Genomic Heterogeneity and O-Antigenic Diversity of *Campylobacter upsaliensis* and *Campylobacter helveticus* Strains Isolated from Dogs and Cats in Germany

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A serotyping scheme based on heat-stable surface antigens was established for 101 *Campylobacter upsaliensis* and 10 *Campylobacter helveticus* strains isolated from 261 dogs and 46 cats of different ages originating from two geographically distinct regions in Germany. The prevalence of *C. upsaliensis* varied between 27.8% in juvenile dogs (<12 months of age) and 55.4% in adult dogs ($P < 0.05$). Of the cats, 19.6% harbored *C. upsaliensis*, whereas 21.7% carried *C. helveticus*. Of the *C. upsaliensis* isolates from both host species, 93.1% belonged to five different serogroups, two of them being prevalent at rates of 47.5 and 27.7%, with different frequencies in both regions. Six (54.6%) of the *C. helveticus* isolates also belonged to serotypes found among *C. upsaliensis* strains, whereas five (45.4%) possessed an O antigen unique for *C. helveticus*. In contrast, a considerable degree of genomic diversity of the isolates was assessed by macrorestriction analyses with the endonucleases *Sma*I and *Xho*I, using pulsed-field gel electrophoresis as well as enterobacterial repetitive intergenic consensus sequence PCR (ERIC PCR). Restriction with *Sma*I pointed towards the existence of clonal groups associated to some extent with serotypes, while restriction with *Xho*I disintegrated these groups to smaller noncoherent subgroups. Analysis of ERIC PCR profiles did not exhibit any associations with serotypes. In conclusion these data demonstrate the genomic heterogeneity among *C. upsaliensis* strains and indicate that the combination of *Sma*I restriction with serotyping is a useful tool to investigate the expansion of clonal groups of *C. upsaliensis*.

Campylobacter upsaliensis, a catalase-negative or weakly positive thermotolerant *Campylobacter* species, was first isolated in 1983 from fecal samples of healthy and diarrheic dogs in Sweden (48). In cats this microorganism was identified a few years later, in 1989 (9). *C. upsaliensis* is also sporadically isolated from procedures done on humans or from human diseases, like abortion (16), bacteremia (39), abscess (10), gastroenteritis (11, 12, 13, 26, 52), and opportunistic infections in immunocompromised hosts (23, 43). However, its genetic characteristics and its possible pathogenic capacity for humans and small animals are far from well defined (5). According to some risk analyses, living with a dog or cat as companion is a considerable risk factor for men (14). Carrier rates of dogs and cats for *C. upsaliensis* between 5 and 66.2% have been reported by other investigators from different countries (3, 6, 7, 17, 30, 34, 48), with significant correlation between *Campylobacter* shedding and age in young diarrheic dogs reported by Burnens et al. (6). To gain further knowledge on the prevalence of this enteric pathogen in pets in Germany, its association to enteric disease, and its genomic diversity, *C. upsaliensis* was isolated from dogs and cats living in two distinct geographic areas in Germany over a period of approximately 1 year (November 1997 to January 1999). Fecal specimens of 261 dogs and 46 cats of different ages (healthy or suffering from enteric disease or

other illness) were presented to two veterinary hospitals and were collected. The two areas of investigation were chosen by their characteristics as metropolitan (Berlin) and a rather provincial place 400 km away (situated in North Rhine-Westphalia [NRW]). With regard to previous studies on the closely related species *Campylobacter helveticus* occurring in cats, which was described for the first time in 1992 (49), this species was included in our investigation.

Genotypic (molecular) methods have successfully been applied to accomplish phenotypic approaches for subtyping *Campylobacter* species (44). Previous reports using pulsed-field gel electrophoresis (PFGE) to characterize bacteria, including *Campylobacter jejuni* (40, 59), *C. upsaliensis* (4), and *Campylobacter hyointestinalis* (47), on the genomic level demonstrated the high discriminatory power of this method and its usefulness for epidemiological studies. Flagellin gene polymorphism (32, 33, 38, 50) or the combination of macrorestriction analyis and serotyping according to heat-stable or heat-labile antigens has also been performed to discriminate *C. jejuni* and *Campylobacter coli* strains from each other (40, 50). In the present study *C. upsaliensis* isolates were characterized with respect to their species by biochemical tests confirmed by PCR. Their heat-stable antigens were determined using indirect hemagglutination (45) for the first time, to our knowledge, and the degree of their genotypic similarities was assessed by analyzing macrorestriction patterns and enterobacterial repetitive intergenic consensus sequence PCR (ERIC PCR) profiles (53).

The aim of this study was to extend knowledge of the relationship between genomic and O-antigenic diversity of the species *C. upsaliensis* and of the mode of expansion of this

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TABLE 1. Bacterial strains used for antiserum preparation

Bacterial strain	Species	Serotype	Origin; characteristics
10935	*C. jejuni*	O:1	CCUG; reference strain
10936	*C. jejuni*	O:2	CCUG; reference strain
10938	*C. jejuni*	O:4	CCUG; reference strain
10945	*C. jejuni*	O:13	CCUG; reference strain
	C. jejuni	O:16	CCUG; reference strain
	C. jejuni	O:43	CCUG; reference strain
L2H	*C. jejuni*	O:37	Germany; wild-type strain; species origin unknown
1834	*C. jejuni*	O:40	Germany; wild-type strain; human origin
TU 429	*C. coli*	O:20	Pig
TU 440	*C. coli*	O:n.t.[a]	Pig
C. lari 68	*C. lari*		Chicken
Ulrike 3	*C. upsaliensis*		Berlin; dog
H5	*C. upsaliensis*		Berlin; dog
H16	*C. upsaliensis*		Berlin; dog
H29	*C. upsaliensis*		Berlin; dog
K1E	*C. helveticus*		NRW; cat

[a] n.t., not typeable.

potential human enteric pathogen carried by animal hosts, especially dogs.

MATERIALS AND METHODS

Collection and cultivation of bacteria. Rectal swabs were collected from 261 randomly selected dogs and 46 cats of different ages with and without gastroenteritic symptoms that were presented to two veterinary hospitals. The specimens were collected using Culturettes (Becton Dickinson) and transferred onto selective media within 24 h after collection. As selective media, cefoperazon-amphotericin β-teichoplanin agar (2), modified *Campylobacter* charcoal differential agar (21), and charcoal-based selective medium (24) were used. The plate contents were incubated for 48 to 72 h under microaerophilic conditions (5%O_2, 10% CO_2, and 85% N_2) at 38°C.

Further cultivation was performed on Mueller-Hinton agar plates containing 5% defibrinated sheep blood (MHB). The bacteria were stored as stock cultures in thioglycolate broth containing 15% glycerol at −70°C.

Species determination. The species of *C. upsaliensis* and *C. helveticus* isolates were determined biochemically according to the literature (20, 22, 35, 36) using the following criteria: gram negativity, spiral shape of rods, requirement of microaerophilic growth conditions, cytochrome oxidase activity, weak or no catalase activity, capacity to reduce selenite, and sensitivity to nalidixic acid and to cephalotin. The results were confirmed using species-specific PCR established for *C. upsaliensis* (8) and *C. helveticus* (27).

Indirect hemagglutination. Sheep erythrocytes were sensitized with bacterial antigen extracted by heat (45). Briefly, bacteria harvested from MHB agar plates were suspended in 150 mM NaCl, adjusted to an optical density at 600 nm (OD_{600}) of 1, and heated at 100°C for 1 h. After centrifugation at 6,000 × *g*, the antigen-containing supernatant was collected and sheep erythrocytes that had been washed with 150 mM NaCl were added to the supernatant: 5 µl of erythrocyte sediment was added to 1 ml of antigen solution, resulting in an OD_{540} of 3.2 to 3.4.

The erythrocyte-antigen mix was incubated at 37°C for 2 h, washed three times with 150 mM NaCl, and resuspended with the original volume of 150 mM NaCl.

The hemagglutination was performed with rabbit antisera elicited as described previously (31) against formaldehyde-inactivated bacteria of *C. jejuni* reference strains O:1, O:2, O:4, O:9, O:13, O:16, and O:43 (Penner); *C. jejuni* wild-type strains O:37 and O:40; two *C. coli* wild-type strains (O:20 and one that was not typeable); one *Campylobacter lari* strain; four wild-type *C. upsaliensis* strains; and one *C. helveticus* wild-type strain (Table 1). The *C. jejuni* reference strains were purchased from the Culture Collection of the University of Göteborg (CCUG), and the *C. jejuni* and *C. coli* wild-type strains were serotyped at the same place. The *C. upsaliensis* and *C. helveticus* strains were not typeable, because an O-antigen serotyping scheme did not exist for them.

The antisera were complement inactivated (30 min, 56°C) and absorbed with washed sheep red blood cells. For hemagglutination the sera were diluted serially in microtiter plates, using 150 mM NaCl (25 µl); the same volume of sensitized erythrocyte suspension was added and incubated for 2 h at 37°C. The titers were

determined macroscopically. Serum titers of 1:80 and less were neglected. In Table 1 the bacterial strains used for antiserum production are listed.

DNA preparation, DNA primers, and PCR amplification. For PCR amplification DNA was extracted by heat. Briefly, bacteria suspended in 150 mM NaCl were adjusted to an OD_{600} of 1.5 and were diluted 1:4 in H_2O before heating at 100°C for 5 min. The supernatant collected after centrifugation (5,700 × *g*) was used as template DNA solution.

The amplification reaction was performed using Ready-to-Go PCR Beads (Amersham Pharmacia Biotech, Freiburg, Germany) in a volume of 25 µl, containing finally 1 µl of DNA, 1.5 U of *Taq* polymerase, 10 mmol liter^{-1} Tris-HCl (pH 9.0), 50 mmol liter^{-1} KCl, 1.5 mmol liter^{-1} MgCl$_2$, 200 µmol liter^{-1} concentrations of each dNTP, and 5 µM concentrations of each primer. Primer sequences were deduced from 23S rRNA genes for thermophilic *Campylobacter* species (8) and *C. upsaliensis* (8) and from 16S rRNA genes for *C. upsaliensis* and *C. helveticus* (27). Primers were synthesized by Amersham Pharmacia Biotech (now MWG Biotech, Ebersberg, Germany). Samples were subjected to 27 cycles of amplification in a DNA thermal cycler (Biometra, Göttingen, Germany) under conditions described by Eyers et al. (8) and Linton et al. (27) with slight modifications. Each cycle consisted of 2 min at 94°C, 1 min at 94°C, 1 min at 52 to 54°C, and 1 min at 74°C. Final elongation was performed at 74°C for 180 s. Amplified samples were analyzed by electrophoresis on 1.2% agarose gels and were visualized by ethidium bromide staining under UV light. PCR product sequence analysis was performed by Gessellschaft für Molekularbiologische Technologie, (Berlin, Germany).

The ERIC PCR was performed in a volume of 25 µl in 0.5-ml tubes with oil overlaying. The PCR mix consisted of 10 µl of bacterial genomic DNA extracted by heat, 2.5 µl of 10× PCR buffer, 3 mM MgCl$_2$, a 25 mM concentration of each dNTP, and 50 pmol of each primer (ERIC I/ERIC II) according to Versalowic et al. (53). Samples were subjected to 95°C for 7 min followed by 30 cycles of amplification in a DNA thermal cycler (model 480; Perkin-Elmer) using the following conditions: 94°C, 1 min; 52°C, 1 min; and 72°C, 8 min. PCR products were separated by electrophoresis using precast gels [6% Poly(Nat), Elchrom Scientific AG, Weiterstadt, Germany] in a special electrophoresis apparatus with buffer recirculation (SEA 2000; Elchrom Scientific AG) at 10°C and were stained with ethidium bromide. Gels were photographed with a digital camera system (Herolab, Wiesloch, Germany). The electrophoretic patterns were analyzed using Gelcompar 4.1 Software (Applied Maths BVBA, Kortrijk, Begium; Herolab). Genetic similarities between isolates were calculated using the Ward algorithm and the Pearson correlation coefficient (for details see the Gelcompar 4.1 manual).

Macrorestriction analysis using PFGE. Bacteria grown on MHB agar plates for 48 h were harvested in 150 mM NaCl. The suspension was adjusted photometrically to an OD_{600} of 1.2. Agar plugs were prepared by adding 500 µl of bacterial suspension to 700 µl of 1.2% agarose DNA-grade gels (Life Technologies, Kartsruhe, Germany), mixing thoroughly, and filling 100 µl in the plug mould. The solidified agarose plugs were incubated in ESP lysis buffer (0.5 mM EDTA, pH 9.5; 1% [wt/vol)] *N*-lauroyl sarcosine; and 1.8 mg of proteinase K [Roche Diagnostics, Mannheim, Germany]/ml) for 36 h at 56°C. To prepare samples for restriction endonuclease digestion, the plugs were cut into three pieces of equal size and were washed extensively in Tris-EDTA buffer (10 mM Tris and 10 mM EDTA, pH 7.5) at 4°C. Then the plug pieces were equilibrated twice with the appropriate digestion buffer at room temperature for 30 min and were incubated in 150-µl digestion buffer containing 20 U of the restriction endonuclease *Xho*I or *Sma*I overnight at temperatures recommended by the manufacturer (Roche Diagnostics). Electrophoretic separation of the DNA fragments in 1.2% (wt/vol) agarose gels was performed in a contour-clamped homogeneous electric field (CHEF DR III; Bio-Rad, Munich, Germany) apparatus under the following conditions: 6 V cm^{-1}, pulse times ramping from 0.3 to 12 s for both enzymes, electrode angle of 120°, and a temperature of 15°C for 24 h. The running buffer contained 0.5× Tris-borate-EDTA (44.5 mM Tris; 44.5 mM boric acid; 1 mM EDTA, pH 8.0). As reference, DNA of the *C. upsaliensis* strain DSM 5365 digested with *Sma*I or *Xho*I was run on each gel. Finally gels were stained with ethidium bromide, viewed under UV light, and documented on Polaroid films. Photographs were scanned, and similarities between profiles, based on band positions, were calculated with Gelcompar 4.1 software (Applied Maths BVBA) using the Ward algorithm and the Dice coefficient (for details, see Gelcompar 4.1 manual) with a maximum tolerance of 1.8% and optimization of 0.5% for both enzymes. Using these parameters, the reproducibility of band profiles generated from eight duplicate strains restricted with *Sma*I was ≥95.5%; that of six duplicate strains restricted with *Xho*I was ≥95.8%. As molecular weight standard, λ concatemers (molecular size, 48.5 kb; Roche Diagnostics) were run on three lanes (both edges and the middle) of each gel.

Statistical calculations. In spite of the fact that the mode of sample collection in a nonrandom manner does not permit calculations of true statistical signifi-

TABLE 2. Prevalence of *Campylobacter* with emphasis on *C. upsaliensis* in dogs and cats in Berlin and NRW, respectively

Host species/geographic origin (illness of host)	Age (no.)	Total no. of animals (n = 307)	Results for investigated samples		
			No. *Campylobacter* positive (%) (n = 131)	No. *C. upsaliensis* positive (%) (n = 97)	No. *C. helveticus* positive (%) (n = 11)
Dog/Berlin (enteritis)	Adult (35)	50	14 (40.0)	13 (37.1)	1
	Juvenile (15)		11 (73.3)	7 (46.6)	
	Total		25 (50.0)	20 (40.0)	1
Dog/Berlin (without enteritis)	Adult (74)	85	19 (25.7)	18 (24.3)	
	Juvenile (11)		7 (63.6)	5 (45.5)	
	Total		26 (30.6)	23 (27.1)	0
Dog/NRW (enteritis)	Adult (9)	14	4 (44.4)	2 (22.2)	
	Juvenile (5)		3 (60.0)	2 (40.0)	
	Total		7 (50.0)	4 (28.6)	0
Dog/NRW (without enteritis)	Adult (87)	112	30 (34.5)	24 (27.6)	
	Juvenile (25)		21 (84.0)	17 (68.0)	
	Total		51 (45.5)	41 (36.6)	0
Cat/Berlin (enteritis)	Adult (8)	12	3 (37.5)	1 (12.5)	1
	Juvenile (4)		2 (50.0)		
	Total		5 (41.6)	1 (8.3)	1
Cat/Berlin (without enteritis)	Adult (7)	9	2 (28.6)		
	Juvenile (2)		2 (100.0)	2 (100.0)	2
	Total		4 (44.4)	2 (22.2)	2
Cat/NRW (enteritis)	Adult (1)	1			
	Juvenile (0)				
	Total		0	0	0
Cat/NRW (without enteritis)	Adult (15)	24	9 (60.0)	4 (26.7)	6
	Juvenile (9)		4 (44.4)	2 (22.2)	1
	Total		13 (54.2)	6 (25.0)	7

cance, the differences of prevalence values were assessed by calculating 95 and 99% confidential intervals.

RESULTS

Prevalence of *C. upsaliensis*. From dogs in Berlin, 135 swabs were taken; 126 swabs were taken from dogs in NRW, near the cities Bielefeld and Paderborn. A total of 109 *Campylobacter* strains were isolated, 51 strains (37.8%) in Berlin and 58 (46.0%) in NRW. The overall prevalence of *Campylobacter* in juvenile dogs (<12 months of age) was 75.0%, compared to 32.7% in adult dogs from both geographic regions ($P < 0.01$) (Table 2). Substantial differences between dogs with and without symptoms of enteritis could not be detected.

Of 109 canine *Campylobacter* isolates, 88 belonged to the species *C. upsaliensis* (80.7%). Only negligible differences in frequency of isolation were detected between the two respective regions (84.3% [Berlin] and 77.6% [NRW]). The prevalence of *C. upsaliensis* among all dogs included in the investigation varied between 55.4% in juvenile and 27.8% in adult animals ($P < 0.05$).

Of the cats, 47.8% (22 of 46) were *Campylobacter* positive, 40.9% (9 of 22) of which harbored the species *C. upsaliensis*. However, 45.5% (10 of 22) of the *Campylobacter*-positive cats harbored *C. helveticus*, whereas this species was identified only in one dog (data not shown).

Determination of O antigens. From 88 dogs and 9 cats, 92 canine and 9 feline *C. upsaliensis* strains were isolated. The strains were subjected to serotyping. Ninety-four of these 101 strains (93.1%) were typed according to their O antigens by using five antisera, four of them directed against *C. upsaliensis* strains and one against a *C. helveticus* strain as listed in Table 1. The positively reacting strains belonged to five different serotypes (preliminarily named O I to O IV and O VI), two of them being prevalent at 47.5% (O III) and 27.7% (O IV). The serotypes O I, O II, and O VI were identified at 8.9, 5.9, and 3.0%, respectively (Table 3). Serotype O II was characterized by a strong reaction with the serotype O I-recognizing antiserum, combined with a weak reaction (1:80) with the *C. jejuni* O:2 (Penner)-recognizing antiserum. Serotype O V was not detected among the *C. upsaliensis* strains tested. Of the *C. upsaliensis* isolates, 6.9% did not react with any antiserum. Beyond the weak reaction of serotype O II with *C. jejuni* O:2-recognizing antiserum, the reactivity of *C. upsaliensis* or *C. helveticus* O antigens with antisera recognizing *C. jejuni* O:1, O:4, O:9, O:13, O:16, O:37, and O:43, two *C. coli* antisera (O:20 and a non-cross-reacting strain), and one *C. lari* antiserum (Table 1) was not detected.

In contrast to *C. upsaliensis*, the total number of 11 *C. helveticus* strains could be characterized with respect to their O antigens. Six isolates belonged to the serotypes O II and O III identified among *C. upsaliensis*, whereas the serotypes O I, O IV, and O VI could not be identified. Five *C. helveticus* isolates possessed an O antigen (V) which seemed to be unique to *C. helveticus*.

TABLE 3. O-antigen specificities among canine and feline *C. upsaliensis* and *C. helveticus* strains in Berlin and NRW, respectively

Host/geographic origin	No. of *C. upsaliensis* or *C. helveticus* isolates[a]	No. of samples yielding each O antigen group (%)						
		I	II	III	IV	V	VI	n.t.[b]
Dog/Berlin	43	4 (9.3)	3 (7.0)	14 (32.6)	12 (27.9)	0	3 (7.0)	7 (16.3)
Dog/NRW	49	5 (10.2)	3 (6.1)	30 (61.2)	11 (22.4)	0	0	0
Dog/Berlin and NRW	92	9 (9.8)	6 (6.5)	44 (47.8)	23 (25.0)	0	3 (3.3)	7 (7.6)
Cat/Berlin	3	0	0	0	3	0	0	0
Cat/NRW	6	0	0	4	2	0	0	0
Cat/Berlin and NRW	9	0	0	4	5	0	0	0
Dog and cat/Berlin	46	4 (8.7)	3 (6.5)	14 (30.4)	15 (32.6)	0	3 (6.5)	7 (15.2)
Dog and cat/NRW	55	5 (9.1)	3 (5.5)	34 (61.8)	13 (23.6)	0	0	0
Dog and cat/Berlin and NRW	101	9 (8.9)	6 (5.9)	48 (47.5)	28 (27.7)	0	3 (3.0)	7 (6.9)
Dog and cat/Berlin and NRW	11	0	3 (27.3)	3 (27.3)	0	5 (45.4)	0	0

[a] Only the bottom row gives *C. helveticus* results.
[b] n.t., not typeable.

Restriction fragment length polymorphism (RFLP). From 101 *C. upsaliensis* and 11 *C. helveticus* strains differentiated by serotyping (see above), 31 strains yielded repeatedly unsatisfactory weak pulsed-field gel electropherograms and were omitted from further investigation. Therefore, 80 strains were subjected to RFLP analysis, 76 belonging to the species *C. upsaliensis* and 4 to *C. helveticus*. Approximately 5% of these strains possessed a very small number of recognition sites for the endonuclease *Sma*I or *Xho*I, yielding fewer than five bands. For example, the *C. helveticus* isolates were not digested by *Sma*I, whereas all of them possessed recognition sites for *Xho*I. These strains were not omitted from further analysis.

RFLP analyses with the endonucleases *Sma*I (recognition site, CCC↓GGG) and *Xho*I (recognition site, C↓TCGAG) (downward-pointing arrows indicate cutting sites) revealed a considerable degree of genomic heterogeneity among the *C. upsaliensis* strains, as shown in the dendrograms of genotypic similarities (Fig. 1A and B). In general, the isolates possessed a number of recognition sites for *Sma*I and *Xho*I, yielding between 10 and 20 DNA bands for either enzyme (molecular mass, up to approximately 450 kb for *Sma*I and 250 kb for *Xho*I). Almost every isolate exhibited its unique restriction pattern. Only in six cases did isolates from unrelated hosts exhibit restriction patterns identical to those of one or two other strains when digested with *Sma*I and in four cases when digested with *Xho*I (Fig. 1A and B). However, none of these pairs or groups of strains yielded identical patterns with both of the enzymes. The *C. upsaliensis* strains H35E, H37E, H38E, H39E, H40E, H41E, and H42E (Fig. 1, brackets and three asterisks) were collected from 8-week-old puppies belonging to one litter. Similarly, K16 and K17 (Fig. 1, bracket and asterisk) were isolated from two kittens of one litter. In contrast, H28 and H29 (Fig. 1, arrows) were isolated from unrelated dogs living together in one household. From four *C. helveticus* isolates (Fig. 1, bracket and two asterisks) grouped in one cluster within the dendrograms, three strains (serogroup O V) originated from cats living together in one family. Based on the similarities of *Sma*I-generated band profiles of ≥89%, four clusters of strains could be determined to exhibit associations to serotypes. Cluster I consisted mainly of serotype O III-

positive strains. In cluster II mainly O IV-positive strains were assembled. Cluster III contained the *C. helveticus* isolates (O III and O V). Cluster IV was the most heterogeneous cluster, containing untypeable strains at a rate of 29%.

Restriction with *Xho*I resulted in six clusters on the basis of ≥90% internal similarity. However, correlations between serotypes of strains and restriction patterns were practically not detected. When strains exhibiting ≥95% band profile similarity were grouped together, 17 clusters consisting of 2 to 10 strains were generated. Only eight of these groups contained strains expressing mainly identical O antigens. These groups were scattered rather randomly within the dendrogram.

An association of the RFLP patterns and geographic origin of the strains or host species has not been detected. The feline *C. upsaliensis* strains were randomly distributed among the canine strains.

ERIC fingerprint analysis. From the collection of 76 *C. upsaliensis* and 4 *C. helveticus* strains characterized by macrorestriction analysis, 74 *C. upsaliensis* and 3 *C. helveticus* strains were typed using ERIC PCR (Fig. 2). All bands, bright or light, were counted according to their positions and strength. Nine clusters were detected when strains exhibiting similarity rates of ≥93% were grouped together.

The *C. helveticus* strains were grouped together in cluster II (Fig. 1, asterisk). Beyond that, an association between the degree of genomic similarities and origin of isolates was not detected, neither regarding the geographic area nor the host species. Furthermore, a correlation between the serotypes or the distribution of strains within the dendrogram generated on the basis of ERIC fingerprinting was not found.

Analysis of 16S rRNA sequence. From the 76 *C. upsaliensis* and 4 *C. helveticus* strains investigated by macrorestriction analysis, 12 strains were randomly selected, three from each of the four groups of the *Sma*I-generated similarity tree (Fig. 1A). The numbers of the selected strains were H23E, H123E, and K12E from group I; H50E, H118, and H28E from group II; K2E, K9E, and K16E from group III (*C. helveticus* group); and H25E, H28, and H95 from group IV. According to Linton et al. (27), the major part of the 16S rRNA gene encoding the DNA sequence was amplified. A DNA segment of 1,169 nu-
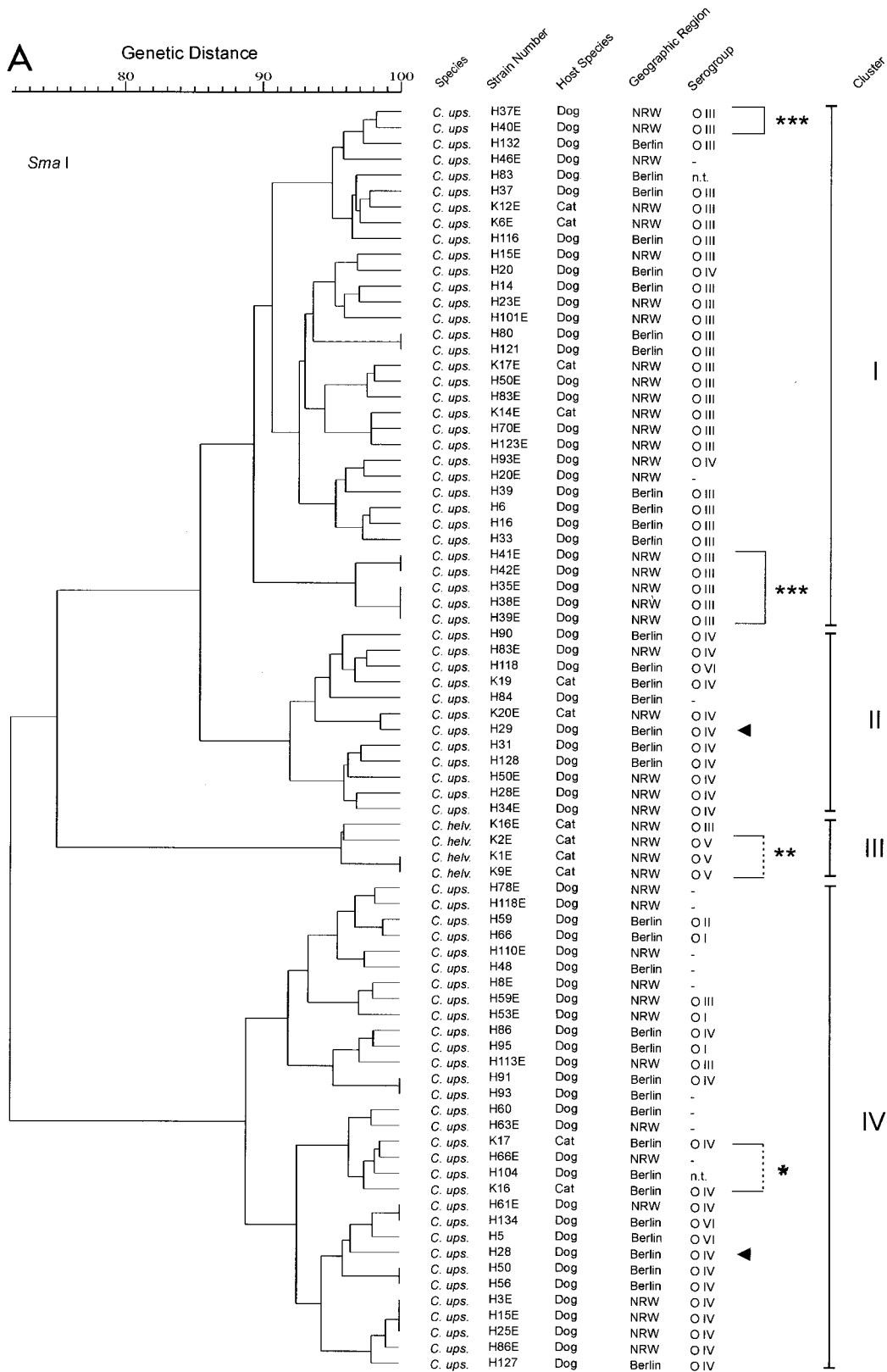
FIG. 1. (A) Genotypic similarities based on macrorestriction profiles, of *C. upsaliensis* and *C. helveticus* strains isolated from dogs and cats in Berlin and NRW, that were generated by the restriction endonuclease *Sma*I. (B) Genotypic similarities based on macrorestriction profiles, of *C. upsaliensis* and *C. helveticus* strains isolated from dogs and cats in Berlin and NRW, that were generated by the restriction endonuclease *Xho*I. Where no serogorup is given (dash), the strain was tested, but not typeable (negative) with the available antisera. The single and triple asterisks indicate respective littermates, and the double asterisks indicate strains from animals living in one household but not genetically related.
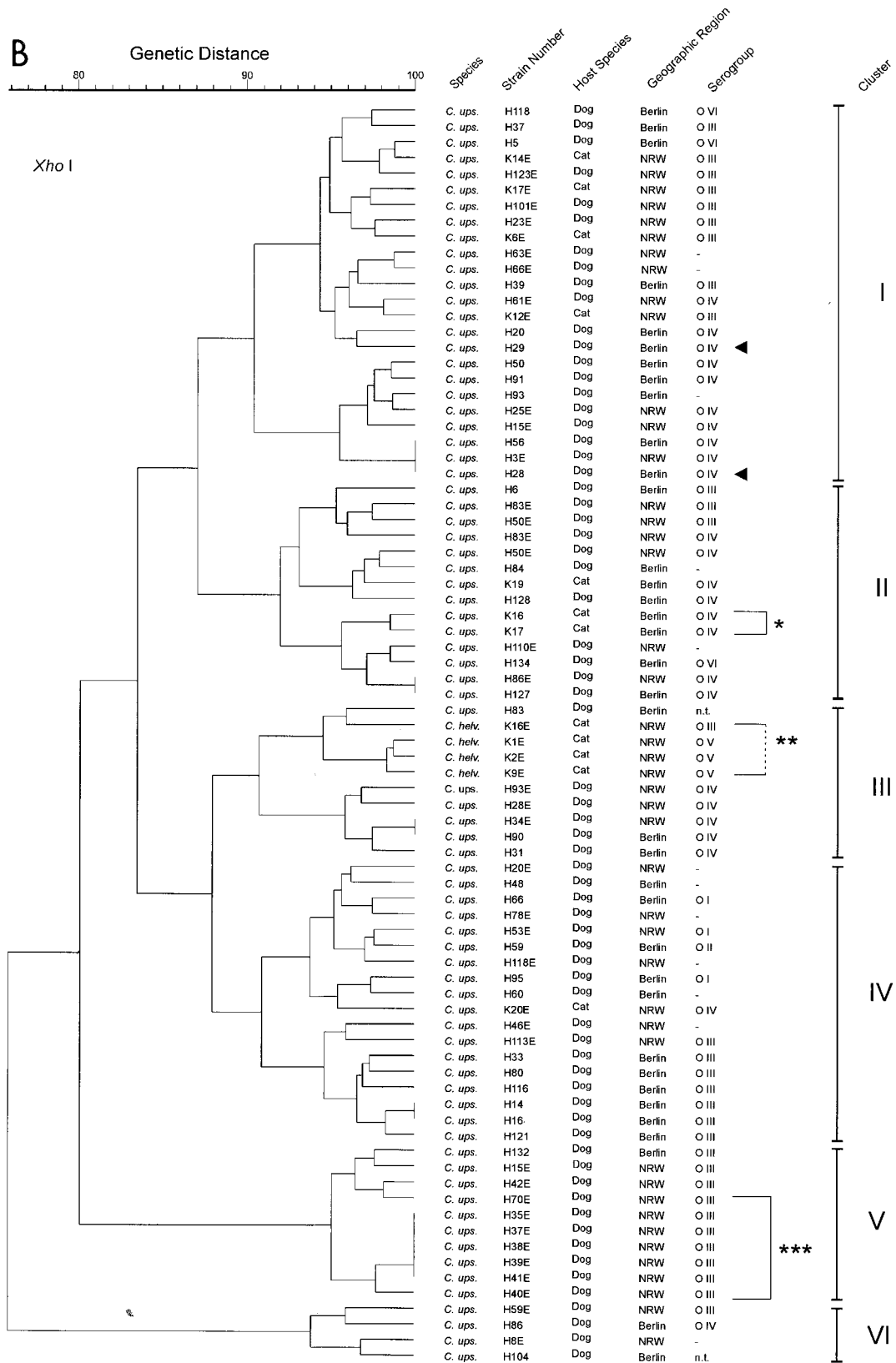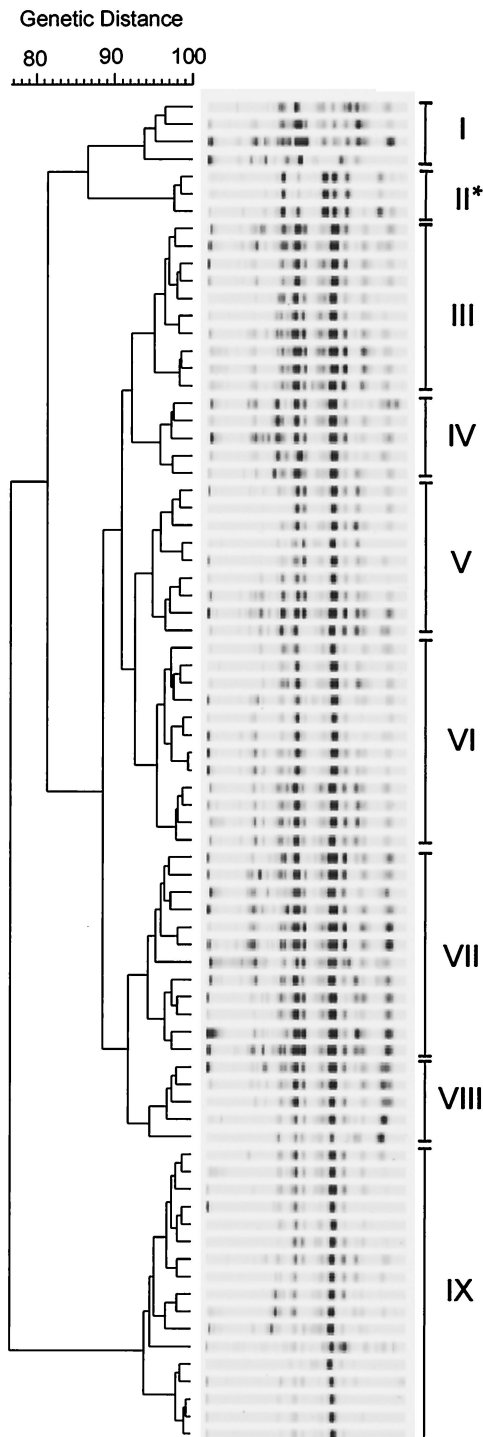
FIG. 1—*Continued.*

Genetic Distance

80   90   100

FIG. 2. ERIC PCR profile analysis of canine and feline *C. upsaliensis* and *C. helveticus* strains isolated in Berlin and NRW. ∗, *C. helveticus* strains. I to IX represent groups of genetic relatedness based on ≥93% similarity of band positions and intensities.

FIG. 3. Differences of 16S rDNA sequence between the *C. upsaliensis* strain K12E and the *C. helveticus* strain K16E.

sessed identical nucleotide sequences compared to strain CCUG 14913, with two exceptions. Strain H95 exhibited C instead of G at nucleotide position 382 (CCUG 14913), and strain K12E showed T instead of C at nucleotide position 642 (CCUG 14913). All three *C. helveticus* strains also possessed identical DNA sequences. However, they differed from the *C. upsaliensis* DNA sequences at several nucleotide positions: G towards C at position 382, identical to the *C. upsaliensis* strain H95, T towards C at position 1195, and 19 changes at positions listed in Fig. 3. The *C. upsaliensis* strain K12E and the *C. helveticus* strain K16E were randomly selected for comparison.

DISCUSSION

Results of serotyping of *C. upsaliensis* have previously been published regarding heat-labile surface antigens (28); however, to our knowledge, typing according to heat-stable antigens has not been performed so far. Therefore, we consider this the first O-antigen typing scheme published for *C. upsaliensis* and *C. helveticus*. O-antigen typing of *C. upsaliensis* strains revealed that in contrast to *C. jejuni* and *C. coli*, which are divided into more than 60 O-antigenic serotypes according to Penner and Hennessy (45), this species seems to possess only a small number of different heat-stable antigens. Of the investigated strains, 93.1% were divided into only five different serotypes. Since crosswise absorption experiments have not been performed, we cannot exclude that the five heat-stable serotypes identified in the present study consist of a somewhat larger number of cross-reacting antigens. Nevertheless, the O-antigen diversity of *C. upsaliensis* seems to be less pronounced than that of *C. jejuni*. Similar to *C. jejuni*, where a limited number of serotypes exceed others in frequency of isolation worldwide (42, 46; W. M. Johnson, D. L. Woodward, R. Khakhria, and L. J. Price, *Campylobacter, Helicobacter* and related organisms. Proc. 9th Int. Workshop, p. 27, 1997), we found that the serotypes O III and O IV, identified at rates of 47.5 and 27.7%, respectively, constituted the most prevalent serotypes within the collection of isolates. After a comparison of the number of strains that exhibited the prevalent serotype O III or the rarely found serotype O VI or were untypeable, a marked difference of geographic distribution could be detected. In NRW 61.8% of all tested strains exhibited serotype O III, while none of them were O VI positive or untypeable. In contrast, of the strains collected in Berlin, only 30.4% belonged to serotype O

cleotides from each of the 12 strains was analyzed in order to determine their genetic distance. This corresponded to 80.1% of the entire gene of *C. upsaliensis* CCUG 14913, comprising 1,460 nucleotides beginning from nucleotide 225 (58; GenBank accession number L14628). All nine *C. upsaliensis* strains pos-

III, 6.5% to serotype O VI, and 15.2% to the group of untypeable strains. These data point towards clonal expansion of *C. upsaliensis*.

Except for serotype O II, which showed a weak cross-reaction with the C. *jejuni* serotype O:2, the *C. upsaliensis* serotypes were unrelated to serotypes of other *Campylobacter* species available for comparison in this study. Nine *C. jejuni*-specific antisera mainly representing O-antigenic serotypes prevalent worldwide, two *C. coli*-specific antisera, and one *C. lari*-specific antiserum were used. The finding that *C. upsaliensis* strains may share some O-antigen specificity with *C. jejuni* or *C. coli* was reported before from strains in South Africa (25). These authors reported that a number of *C. upsaliensis* strains isolated from humans did not react with *C. jejuni*-specific antisera; however, they reacted strongly with antiserum against *C. coli* O:28. Due to the lack of availability of more than our two *C. coli* antisera, we were not able to confirm this reactivity in our strain collection. In contrast, 54.6% of the *C. helveticus* isolates shared O-antigenic structures (serotypes O II and O III) with *C. upsaliensis*.

In contrast to serotyping, genotyping methods (PFGE and ERIC PCR) revealed a high degree of genomic heterogeneity within the species *C. upsaliensis*. These data are in accordance with previous reports (4, 39, 57) and may be due to some properties of *Campylobacter*, which enable them to take up DNA from the environment and integrate it into the genome or undergo changes within the genome by rearrangement of autogenous DNA (15, 18, 29, 54, 55, 56). Beyond that, macro-restriction analysis with *Sma*I revealed a certain degree of association between serotypes and genotypic similarities. The concentration of serotype O III-positive strains in cluster I of the dendrogram especially points towards clonal expansion of at least certain subgroups within the species *C. upsaliensis*. In *C. jejuni* NCTC 11168, whose genome is totally characterized by DNA sequence analysis (41), 9 of 15 specific recognition sites of the endonuclease *Sma*I are located within the three copies of the 16S rRNA genes. Therefore, *Sma*I seems to be a very useful endonuclease for studies of the clonality of *C. jejuni* (19, 37, 40). The same may hold true for *C. upsaliensis*.

In contrast, restriction with the endonuclease *Xho*I caused the disintegration of these serotype-associated groups to smaller noncoherent subgroups. Therefore, due to their specific recognition sites, certain restriction endonucleases are more suitable tools to answer questions concerning clonality and evolution, while others may be more suitable for epidemiological studies due to their high discriminatory capacity.

The *C. helveticus* isolates were grouped together when restricted with either enzyme, despite belonging to different serotypes. As they were not digested by *Sma*I, they formed a separate group within the *Sma*I-generated dendrogram. In contrast, they possessed recognition sites for *Xho*I; therefore, they form only a subgroup within the *Xho*I-generated dendrogram.

Beyond serotypes, associations of genotypic similarities to other characteristics common for certain strain groups, like geographic or host origin (dog or cat) or enteric health status of their hosts, were not detected. These data are partly in agreement with those of Stanley et al. (51), who reported when using 16S rRNA ribotyping that *C. upsaliensis* isolates from healthy and diarrheic dogs were not distinguishable. However,

they detected genotypic differences between human and canine strains. Unfortunately, due to the lack of human isolates, we were not able to compare canine with human isolates. These data may indicate that the pathogen is ingested by dogs and cats from the same sources, in contrast to the pattern for humans. On the other hand, it may also be a matter of adaptation of certain strains from general sources to different hosts in order to cause and maintain an infection.

ERIC sequence profile analysis demonstrated the high discriminatory potential of this method, as described before (57). However, the profiles did not reveal associations to any of the above-mentioned characteristics of the strains. Again the investigated *C. helveticus* isolates were grouped together.

The high degree of 16S rDNA sequence similarity among the nine *C. upsaliensis* strains selected from the whole amplitude of the *Sma*I-generated tree of genetic similarity is in marked contrast to the high degree of heterogeneity of the whole genome and confirms that all the *C. upsaliensis* strains belong to one species. Only 1 nucleotide each was changed within the whole length of 1,169 nucleotides in two strains. With these exceptions, all 12 strains exhibited 16S rDNA sequences identical to that for the *C. upsaliensis* strain CCUG 14913 supplied in the database (58). The three *C. helveticus* strains analyzed also possessed identical sequences, compared with each other. The small number of nucleotide exchanges compared to the 16S rDNA sequence of *C. upsaliensis* underlines the relatedness of the two *Campylobacter* species. Overall, 21 of 1,169 nucleotides were changed with 19 of them between the positions 961 and 1345, compared to the 16S rDNA sequence of strain *C. upsaliensis* CCUG 14913.

From all these genotypic investigations, the strains which did not give satisfactory electropherograms, perhaps due to extracellular DNases or other factors, were excluded from analysis without further investigation. Therefore, the degree of genomic heterogeneity within the two species under investigation may be even higher than demonstrated in this work.

Thermophilic *Campylobacter* species were isolated from 41.8% of the canine fecal specimens from two distinct geographic regions in Germany, an isolation rate in accordance with other reports, where 13.8 and 50% of the samples were positive (1, 3, 6, 7, 17, 30, 34, 48). In the present study 80.7% of the isolates were identified as *C. upsaliensis,* and only 19.3% were identified as *C. jejuni* or other catalase-positive thermophilic *Campylobacter* or related species. Baker et al. (3) reported similar *C. upsaliensis* isolation rates in dogs in southern Australia. Other authors, however, isolated *C. upsaliensis* only at rates of 15.7 (6), 19 (17), and 7.1% (30), whereas *C. jejuni* was isolated at 19.2 (6), 76 (17), and 33.9% (30). Loss of viability of bacteria due to mailing conditions or the selective media used for *Campylobacter* isolation may contribute to the rarity of those results. In order to get reliable results, we focused on freshly collected samples, which were streaked on plates within 24 h, and did not include samples sent from elsewhere to the laboratory. Investigating two geographically separated groups of isolates, we sought to analyze differences in expansion of genomic characteristics in different bacterial populations and to prevent misinterpretations caused by generalizing certain findings which would have emerged potentially only in one group of isolates.

The prevalence of *Campylobacter* in juvenile and adult dogs

differed significantly, in that young animals carried *Campylobacter* at an average isolation rate of 75.0%, whereas adults were positive only at a rate of 32.7% ($P < 0.01$). Similarly *C. upsaliensis* was isolated more than twice as often in juvenile dogs as in adult dogs (55.4 versus 27.8%; $P < 0.05$). Regarding the presence or absence of enteric symptoms, there was a significant difference only for the *Campylobacter* isolation rates detected for juvenile and adult healthy dogs, with higher frequencies in juvenile dogs. In enteritic dogs there existed only a nonsignificant tendency to higher *Campylobacter* rates (in juvenile animals). In general, in contrast to the findings of Burnens et al. (6), substantial differences between dogs with and without enteritic symptoms were not detected.

Taking these results together, significant differences in the incidence of *Campylobacter*, especially in *C. upsaliensis* isolation, were detected between juvenile and adult dogs without gastroenteritic symptoms, whereas a significant association with enteritis could not be found in either age group.

The number of feline isolates obtained in this study was too small to allow any further interpretation concerning associations of pathogen carriage with age or enteric health.

The potential significance of *C. upsaliensis* for humans is increasingly realized. The diversity of the genome renders epidemiological studies difficult to perform. Combinations of phenotypic methods, including O-antigen typing and well-considered genotypic methods, may help to follow the route of the pathogen during infection.

ACKNOWLEDGMENT

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