various pathogenicity characteristics. In routine diagnostic laboratories, typing of *Campylobacter* is usually restricted to one colony per stool sample. In humans, simultaneous infection with more than one *Campylobacter* strain is found to be rare and is not considered to impair epidemiological analyses (15). With respect to dogs, however, no studies have been described that investigated the simultaneous presence of multiple species or strains. As the possibility to discriminate by colony morphology between *Campylobacter* species (13) or even between *Campylobacter* and *Helicobacter* species (6, 17) is limited, the prevalence of coinfection in companion animals could well be underestimated. Because the simultaneous presence of multiple strains or species is crucial to establish the role of *Campylobacter* in clinical disease as well as for epidemiological studies, we examined multiple colonies from a total of 30 fecal samples from diarrheic and nonsymptomatic dogs. We used a variety of culture media, as antimicrobials present in selective medium may selectively inhibit distinct *Campylobacter* species or strains. As *Campylobacter* is sensitive to oxygen and dryness, we also assessed the effect of the time interval between sampling and processing (transport time) on the isolation of *Campylobacter*.

Samples were obtained from September 2000 until February 2001 from household dogs of different ages (see Table 2) and were transported to the laboratory without cooling. The average sample size was about 25 g (minimum, 5 g). The isolation methods employed included a filtration method and three different selective media. Before processing the samples were homogenized and directly plated to the selective medium. In the filtration method, 10 to 12 drops of a fecal suspension in brain heart infusion were placed on a 0.65-μm-pore-size cellulose acetate filter (Sartorius, Goettingen, Germany) on blood agar base no. 2 (Oxoid) supplemented with 5% sheep blood. After 1 h of incubation at 37°C under aerobic conditions, the filter was removed and the plates were incubated microaerobically. We used as selective agar plates the following media: (i) modified-charcoal cefoperazone deoxycholate agar (mCCDA), a blood-free selective agar base supplemented with 32 μg of cefoperazone/ml and 10 μg of amphotericin/ml (Oxoid CM 739 with Oxoid supplement SR 155) which is widely used in medical, veterinary, and food microbiology laboratories for the isolation of *Campylobacter*; (ii) cefoperazone amphotericin teicoplanin selective medium (CAT), a blood-free charcoal based agar containing 8 μg of cefoperazone/ml, 4 μg of teicoplanin/ml, and 10 μg of amphotericin/ml (Oxoid supplement SR 174); and (iii) Karmali, a blood-free charcoal-based agar containing 32 μg of cefazolin/ml, 20 μg of vancomycin/ml, and 100 μg of cycloheximide/ml (Oxoid supplement SR 167). The latter two types of plates were used because they are recommended for the detection of *Campylobacter upsaliensis* and *Campylobacter helveticus* (2, 10). All plates were incubated at 37°C under microaerobic conditions in jars (Anoxomat, Mart, Lichtenvoorde, The Netherlands) (5% O_2, 10% CO_2, 85% H_2) and examined daily for growth for 4 to 6 days. From plates with growth of *Campylobacter* suspected (on the basis of colony morphology, catalase, oxidase, and Gram staining results), multiple subcultures of separate colonies (an average of 12 per sample) were grown on blood agar base no. 2 (Oxoid) supplemented with 5% sheep blood. The cultures were identified by PCR-restriction fragment length polymorphism with a method that distinguishes *Campylobacter*, *Arcobacter*, and *Helicobacter* by analysis of the 16S rRNA gene (11). *Campylobacter* species were identified by PCR-restriction fragment length polymorphism analysis of a highly polymorphic part of the 23S rRNA gene (5) and of the 16S rRNA gene (11). The patterns were compared with those of reference strains obtained from the CCUG/LMG (Culture Collection Universiteit of Ghent, Ghent, Belgium).

Efficiency of isolation. The use of different kinds of growth media increased the sensitivity of *Campylobacter* isolation (Table 1), as described before (4, 14). Overall, 23 (77%) of the samples were found positive for *Campylobacter* by one or more of the methods. Of the 23 positive-testing dog samples, 16 (70%) harbored *C. upsaliensis*, 12 (52%) harbored *C. jejuni*,

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TABLE 1. Incidence of *Campylobacter* species grown from dog feces as determined by four isolation protocols

Strain	No. (%) of fecal samples positive for *Campylobacter* (no. of samples tested = 30) by:				
	All four methods	Filter	mCCDA	CAT	Karmali
C. upsaliensis	16 (53)	10 (33)	15 (50)	10 (33)	12 (40)
C. jejuni	12 (40)	3 (10)	5 (17)	7 (23)	10 (33)
C. lari	3 (10)	0	0	2 (7)	1 (3)
Total	23 (77)	13 (43)	19 (63)	17 (57)	18 (60)

and 3 (13%) harbored *C. lari*. This distribution is consistent with other reports on dogs (3, 9, 12, 16).

Coinfection. By analysis of an average of 12 single colonies from each positive-testing sample, multiple *Campylobacter* species were observed in 6 (26%) of the 23 positive-testing stool samples (Table 2). In two samples, as many as three species could be isolated. No systematic study on coinfection with *Campylobacter* species in dogs has been reported before to our knowledge, although the presence of two *Campylobacter* species (7, 20) or serotypes (7) in a single sample has been mentioned. However, our results are comparable with those found in investigations of cats, for which it has been demonstrated that 34% of the *Campylobacter*-positive samples from healthy animals contained more than one *Campylobacter* species (17).

Healthy versus diarrheic animals. We examined fecal samples from diarrheic animals ($n = 8$) as well as from healthy animals ($n = 22$). Multiple *Campylobacter* species were detected only in samples from healthy animals (Table 2). No conclusions can be drawn, however, because of the limited number of samples and since most samples of diarrheic animals had a sampling-to-processing time that exceeded 4 h.

Media. The selective media showed comparable isolation rates, whereas the filtration method was less sensitive (Table 1), possibly because samples with low numbers of bacteria have been shown to give negative results (8, 18). The distribution of species isolated with the various methods is shown in Table 2. Our results do not confirm that CAT and Karmali media are better suited for detection of *C. upsaliensis*, notably, as we found a higher number of mCCDA plates positive for *C. upsaliensis* than resulted using the Karmali method, CAT method, and filtration method, which were specifically introduced for the detection of this *Campylobacter* species (2, 10). Although the filtration method is recommended for the detection of *Campylobacter* strains that might be inhibited by anti-

TABLE 2. *Campylobacter* species detected in fecal samples from dogs by filter, mCCDA, CAT, and Karmali

Group and animal	Method detecting[a]:			h between sampling and processing	Clinical status[b]	Age of animal
	C. upsaliensis	*C. jejuni*	*C. lari*			
Single species detected:						
1	a b d			2	N	9 mo
2	a b c d			2	N	3 yr
3	a b c d			4	N	7 mo
4	b c d			4	N	4 yr
5	a			36	D	3 mo
6	a b d			36	D	5 yr
7	a b c d			60	N	14 yr
8	b d			84	D	Adult[c]
9	b c			84	N	Juvenile[c]
10	a b c d			84	D	Adult[c]
11	a b c			84	N	Adult[c]
12		c		4	N	7 yr
13		a c d		4	N	8 yr
14		b c d		4	N	Adult[c]
15		a b c d		6	D	Adult[c]
16		b c		6	N	7 mo
17		a b d		108	N	Adult[c]
Two species detected:						
18	b	cd		4	N	2 yr
19	b c d	c d		4	N	4 yr
20	a b c d	b d		4	N	4 yr
21		d	d	4	N	2 yr
Three species detected:						
22	b d	d	c	4	N	3 yr
23	a b c d	d	c	4	N	10 yr

[a] Methods: a, filter; b, mCCDA; c, CAT; d, Karmali.
[b] N, healthy; D, diarrheic.
[c] Exact age not specified.

biotics present in selective media, we did not detect additional *Campylobacter* species using this method.

Karmali medium performed best in the detection of multiple species from single samples, as two species were recovered from 5 (28%) of the 18 positive-testing samples. The filter method failed to detect any coinfection. As expected, the chance of detecting coinfection was increased by the use of multiple isolation methods.

Transport time. An influence of the time interval between sampling and processing of the sample in the laboratory was observed, as coinfection was detected only in samples processed within 4 h after collection (Table 2). Some samples yielded a single species even after 3 days, on the other hand, indicating a considerable variation in the survival times of campylobacters between samples and/or differences in the viability of *Campylobacter* species.

In conclusion, despite a relatively low number of samples our data clearly show that coinfections of different *Campylobacter* species are quite common in dogs. The possibility cannot be excluded that the diversity might be even more extensive, as different strains might exist within a single species. This notion is important for epidemiological studies, e.g., the tracing of sources of human infections and studies on the pathogenicity of *Campylobacter* in dogs.

For studying *Campylobacter* infections in dogs and cats multiple colonies should be examined, preferably from a combination of media and from specimens that are processed within 4 h after sampling.

The help of the technicians from the Veterinary Microbiological Diagnostic Center of the Veterinary Faculty is gratefully acknowledged. We also thank Alan Rigter for the helpful assistance in the speciation of the strains and Jos van Putten for his critical reading of the manuscript.

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