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Short communication

# Strain variation within *Campylobacter* species in fecal samples from dogs and cats

Miriam G.J. Koene [a,c,d,*], Dirk J. Houwers [a], Jeroen R. Dijkstra [b,1], Birgitta Duim [b,2], Jaap A. Wagenaar [a,b,c,d]

[a] *Department of Infectious Diseases and Immunology, Faculty of Veterinary Medicine, Utrecht University, P.O. Box 80165, 3508 TD Utrecht, The Netherlands*
[b] *Central Veterinary Institute of Wageningen UR, P.O. Box 65, 8200 AB Lelystad, The Netherlands*
[c] *OIE Reference Laboratory for Campylobacteriosis, The Netherlands*
[d] *WHO Collaborating Centre for Campylobacter, The Netherlands*

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**Abstract**

To investigate the incidence of co-colonization of different strains of *Campylobacter* species present in canine and feline stool samples, isolates were recovered by culture from 40 samples from dogs ($n = 34$) and cats ($n = 6$). Animals were of different ages, with diarrhoea or without clinical signs. Three isolation procedures were used: two selective agars and a filtration method. In each stool sample, multiple colonies were identified to the species level by PCR, subsequently genotyped by Amplified Fragment Length Polymorphism (AFLP) and pattern similarities (451 isolates) were calculated to investigate their phylogenetic relationships.

Genetic heterogeneity of strains in individual stool samples was detected within the species *Campylobacter jejuni*, *C. upsaliensis* and *C. helveticus*, though to a different degree in dogs and cats. In 3 of the 34 (9%) canine samples, more than one genotype of the same *Campylobacter* species was present, while strain variation was detected in four of the six feline samples. The results show that preferably, multiple colonies should be analyzed in molecular epidemiological and aetiological studies.


*Keywords:* *Campylobacter jejuni*; *Campylobacter upsaliensis*; *Campylobacter helveticus*; Co-colonization; AFLP; Strain variation; Dogs; Cats

* Corresponding author. Present address: Central Veterinary Institute of Wageningen UR, P.O. Box 65, 8200 AB Lelystad, The Netherlands. Tel.: +31 320 238425; fax: +31 320 238961.
*E-mail address:* miriam.koene@wur.nl (M.G.J. Koene).
[1] Present address: Department of Pathology, Radboud University Nijmegen Medical Centre, Nijmegen, The Netherlands.
[2] Present address: Academic Medical Centre, Department of Medical Microbiology, Amsterdam, The Netherlands.

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## 1. Introduction

Most *Campylobacter* species, like *C. jejuni*, *C. coli*, *C. lari* and *C. upsaliensis*, show a genetically diverse population. Natural transformability and intragenomic DNA rearrangements contribute to this diversity (Boer et al., 2002). Co-colonization of multiple genotypes of a *Campylobacter* species has been shown to occur in humans, pigs and poultry (Richardson et al., 2001; Schouls et al., 2003; Weijtens et al., 1999) and is thought to facilitate DNA exchange and therewith increase the genetic diversity.

In both dogs and cats, long-term colonization of *Campylobacter* (Bender et al., 2005; Hald et al., 2004) and co-colonization of distinct *Campylobacter* species (Koene et al., 2004; Shen et al., 2001) have been described. However, data on the incidence and extent of genotypic variation of *Campylobacter* species in companion animals are currently lacking.

Such information is important for epidemiological purposes and could help in assessing the role of *Campylobacter* as a pathogen in these animals. *Campylobacter* is regarded as a possible cause of diarrhoea in dogs and cats, although prevalence studies, experimental infections and response to antibiotic therapy have been inconclusive (Fox et al., 1983; Hald and Madsen, 1997; Sandberg et al., 2002; Weber et al., 1983a,b). In addition to its clinical relevance, the presence of *Campylobacter* in companion animals is of interest in view of public health. Contact with pet animals has been shown to be a risk factor for *Campylobacter* infections in humans and actual transmission has been demonstrated (Wolfs et al., 2001).

The presence of multiple strains of a *Campylobacter* species in a host should be taken in consideration when performing molecular epidemiological and aetiological studies. The aim of this survey is to study the extent of co-colonization of different genotypes of *Campylobacter* in stool samples from dogs and cats by using Amplified Fragment Length Polymorphism (AFLP).

## 2. Material and methods

A total of 40 *Campylobacter*-culture positive fecal samples from dogs ($n = 34$) and cats ($n = 6$) were examined that were either submitted for routine bacteriological analysis (diarrhoeic animals), samples taken from dogs in a boarding kennel on their day of arrival, and from a veterinary practice (healthy animals). Age distribution of animals was as follows: 22 dogs (65%) were adult, 12 dogs (35%) were younger than 1 year. Cat samples were obtained from one adult, three juveniles, and two for which no information about their age was provided. Diarrhoea was reported in 16 dogs (47%) and 5 cats (83%). The remaining animals had no clinical signs reported by their owner.

Samples, with average size of about 25 g (minimum, 5 g), were transported to the laboratory without transport medium or cooling and were collected over a period of 2 years (August 1999–July 2001). The time interval between sampling and processing of the samples ranged from 2 h to several days (Tables 1a and 1b). Species identification of *Campylobacter* isolates in some of these samples was reported previously (Koene et al., 2004), and these samples are indicated in Table 1a.

Three procedures for the isolation of *Campylobacter* were used, (i) modified charcoal cefoperazone deoxycholate agar (mCCDA), a blood-free selective agar base supplemented with 32 μg/ml cefoperazone and 10 μg/ml amphotericin (Oxoid CM 739 with Oxoid supplement SR 155), (ii) Karmali, a blood-free charcoal based agar containing 32 μg/ml cefazolin, 20 μg/ml vancomycin, and 100 μg/ml cycloheximide (Oxoid supplement SR 167), and (iii) a filtration method, for which 10–12 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. All plates were incubated in jars under microaerobic conditions (6% $O_2$, 7% $CO_2$, 7% $H_2$ and 80% $N_2$) (Mart, Anoxomat, Lichtenvoorde, The Netherlands) at 37 °C for up to 6 days. From plates showing growth suspect of *Campylobacter* (colony morphology and Gram stain), multiple separate colonies (2–33), depending on the number of colonies found in the primary isolation, were subcultured on blood agar base no. 2 (Oxoid) supplemented with 5% sheep blood. These isolates were stored as glycerol stocks at −80 °C until further analysis.

Table 1a
Genotyping results by AFLP from *Campylobacter* positive samples from dogs

| Sample ID | No. of isolates tested | AFLP-type isolated (number of isolates) | | | | Time interval sampling-processing (h) | Diarrhoea | Age |
|---|---|---|---|---|---|---|---|---|
| | | *C. upsaliensis* | *C. jejuni* | *C. lari* | *C. hyointestinalis* | | | |
| P1 | 11 | U21 (3), U22 (1), U25 (6) | | | Hy1 (1) | 8 | No | 9 months |
| N3[a] | 11 | U1 (6), U2 (1) | J11 (3) | L2 (1) | | 4 | No | 3 years |
| N5[a] | 18 | U15 (14) | J11 (3) | L1 (1) | | 4 | No | 10 years |
| N4[a] | 10 | U18 (1) | J11 (9) | | | 4 | No | 2 years |
| N9[a] | 23 | U4 (18) | J2 (5) | | | 4 | No | 4 years |
| N8[a] | 19 | U16 (19) | | | | 4 | No | 4 years |
| N10[a] | 18 | U7 (18) | | | | 4 | No | 7 months |
| N11[a] | 7 | U8 (7) | | | | 2 | No | 9 months |
| N12[a] | 9 | U17 (9) | | | | 2 | No | 3 years |
| P2 | 12 | U20 (12) | | | | 8 | No | 9 months |
| D2 | 27 | U19 (27) | | | | 12 | Yes | 3 years |
| D4 | 13 | U14 (13) | | | | 36 | Yes | 4 months |
| D9 | 17 | U9 (17) | | | | 36 | Yes | 1 year |
| D10 | 2 | U10 (2) | | | | 36 | Yes | 3 years |
| D11 | 12 | U11 (12) | | | | 12 | Yes | 3 months |
| D13 | 33 | U24 (33) | | | | 36 | Yes | 6 months |
| D15[a] | 5 | U13 (5) | | | | 36 | Yes | 5 years |
| D16[a] | 3 | U23 (3) | | | | 36 | Yes | 3 months |
| G57[a] | 9 | U12 (9) | | | | 60 | No | Adult |
| G61[a] | 4 | U5 (4) | | | | 108 | Yes | Adult |
| G70[a] | 6 | U6 (6) | | | | 60 | No | Adult |
| D12 | 9 | | J3 (8), J10 (1) | | | 36 | Yes | 5 months |
| N2[a] | 2 | | J16 (2) | | | 4 | No | 7 years |
| N7[a] | 8 | | J11 (8) | | | 4 | No | 4 years |
| N15[a] | 2 | | J1 (2) | | | 6 | No | 7 months |
| D3 | 2 | | J7 (2) | | | 60 | Yes | 10 years |
| D6 | 12 | | J5 (12) | | | 84 | Yes | 2 months |
| D7 | 21 | | J6 (21) | | | 12 | Yes | 1 year |
| D8 | 24 | | J8 (24) | | | 12 | Yes | 1 year |
| D14 | 4 | | J11 (4) | | | 84 | Yes | 6 months |
| D17[a] | 8 | | J1 (8) | | | 6 | Yes | Adult |
| G50-1 | 6 | | J4 (6) | | | 60 | No | Adult |
| G48[a] | 7 | | J12 (7) | | | 108 | No | Adult |
| N1[a] | 2 | | | L1 (2) | | 4 | No | 2 years |

[a] Speciation of the isolates by PCR is described in Koene et al. (2004).

Table 1b
Genotyping results by AFLP from *Campylobacter* positive samples from cats

| Sample ID | No. of isolates tested | AFLP-type isolated (number of isolates) | | | Time interval sampling-processing (h) | Diarrhoea | Age |
|---|---|---|---|---|---|---|---|
| | | *C. upsaliensis* | *C. jejuni* | *C. helveticus* | | | |
| G44-1 | 6 | U3 (6) | | | 48 | No | Adult |
| K1 | 21 | | J13 (1), J14 (20) | | 36 | Yes | 6 weeks |
| K2 | 13 | | J3 (11), J9 (2) | | 12 | Yes | Not reported |
| K7 | 21 | | J15 (21) | | 36 | Yes | 3 months |
| K3 | 2 | | | He1 (1), He4 (1) | 12 | Yes | Not reported |
| K5 | 12 | | | He2 (1), He3 (11) | 12 | Yes | 3 months |

Chromosomal DNA was isolated using a commercial kit (Promega, Madison, WI, USA) and DNA concentrations were estimated by agarose gel electrophoresis.

Species were identified through PCR-Restriction Fragment Length Polymorphism analysis, using two methods. PCR-RFLP of the 16S rRNA (Marshall et al., 1999) identified *C. helveticus*, *C. hyointestinalis*, *C. lari* and *C. upsaliensis*. *C. jejuni* was identified by amplifying a highly polymorphic part of the 23S rRNA gene and subsequently digesting the PCR product with two restriction enzymes, AluI and Tsp509I (Fermer and Engvall, 1999).

*Campylobacter* isolates were genotyped using Amplified Fragment Length Polymorphism analysis as described by Duim et al. (1999). After electrophoresis, the AFLP patterns were recorded and evaluated with Gene Scan software (PE Applied Bio systems). Densitometric values were transferred to the Bionumerics 3.5 software (Applied Maths, Sint-Martens-Latem, Belgium), and gels were normalized using an internal size standard that was added to each lane. A similarity matrix was created by determining the Pearson product moment correlation coefficient ($r$). The unweighted pair group method using average (UPGMA) linkage was used to cluster the patterns. A cut-off level of 90% homology was used to discriminate different genotypes.

## 3. Results

Statistical analysis of the AFLP results of 451 isolates from 40 *Campylobacter* positive stools (34 dogs and 6 cats) showed the different *Campylobacter* species forming distinct clades correlating with the patterns from reference strains and this was confirmed by the results of the species identification by PCR-RFLP (data not shown).

Of the 34 examined dogs, three were shown to be colonized with more than one *Campylobacter* genotype per species while this was found in four of the six cats. In samples with multiple genotypes, next to a predominant genotype, other genotypes of the same *Campylobacter* species were present at a lower frequency as identified by AFLP. In canine samples, *C. upsaliensis* was represented by three AFLP genotypes in sample P1 and by two genotypes in sample N3. *C. jejuni* showed two different genotypes in one sample (D12) (Table 1a). In four of the six stool samples from cats, strains of distinct *Campylobacter* species showed variation in genetic fingerprints. This involved *C. jejuni* (K1, K2) and *C. helveticus* (K3, K5) (Table 1b).

A total of 48 unique AFLP genotypes (showing less than 90% homology) were recovered as shown in Fig. 1, represented by *C. upsaliensis* (U1–U25), *C. jejuni* (J1–J16), *C. lari* (L1–L2), *C. helveticus* (He1–He4) and *C. hyointestinalis* (Hy1). Occasionally, isolates with identical or closely related AFLP patterns (90–100% homology) were isolated from epidemiological unrelated animals as shown in Tables 1a and 1b. This included three *C. jejuni* genotypes (J1, isolated from sample N15 and sample D17; J3, isolated from samples D12 and K2; and J11, from samples N3–N5 and D14) and one *C. lari* genotype (L1, isolated from samples N5 and N1).

The use of multiple isolation methods improved the detection of genetic diversity. In samples with multiple genotypes, variants were distributed over different media, rather than being limited to a single method (data not shown).

No correlation was demonstrated between the occurrence of strain variation and clinical status, age of the host or the time interval between sampling and processing of the samples.

## 4. Discussion

We isolated 48 unique *Campylobacter* genotypes from 40 stool samples (Fig. 1). Analysis of the genotypic results indicated that the *Campylobacter* population in the studied dogs and cats is genetically highly diverse, which is in agreement with studies performed elsewhere (Hald et al., 2004; Moser et al., 2001; Shen et al., 2001; Stanley et al., 1994).

Four homologous *Campylobacter* genotypes were isolated from multiple samples; *C. lari* genotype 1 (L1) was isolated from two epidemiologically unrelated, clinically healthy dogs. In three cases, homologous genotypes of *C. jejuni* (J1, J3 and J11) were isolated from epidemiologically unrelated samples. J1 and J11 were isolated from both diarrhoeic and healthy dogs, while J3 was cultured from two clinical samples from a dog and a cat.

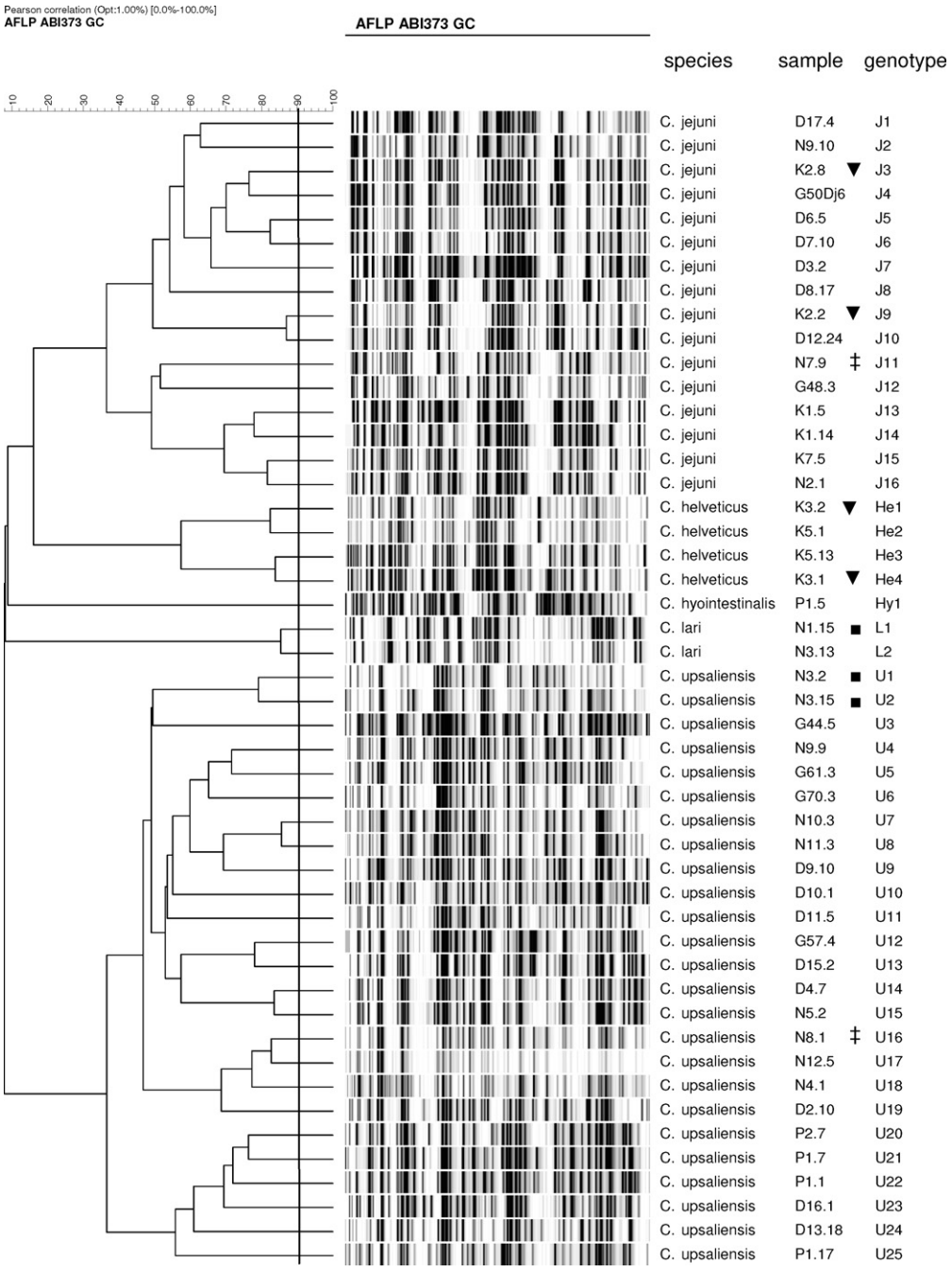


Fig. 1. Representative AFLP patterns of *Campylobacter* species from individual samples isolated from fecal samples of dogs and cats. The black line indicates the cut-off level (less than 90% genetic similarity) for pattern differentiation. Epidemiologically related animals – by sharing the same household – are indicated by symbols (■, ▼, ‡).

Interestingly, in three cases of animals sharing the same household, each individual animal shed *Campylobacter* with distinct genotypic patterns, or even different *Campylobacter* species (samples N1 + N3, N7 + N8 and K2 + K3) as is shown in Fig. 1. This may indicate that either no animal to animal transmission of strains occurs or that strains alter during prolonged colonization in different individuals.

The genetically diverse population shown by most *Campylobacter* species like *C. jejuni*, *C. coli*, *C. lari* and *C. upsaliensis* is affected by the occurrence of natural transformation and intragenomic rearrangements. In the present study, in 3 of the 34 (9%) canine samples, more than one genotype of the same *Campylobacter* species was present, while strain variation was detected in four of the six feline samples. Co-colonization of strains within one host may lead to an increase in the genetic diversity if DNA exchange takes place and new chimeric strains can develop. Both in humans, and in animals like chickens (Boer et al., 2002; Hanninen et al., 1999), pigs (Weijtens et al., 1999) and dogs (Hald et al., 2004) time related differences in molecular fingerprints have been described.

It has been shown that host species differ with regard to strain variation within a single host. Mixed infections of *Campylobacter* in human patients are considered unusual (Richardson et al., 2001; Steinbrueckner et al., 2001) in contrast to the situation in poultry and pigs (Camarda et al., 2000; Jensen et al., 2006; Schouls et al., 2003; Weijtens et al., 1999). However, the mechanisms behind these differences in host–microbe interaction remain largely unidentified.

We found no obvious correlation between clinical status and the shedding of multiple strains of *Campylobacter*. Little data is available on the nature and duration of immunity after *Campylobacter* colonization in pets, but long-term infection and reinfection with different strains without clinical symptoms (Hald et al., 2004; Stanley et al., 1994) in dogs and in cats (Bender et al., 2005) indicates a lack of protective immunity.

Although the number of positive cats in this study is limited, the results suggest that *Campylobacter* strains in feline fecal samples possess a higher degree of genetic diversity than those found in dogs, as strain variation was detected in four of the six samples whereas in canine samples *Campylobacter* species were generally represented by a single genotype (Tables 1a and 1b). This difference cannot be explained by technical factors such as differences in time interval between sampling and processing of samples or age of the animals. It may indicate that cats are subject to a higher infection pressure than dogs or that there may be differences in host immune responses or colonization dynamics. Due to the limited number of samples, this warrants further investigation.

The detection of genetic diversity was enhanced by the use of multiple isolation methods, but none of the employed methods was superior in detecting different species or genotypes. For routine diagnosis the use of multiple media is not practical and unnecessarily expensive. However, for specific epidemiological studies, the use of multiple media is strongly recommended.

## 5. Conclusions

We conclude that genetic diversity among isolates of *C. jejuni* and *C. upsaliensis* within one host occurs, and that the degree may differ in dogs and cats. In all cases in which strain diversity was detected, one genotype dominated, which means that co-colonization can be missed even if multiple colonies are analyzed. This may frustrate epidemiological, aetiological and pathogenetic studies of *Campylobacter* in dogs and cats, particularly in relation to zoonotic issues.

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