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Molecular Characterization of *Capnocytophaga canimorsus* and Other Canine *Capnocytophaga* spp. and Assessment by PCR of Their Frequencies in Dogs▽†

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***Capnocytophaga canimorsus* can be a virulent pathogen, whereas *C. cynodegmi* is of low virulence. Heterogeneity within these species, their frequency in dogs, and pathogenicity factors are largely unknown. Strains from blood cultures from patients presumptively identified as *C. canimorsus* ($n = 25$) and as *C. cynodegmi* by *rrs* analysis ($n = 4$), blood cultures from dogs ($n = 8$), blood cultures from cats ($n = 2$), and cultures from swabs from dog mouths ($n = 53$) were analyzed. PCR-restriction fragment length polymorphism (PCR-RFLP), a species-specific PCR on *rpoB*, and *rrs* sequencing were used. All 29 strains from human blood cultures could be grouped into three PCR-RFLP types. One included the *C. canimorsus* type strain, and the other types were closely related. Two canine strains were *C. canimorsus* and grouped into the least common RLFP pattern group. Five were *C. cynodegmi* and clustered with the reference strain. One canine and both feline strains were distinct. Four human strains that presumptively had been identified as *C. cynodegmi* by RNA gene sequence analysis clustered with the *C. canimorsus* strains by both PCR-RFLP and the sequence-specific PCR of the *rpoB* gene. *C. canimorsus* DNA was present in 73% (range, 61 to 85%) of dogs' mouths, and *C. cynodegmi* DNA was present in 96% (range, 94 to 100%) of dogs' mouths. As defined by *rpoB* PCR-RFLP and by PCRs using specific primers, all strains from human blood were *C. canimorsus*. The sequencing of *rrs* genes suggested the presence of different gene copies in a few strains, indicating that the method is less appropriate for species identification. Both species are present in the majority of dogs. Additional *Capnocytophaga* species occur in dogs' and cats' mouths.**

Capnocytophaga canimorsus, originally described as DF-2, can infect humans after bites from dogs and occasionally from cats. The usual clinical presentation of infection with this bacterium is fulminant sepsis, which has a high mortality. Infection with *C. canimorsus* also can lead to endocarditis, meningitis, eye infections, and mycotic aneurysms. Although high-risk groups such as patients after splenectomy or with alcoholism have been defined, the infection also can occur in healthy persons (14, 19).

A second *Capnocytophaga* species, *Capnocytophaga cynodegmi*, also has been cultured from the oral flora of dogs (3). This species, originally referred to as DF-2-like, has been cultured only infrequently from patients with infections. In the initial report characterizing this species, six strains originating from wound or eye infections are reported (3). Since then, only one patient with a wound infection (9) and two cases of bacteremia (11, 20) with this species have been described. In the latter two cases, only biochemical determination was performed; one of the two strains was identified only on the basis of a positive nitrate test, which is not a common feature of this species. Therefore, *C. cynodegmi* appears to be an extremely rare pathogen with low virulence.

In the original publication in which both species were described, discrimination was based mainly on DNA-DNA hybridization experiments, since strains of both species had biochemical features in common (3). Although nowadays sequencing the 16S rRNA gene is a tool that is frequently used to identify bacterial species, especially those species that are difficult to identify by conventional methods, a homology of more than 97% between *C. canimorsus* and *C. cynodegmi* is within the range of one species. The *rpoB* gene is used frequently for differentiating species that are closely related on the basis of the 16S rRNA gene (5, 12, 13, 18) and has a homology of only 83% in both species; therefore, it is a more suitable target for their genetic characterization. An alternative molecular method to sequencing for distinguishing between and within species is PCR-restriction fragment length polymorphism (PCR-RFLP). This technique has been applied successfully to identify other *Capnocytophaga* species originating from the human mouth (4).

It is unclear why *C. canimorsus* infections are so infrequent.

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One possibility is that the bacterium occurs in low frequency in dogs. *C. canimorsus* has been described routinely as a commensal microorganism in dog mouths. This is based on three different studies, which report a frequency of this bacterium of 8% (1), 24% (23), and 25% (2). Bailie et al. (1) published long before *C. cynodegmi* was identified and cannot have been able to discriminate between the species. In their description of the methods to identify *C. canimorsus*, Blanche et al. (2) refer to a publication that also did not differentiate between *C. canimorsus* and *C. cynodegmi.* Therefore, in both studies the occurrence of *C. canimorsus* may have been overestimated. In addition, in neither of these two studies was a selective medium was used, and this may have resulted in an underestimation of the colonization rate of dogs by *C. canimorsus*. In contrast, Westwell et al. (23) used a selective medium to isolate *Capnocytophaga* species strains and discriminated between DF-2 and DF-2-like strains. DF-2-like strains were identified in 11% of the dogs. The authors did not report whether they also looked for double infections by screening multiple colonies from every dog. In a very recent paper, *Capnocytophaga* species strains, characterized as *C. canimorsus* by *rrs* sequence analysis, were cultured from 61% of 103 dogs (15). However, *rrs* sequence analysis might not be the most appropriate way to distinguish between *C. canimorsus* and *C. cynodegmi.*

Another explanation is a difference in virulence among *C. canimorsus* strains. It has been described that *C. canimorsus* produces a toxin affecting macrophages that is not present in *C. cynodegmi* (7). One possibility is that not all *C. canimorsus* strains produce this toxin. It also could be that strains from dogs are genetically more diverse, and that only a subpopulation of these strains is pathogenic for humans.

The aim of the present study was to characterize oxidase-positive *Capnocytophaga* species strains in more detail, to develop species-specific real-time PCRs for *C. canimorsus* and *C. cynodegmi*, and to assess the presence of both pathogens in the oral flora of dogs with this technique without culturing.

MATERIALS AND METHODS

Bacterial isolates. Reference strains for *C. canimorsus* (ATCC 35979 T) and *C. cynodegmi* (ATCC 49045) were obtained from the ATCC. Reference strains for *C. gingivalis* (DSM 3290 T), *C. sputigena* (DSM 7273 T), *C. haemolytica* (DSM 11385 T), *C. granulosa* (DSM 11449 T), and *C. ochracea* (DSM 7271 T) were obtained from the German Collection of Microorganisms and Cell Cultures. Twenty-eight other oxidase-positive *Capnocytophaga* species strains cultured from the blood of human patients were kindly provided by different clinical laboratories from The Netherlands. Four of these strains (98-196, 02-195, 01-351, and 02-39) had been identified presumptively as *C. cynodegmi* by the partial sequencing of the *rrs* gene, whereas the other 24 strains had been identified as *C. canimorsus* by this method (Table 1). Sixteen bacterial and yeast strains from other species (*Haemophilus influenzae*, *Staphylococcus aureus*, *Neisseria meningitidis*, *Pseudomonas aeruginosa*, *Salmonella enteritidis*, *Salmonella enterica* serovar Typhimurium, *Bordetella pertussis*, *Acinetobacter baumannii*, *Moraxella catarrhalis*, *Streptococcus bovis*, *Streptococcus salivarius*, *Chlamydia pneumoniae*, *Bacillus cereus*, *Serratia marcescens*, *Escherichia coli*, and *Candida albicans*) were obtained from our collections for specificity testing.

Additionally, *Capnocytophaga* species strains were cultured from swabs from the gums of 14 dogs and 2 cats. Swabs were stored at 4°C in Stuart medium up to 2 days before inoculation, with the exception of three swabs that were stored for 7 to 9 days. Swabs were inoculated onto culture plates containing 5% sheep blood agar and gentamicin (BioMérieux) for 1 week at 37°C in the presence of 5% CO_2. Oxidase-positive strains that were gram-negative rods by Gram staining were further identified by API Rapid ID32 A, (BioMérieux, Lyon, France). By this approach, 11 strains were obtained from dogs and 2 from cats.

Canine samples for PCR and culture. Fifty-three dogs attending a veterinary clinic for various reasons had their mouths swabbed with informed consent from the owners. Cotton-tip swabs were rubbed on the gums or on the tongue and suspended in phosphate-buffered saline and stored at −20°C until processed for PCR. The age and sex of 43 of these dogs were reported; their mean age was 6 years (range, 2 months to 15 years). Twenty-seven dogs (63%) were male.

Culture. To obtain strains and to compare culture and PCR results, cultures were performed initially from specimens from eight dogs; cultures were performed on the same day on sheep blood agar plates (BioMérieux) containing gentamicin. Colonies that were oxidase and catalase positive and showed gram-negative rods on Gram stain were further analyzed by both *rpoB* PCRs as described below.

Since the initial yield was only four strains (three *C. cynodegmi* and one *C. canimorsus* strain), samples from five additional dogs were obtained and cultured in the same way. This resulted in an additional three *C. cynodegmi* strains and one strain resembling a *Capnocytophaga* species, although it was oxidase negative. An additional attempt to obtain more *C. canimorsus* strains was performed by culturing additional samples from nine dogs on sheep blood agar plates with gentamicin and performing the *C. canimorsus* PCR as described below on the mixture of colonies that appeared on the plates. These samples had been stored for 2 to 9 days in Stuart transport medium. A positive signal was obtained from only three culture plates; after subculture, a *C. canimorsus* strain was grown from only one dog, which later appeared to be the same dog as the one from which we had cultured a *C. canimorsus* strain earlier.

DNA extraction. DNA was extracted from all strains and all canine specimens with the use of the QIAamp DNA mini kit (Qiagen, Hilden, Germany). For comparison, the MagNa Pure LC instrument also was used to extract DNA from the first 38 canine specimens. The efficiency of the extraction of nucleic acids from capnocytophagae by these two methods was comparable (data not shown).

Development of PCR-RFLP on *rpoB* for characterization of strains. For PCR-RFLP, the chosen *rpoB* primers were based on sequences published in the NCBI database: for *C. canimorsus* CIP103936 T and *C. cynodegmi* CIP103937 T, the accession numbers were AY643069 and AY643070, respectively. The primers 5′-CAGTTTATGGATCAAACCAATCC-3′ and 5′-CCATCTCTCCRAAACG CTGAC-3 were expected to amplify a 2,190-bp fragment. Sequences were chosen using Beacon Designer software (Premier Biosoft International, Palo Alto, CA). PCR was performed for 40 cycles at an annealing temperature of 58°C. Ten microliters of the amplicon subsequently was digested with StyI (10 U/μl) for 3 h or MseI (5 U/μl) for 1 h. Restriction fragments were analyzed on an standard 1.5% agarose gel or a 4.0% NuSieve GTG agarose (Invitrogen) gel, respectively.

Development of species-specific *rpoB* PCRs. Two different species-specific PCRs for *C. canimorsus* and *C. cynodegmi* were designed based on the above-mentioned NCBI *rpoB* gene sequences, with specific primers for *C. canimorsus* (5′-TTTCAG CTTCATTAATTCCTTTCC-3′ and 5′-GCCTGACGCATCATATTCG-3′) and *C. cynodegmi* (5′-GAATTTCGGCTTCATTGATTCC-3′ and 5′-CGCATCATATTT GACCCCATC-3′). The 6-carboxyfluorescein-490-labeled probes were 5′-CGATG ATGCGAACCGTGCGTTGAC-3′ for *C. canimorsus* and 5′-CTTGGAACACGA TGATGCGAACCG-3′ for *C. cynodegmi.* PCR amplification was performed in 50 cycles with an annealing temperature of 62°C in the presence of 3.5 mM $MgCl_2$ for the PCR for *C. cynodegmi* and 4.5 mM $MgCl_2$ for the PCR for *C. canimorsus.* In previous experiments, a range of $MgCl_2$ concentrations was used to optimize sensitivity and specificity for both PCRs, and these $MgCl_2$ concentrations resulted in optimal sensitivity and specificity for both species.

Nucleotide sequencing of the 16S rRNA of cultured strains. The partial nucleotide sequencing of nucleotide (nt) 38 to 492 of the 16S rRNA was performed by PCR amplification using primers 5′-TAACACATGCAAGTCGAGGG-3′ and 5′-ATCTCCGTATTACGGCGGC-3′ and subsequent sequence analysis on an ABI Prism 3100 Genetic Analyzer (Applied Biosystems, Inc.). The entire *rrs* gene of a number of strains, including those strains previously identified as *C. cynodegmi* strains from patients, was sequenced using the forward primers 16S8F (5′-AGA GTT TGA TCM TGG YTC AG-3′), 16S339F (5′-CTC CTA CGG GAG GCA GCA G-3′), 16S518F (5′-CCA GCA GCC GCG GTA ATA-3′), 16S785F (5′-GGA TTA GAT ACC CTG GTA G-3′), and 16S912F (5′-CAA AKGA ATT GAC GGG GGC-3′). The reverse primers were 16S575R (5′-CTT TAC GCC CAR TRA WTC CG-3′), 16S802R (5′-CTA CCA GGG TAT CTA ATC C-3′), 16S930R (5′-GCC CCC GTC AAT TCM TTT G-3′), 16S1115R (5′-AGG GTT GCG CTC GT RC G-3′), and 16S1512R (5′-ACG GHT ACC TTG TTA CGA CTT-3′).

RESULTS

Characterization of oxidase-positive *Capnocytophaga* species strains by PCR-RFLP. The amplification of the *rpoB* gene was

TABLE 1. Characteristics of *Capnocytophaga* species strains used in this study

Strain	Origin	Typing by[a]:					
		rpoB RFLP			Sequence analysis (16S rRNA; nt 38–492)		*rpoB* PCR
		Pattern		Type	Type (according nomenclature of Fig. 2)	Accession no.	
		StyI	MseI				
ATCC 49045	Patient, wound (reference strain)	4	4	Ccyn1	CcynA	AY643076.1	*C. cynodegmi*
ATCC 35979	Patient, blood (reference strain)	2	2	Ccan2	CcanA	AY643075.1	*C. canimorsus*
03-349	Patient, blood	1	1	Ccan1	CcanA	FJ669146	*C. canimorsus*
03-308	Patient, blood	1	1	Ccan1	ND	ND	*C. canimorsus*
03-620	Patient, blood	1	1	Ccan1	CcanB	FJ669152	*C. canimorsus*
03-612	Patient, blood	2	2	Ccan2	CcanB	FJ669151	*C. canimorsus*
05-80	Patient, blood	3	3	Ccan3	CcanA	EU124407	*C. canimorsus*
05-29	Patient, blood	1	1	Ccan1	CcanB	FJ669153	*C. canimorsus*
97-16	Patient, blood	1	1	Ccan1	CcanA	FJ669154	*C. canimorsus*
601231	Patient, blood	NA	NA		ND	ND	*C. canimorsus*
602032	Patient, blood	NA	NA		ND	ND	*C. canimorsus*
601232	Patient, blood	1	1	Ccan1	ND	ND	*C. canimorsus*
601233	Patient, blood	2	2	Ccan2	ND	ND	*C. canimorsus*
601234	Patient, blood	1	1	Ccan1	ND	ND	*C. canimorsus*
601251	Patient, blood	2	2	Ccan2	CcanC	EU124411	*C. canimorsus*
602031	Patient, blood	1	1	Ccan1	CcanB2	EU124410	*C. canimorsus*
602035	Patient, blood	2	2	Ccan2	ND	ND	*C. canimorsus*
602091	Patient, blood	1	1	Ccan1	ND	ND	*C. canimorsus*
602241	Patient, blood	2	2	Ccan2	ND	ND	*C. canimorsus*
603292	Patient, blood	1	1	Ccan1	ND	ND	*C. canimorsus*
603231	Patient, blood	1	1	Ccan1	ND	ND	*C. canimorsus*
603232	Patient, blood	2	2	Ccan2	ND	ND	*C. canimorsus*
603233	Patient, blood	1	1	Ccan1	ND	ND	*C. canimorsus*
603234	Patient, blood	1	1	Ccan1	ND	ND	*C. canimorsus*
603235	Patient, blood	1	1	Ccan1	ND	ND	*C. canimorsus*
604051	Patient, blood	1	1	Ccan1	ND	ND	*C. canimorsus*
98-196	Patient, blood	1	1	Ccan1	CcynA4	FJ669146	*C. canimorsus*
02-195	Patient, blood	1	1	Ccan1	CcynA3	FJ669149	*C. canimorsus*
01-351	Patient, blood	1	1	Ccan1	CcynA1	FJ669147	*C. canimorsus*
02-39	Patient, blood	1	1	Ccan1	CcynA2	FJ669148	*C. canimorsus*
LUMC-HC 1	Dog mouth	4	5	Ccyn2	CcynA7	EU124420	*C. cynodegmi*
LUMC-HC 2	Dog mouth	4	5	Ccyn2	CcynA6	EU124413	*C. cynodegmi*
LUMC-HC 3	Dog mouth	2	3	Ccan4	CcanA	EU124409	*C. canimorsus*
LUMC-HC 8	Dog mouth	4	5	Ccyn2	CcynA9	EU124418	*C. cynodegmi*
LUMC-HCJ15	Dog mouth	3	3	Ccan3	CcanA	EU124408	*C. canimorsus*
LUMC-HJB21	Dog mouth	4	5	Ccyn2	ND		*C. cynodegmi*
LUMC-DogNido1	Dog mouth	6	7	C_dog	Cnew	EU124412	Negative
LUMC-DogNido3	Dog mouth	4	5	Ccyn2	CcynA6	EU124414	*C. cynodegmi*
LUMC-HA1	Dog mouth	4	4	Ccyn1	ND	ND	*C. cynodegmi*
LUMC-HA2	Dog mouth	4	4	Ccyn1	CcynA5	EU124415	*C. cynodegmi*
LUMC-HA3	Dog mouth	4	4	Ccyn1	CcynA8	EU124419	*C. cynodegmi*
LUMC-CatG1	Cat mouth	6	6	C_cat	Ccyncat	EU124417	Negative
LUMC-CatE l1	Cat mouth	6	6	C_cat	Ccyncat	EU124416	Negative

[a] NA, not amplifiable; ND, not done.

successful for 40/43 *Capnocytophaga* species strains included in the study (Table 1), as well as for both feline isolates (LUMC-CatG1 and LUMC-CatE1) and the oxidase-negative strain (LUMC-DogNido1). The reference strains from *C. ochracea*, *C. haemolytica*, *C. sputigena*, *C. granulosa*, and *C. gingivalis* gave no amplification product with the primers for the *rpoB* gene. Subsequent digestion with the restriction enzymes StyI and MseI resulted in different RFLP types, as shown in Fig. 1. For StyI digestion, one of the patterns was in agreement with the pattern predicted by the in silico digestion of the amplification product of strain 24231, described as *C. canimorsus* in GenBank (accession no. AY643071), and consisted of fragments of 1,127, 756, 169, and 136 bp (StyI type Ccan1) (Fig. 1a, lane 4). The second pattern of strain ATCC 35979 (Fig. 1a, lane 5) consisted of fragments of 788, 756, 339, 169, and 136 bp (StyI type Ccan2) and was in accordance with the sequence of this strain (AY643069). We also found a third pattern in which, compared to the sequence of type 2, one StyI restriction site apparently was not present, resulting in pattern 3 (892, 788, 339, and 169 bp; StyI type Ccan3) (Fig. 1a, lane 6). We also could distinguish three different MseI patterns, of which patterns Ccan1 and Ccan2 corresponded to predicted patterns based on the in silico digestion of the published sequences, and the third pattern showed a number of differences (Fig. 1b).

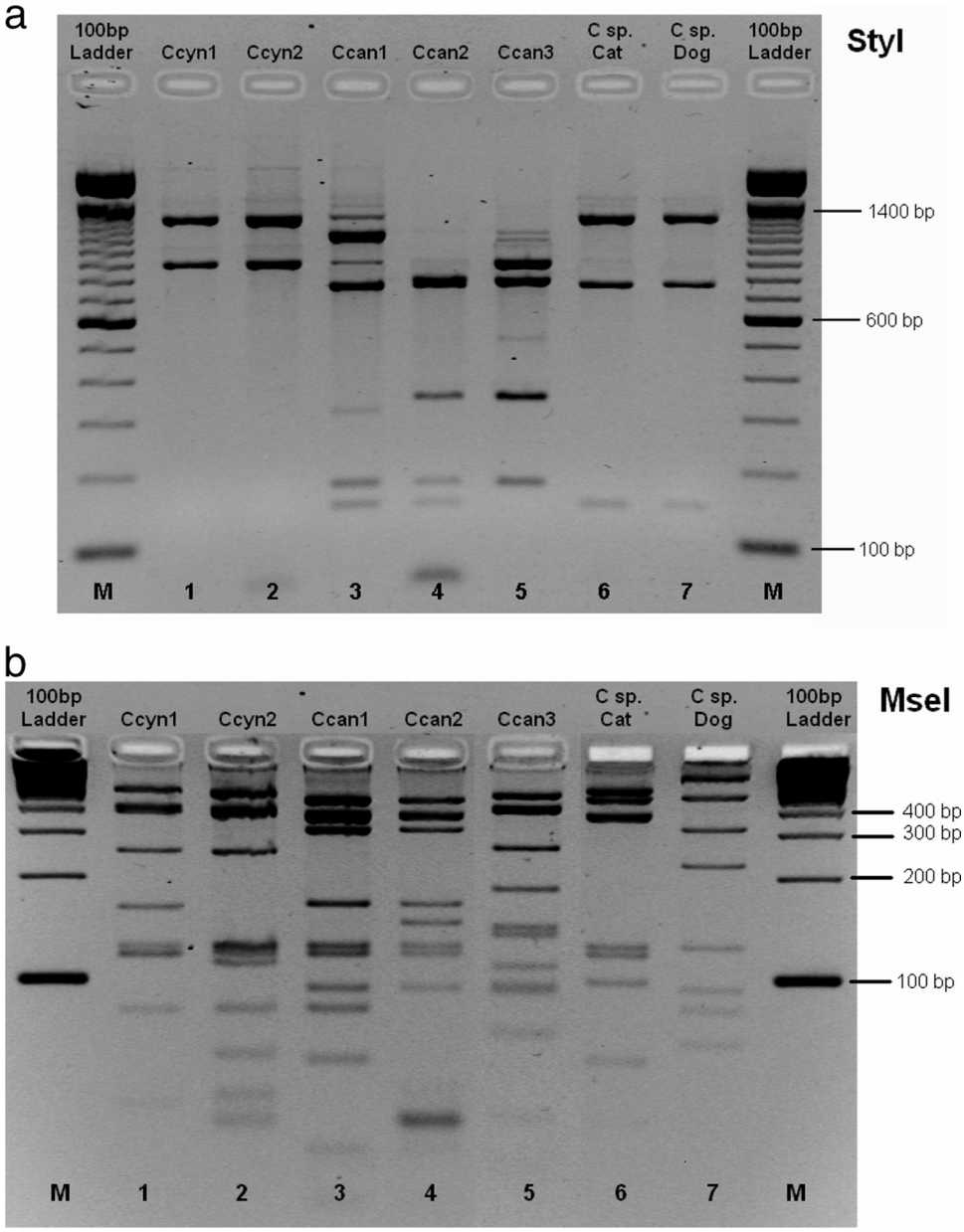

FIG. 1. StyI and MseI restriction patterns of the PCR amplification product of the *rpoB* gene (see Materials and Methods). DNA fragments were separated on a 1.5% standard agarose gel after StyI digestion (a) or on a 4% NuSieve agarose gel after MseI digestion (b). Lanes M, 100-bp marker; lanes 1, *C. cynodegmi* ATCC 49045; lanes 2, *C. cynodegmi* LUMC-Ccyn01; lanes 3, *C. canimorsus* 601232; lanes 4, *C. canimorsus* ATCC 35979; lanes 5, *C. canimorsus* LUMC-HCJ15; lanes 6, *Capnocytophaga* sp. strain LUMC-CatG1; lanes 7, *Capnocytophaga* sp. strain LUMC-DogNido1.

Fifteen human *C. canimorsus* strains fell into type 1 (Table 2). Interestingly, the four strains 98-196, 02-195, 01-351, and 02-39, presumptively identified as *C. cynodegmi* based on the partial sequencing of the *rrs* gene, also clustered in this PCR-RFLP group. Eight *C. canimorsus* isolates belonged to type 2. Only two *C. canimorsus* isolates from patients, as well as one of the dog isolates, belonged to type 3. The second dog isolate had StyI pattern type 2 and MseI pattern type 3 and is classified as type 4 in Table 2.

C. cynodegmi strains could be grouped in two different types, differing only in their MseI digestion patterns: type 4, which fit the pattern of strain ATCC 49045 (Fig. 1, lanes 1) and in accordance with the prediction on the basis of sequence AY643070 (strain CIP103937 T = ATCC 49044 T), and type 5, showing one or several additional restriction sites in a 157-bp fragment (Fig. 1, lanes 2). Both types showed the same StyI pattern as that predicted (1,296 and 892 bp). The reference strain, which is a strain from a wound of a patient after a dog bite, clustered with one of these types. The two cat *Capnocytophaga* species strains showed identical PCR-RFLP patterns that differed from the sequences of the other strains. Compared to the sequence of *C. cynodegmi*, one additional StyI

TABLE 2. Distribution of human and canine *Capnocytophaga* strains and types as defined by StyI and MseI digestion patterns

Species	Type	Origin	*n*	Digestion pattern (no.)	
				StyI	MseI
C. canimorsus	1	Patient[a]	19	1	1
	2	Patient	8	2	2
	3	Patient	1	3	3
	3	Dog	1	3	3
C. canimorsus	4	Dog	1	2	3
C. cynodegmi	1	Patient[b]	1	4	4
	1	Dog	3	4	4
C. cynodegmi	2	Dog	5	4	5
Putative new species	1	Cat	2	6	6
Putative new species	2	Dog	1	6	7

[a] Including the strains 98-196, 02-195, 01-351, and 02-19, which were identified presumptively as *C. cynodegmi* earlier (see the text).
[b] Strain ATCC 49045.

restriction site is present, resulting in fragments of 1,296, 756, and 136 bp (type 6). The strain Nido1, belonging to a newly discovered canine *Capnocytophaga* species, had an StyI digestion pattern that was identical to that of *C. cynodegmi*, but it showed a difference after MseI digestion (Table 2).

***rrs* gene analysis of *Capnocytophaga* species strains.** The partial sequencing of the *rrs* gene (nt 38 to 492) was performed on nine *C. canimorsus* strains cultured from human patients, including strains for each *C. canimorsus* type as described above, on the ambiguous four isolates 98-196, 02-195, 01-351, and 02-39 and on all 10 cultured *Capnocytophaga* species strains from dogs' and cats' mouths. Algorithms for similarity and phylogenetic trees treat ambiguous nucleotides as different features, resulting in different clusters in cases of differences between multiple copies, therefore the nucleotides that differ between strains are shown in Fig. 2. The sequence of the reference strain was designated type CcanA. Eight out of nine strains from patients as well as both strains typed as *C. canimorsus* from dogs had, at most, two nucleotide substitutions compared to the sequence of the reference strain (types B and B2). One strain showed 11 substitutions and was designated CcanC. The reference *C. cynodegmi* strain ATCC 49045 showed 20 nucleotide substitutions compared to the sequence of the *C. canimorsus* reference strain. The sequences of the six canine *C. cynodegmi* strains were highly related to that of the reference strain, showing only two to five nucleotide substitutions. Four strains from patients (98-196, 02-195, 01-351, and 02-39) showed a mixture of 2 nt at 21 positions (Fig. 2). At these positions, both a nucleotide corresponding to the reference sequence of *C. canimorsus* and a nucleotide corresponding to the reference sequence of *C. cynodegmi* were found. Interestingly, two sequences from GenBank, described as *C.*

Sequence	Position number according to AY 643075																																				
	45	46	47	48	57	58	60	71	85	145	151	154	155	156	165	167	168	181	182	222	235	246	260	271	356	408	411	412	424	426	431	450	451	454	
Ccan ATCC35979	G	A	G	T	A	C	T	C	A	A	G	T	G	G	C	G	A	C	T	T	T	C	C	G	T	A	C	A	G	C	A	T	G	T	CcanA
LUMC-HC3	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	CcanA
LUMC-HCJ15	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	CcanA
C.can cl 1*	.	G	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	CcanB1
602031	_	_	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	.	.	.	.	.	C	.	.	.	CcanB2
03-349	.	G	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	CcanA
03-612	.	G	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	CcanB
03-620	.	G	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	CcanB
05-29	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	CcanB
05-80	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	CcanA
97-16	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	CcanA
601251	T	.	T	A	.	A	A	.	.	A	A	.	.	A	T	.	.	T	.	.	.	.	.	.	.	.	.	G	.	.	T	.	.	.	CcanC
C.can cl 1*	.	G	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	CcanB1
02-39	.	.	.	.	R	.	.	Y	R	.	R	.	R	.	T	R	R	M	Y	W	Y	.	Y	K	.	R	Y	R	.	.	Y	Y	R	Y	Ccan/cyn
01-351	.	.	.	.	.	.	.	Y	R	.	R	.	R	.	Y	R	R	M	Y	W	Y	.	Y	K	.	R	Y	R	.	.	Y	Y	R	Y	Ccan/cyn
02-195	.	.	.	.	R	.	.	Y	R	.	R	.	R	.	T	R	R	M	Y	W	Y	.	Y	K	.	R	C	R	.	.	Y	Y	R	Y	Ccan/cyn
98-196	.	.	.	.	R	.	.	Y	R	.	R	.	R	.	T	R	R	M	Y	W	Y	.	Y	K	.	G	T	G	.	.	Y	Y	R	Y	Ccan/cyn
*C.can cl 1+4**	.	*R*	.	.	.	.	.	*Y*	*R*	.	*R*	.	*R*	.	*Y*	*R*	*R*	*M*	*Y*	*W*	*Y*	.	*Y*	*K*	.	*R*	*Y*	*R*	.	.	*Y*	*Y*	*R*	*Y*	*Ccan/cyn*
C.can cl 4*	.	.	.	.	.	.	.	T	G	.	A	.	A	.	T	A	G	A	C	A	C	.	T	T	.	G	T	G	.	.	T	C	A	C	CcynA
Ccyn ATCC49045	.	.	.	.	.	.	.	T	G	.	A	.	.	.	T	A	G	A	C	A	C	.	T	T	.	G	T	G	.	.	T	C	A	C	CcynA
LUMC-HA2	.	.	.	.	.	.	.	T	G	.	A	.	.	.	T	A	.	A	C	A	C	.	T	T	.	G	T	G	.	.	T	C	A	C	CcynA5
LUMC-HC2	.	G	.	.	.	.	.	T	G	.	A	.	.	.	T	A	.	A	C	A	C	.	T	T	.	G	T	G	.	.	T	C	A	C	CcynA6
LUMC-DogNido3	.	G	.	.	.	.	.	T	G	.	A	.	.	.	T	A	.	A	C	A	C	.	T	T	.	G	T	G	.	.	T	C	A	C	CcynA6
LUMC-HC1	.	.	.	.	G	.	.	T	G	.	A	.	.	.	T	.	.	A	C	A	.	.	T	T	.	G	T	G	.	.	T	C	A	C	CcynA7
LUMC-HA3	.	.	.	.	G	.	.	T	G	.	A	.	.	.	T	.	.	A	C	A	C	.	T	T	.	G	T	G	.	.	T	C	.	C	CcynA8
LUMC-HC8	.	.	.	.	G	.	.	T	G	.	A	.	.	.	T	A	.	A	C	A	C	.	T	T	.	G	T	G	.	.	T	C	A	C	CcynA9
LUMC-CatG1	.	.	.	.	G	.	.	T	G	.	A	G	.	A	T	T	.	A	C	A	C	T	T	T	.	G	T	G	A	T	T	C	A	C	Ccyncat
LUMC-CatE1	.	.	.	.	G	.	.	T	G	.	A	G	.	A	T	T	.	A	C	A	C	T	T	T	.	G	T	G	A	T	T	C	A	C	Ccyncat
Uncult.Capn.cat*.	.	.	.	.	.	.	.	T	G	.	A	G	.	A	T	T	.	A	C	A	C	.	T	T	.	G	T	G	A	T	T	C	A	C	Ccyncat
LUMC-DogNido1	A	G	C	.	.	G	C	.	G	.	A	.	T	.	.	.	G	A	A	.	.	.	T	T	.	G	G	G	.	G	T	.	C	C	Cnew**

FIG. 2. *rrs* sequence comparison (nt 38 to 492) of reference sequences of *C. canimorsus* (ATCC 35979 T, AY643075), *C. cynodegmi* (ATCC49044 T, AY643076), and a number of *Capnocytophaga* species strains used in the present study. Nucleotides differing from the reference sequence are shown. A dot indicates identity to the sequence of *C. canimorsus*, and a dash indicates a deletion. Sequences AY643077 (*C. canimorsus* 24231 clone 1), AY643078 (*C. canimorsus* 24231 clone 4), and AF426105 (an uncultivable *Capnocytophaga* species strain from cat) (marked with asterisks) are shown for comparison. Strain LUMC-DogNido1 (marked with a double asterisk) has 27 additional nucleotide changes from the sequence of the reference strain ATCC 35979, which are not shown in the figure. Nucleotide positions are according to the sequence of accession number AY643075.

	Type nt 38–492	1073–1109	1208
ATCC 35979	CcanA	TGTTAGTTGCTAGCGAGTC--AAGTCGAGCACTCTAACG	C
05-80	CcanA	GAA.....C.T.--A.T--....T.G.....TTT	.
03-349	CcanA	GAA.....C.T.--A.T--....T.G.....TTT	.
03-612	CcanB	GAA.....C.T.--A.T--....T.G.....TTT	.
03-620	CcanB	GAA.....C.T.--A.T--....T.G.....TTT	.
05-29	CcanA	GAA.....C.T.--A.T--....T.G.....TTT	.
97-16	CcanA	GAA.....C.T.--A.T--....T.G.....TTT	.
01-351	Ccan/cyn	KRW.....Y.K..M.ATCT.....WYK	W
02-39	Ccan/cyn	KRA.....Y.T.--A.T--....T.G.....TTT	A
02-195	Ccan/cyn	GAA.....C.T.--A.T--....T.G.....TTT	A
98-196	Ccan/cyn	KAA.....C.T.--A.T--..R.T.G.M.....TTT	A
C.can cl 1*	CcanB1	GAA.....C.T.--A.T--....T.G.....TTT	.
C.can cl 4*	CcynA	GAA.....C.T.--A.T--....T.G.....TTT	.
ATCC 49045	CcynA	.....--.....	A

FIG. 3. *rrs* sequence comparison (nt 493 to 1350) of selected *Capnocytophaga* species strains. Only nucleotides differing from the reference sequence are shown. For further information, see the legend to Fig. 2.

canimorsus 24231 clone 1 and clone 4, have been published, of which clone 1 is almost identical to the *C. canimorsus* reference sequence, and clone 4 is almost identical to the *C. cynodegmi* reference sequence. A mixture of these two sequences would result in almost the same sequence as the one we found. Since the sequence analysis of the 16S rRNA gene includes all copies of this gene on the chromosome, base pair differences in the various copies may result in ambiguous nucleotides in the consensus sequence. The two feline strains also were related to the *C. cynodegmi* sequence, but they showed four additional nucleotide differences and their sequences were almost identical to sequence AF426105, which originated from an uncultivable strain from a cat with sinusitis and rhinitis (8). These strains probably represent a new *Capnocytophaga* species, of which the pathogenicity is unknown. Finally, one strain, DogNido1, from a dog, had more than 40 nucleotide differences and was more similar to *C. haemolytica* than to other species. It should be noted that this strain was oxidase negative and probably represents a newly identified commensal canine *Capnocytophaga* species.

In addition, the complete sequence analysis of the *rrs* gene was performed for six human *C. canimorsus* strains as well as for the ambiguous strains 01-351, 02-39, 02-195, and 98-196 (Fig. 3). Homology for this region of the gene was even greater than the homology in the 5′ region of the gene. There was only one major difference in nt 1075 to 1112, in which all sequenced strains except strain 01-351 differed from the reference sequences of *C. canimorsus* and *C. cynodegmi*, which are identical in this region. The nine strains had 12 mutations and 2 deletions compared to the reference sequences. The same mutations were found in clones 1 and 4 of the above-mentioned *C. canimorsus* strain 24231 (AY643077 and AY643078, respectively). Nine of the 10 ambiguous nucleotides in this part of the sequence of the remaining strain 01-351 are a consensus of strain 24231 and of the type strains of *C. canimorsus* and *C. cynodegmi*.

We used the Ribosomal Database Project II-release10 tools Seqmatch and Tree Builder (http://rdp.cme.msu.edu/) to generate a phylogenetic tree (see the supplemental material). Whereas *C. cynodegmi* and *C. canimorsus* grouped into two different branches, a number of strains grouped between those branches as an independent group that could not be assigned to either species, although by *rpoB* RFLP and specific real-time PCR these strains clearly were *C. canimorsus*.

Development of specific real-time PCRs for *C. cynodegmi* and *C. canimorsus*. Real-time PCRs to detect *C. canimorsus* and *C. cynodegmi* were developed using the *rpoB* gene as the target gene. Amplification efficiency was optimal at an $MgCl_2$ concentration of 4.5 mM for the *C. canimorsus* PCR and 3.5 mM for the *C. cynodegmi* PCR. Optimal specificity was reached at 62°C (data not shown). At this temperature, 13 fg *C. cynodegmi* DNA still was detectable, whereas 70 pg *C. canimorsus* DNA gave no signal at all. The *C. canimorsus* PCR still was positive at an input of 7 fg. At an input of 130 pg, *C. cynodegmi* DNA resulted in a very weakly positive signal in the PCR, but a smaller amount gave no signal.

DNA of *C. ochracea*, *C. sputigena*, *C. gingivalis*, *C. haemolytica,* and *C. granulosa*, as well as DNA from 16 different bacterial and yeast species (see Materials and Methods), gave no reaction in PCRs for both *C. cynodegmi* and *C. canimorsus*.

We tested DNA from 25 *C. canimorsus* isolates from patients, from the 2 *C. canimorsus* strains from dogs, and from the 4 strains 98-196, 02-39, 02-195, and 01-351. All 31 strains gave a positive reaction in the *C. canimorsus* PCR only. Eight *C. cynodegmi* strains cultured from dogs all reacted in the *C. cynodegmi* PCR. The two strains from cats and the strain Nido1 did not react in either PCR.

Frequency of *C. canimorsus* and *C. cynodegmi* in dog mouths as assessed by real-time PCR. *C. canimorsus* DNA was detected in 39 out of 53 dogs (73%; 95% confidence interval [CI], 61 to 85%) in either a gum or a tongue swab, whereas *C. cynodegmi* DNA was detected in 51 out of 53 dogs (96%; 95% CI, 94 to 100%) (Table 3). Negative controls containing buffer only, which were tested regularly among the canine samples, reacted uniformly negatively in both PCRs.

Specimens from the tongue were slightly more frequently positive for *C. canimorsus* DNA. Quantitative results showed no differences between average cycle threshold (C_T) values of positive tongue and gum samples or between average C_T values in the PCRs for *C. canimorsus* and *C. cynodegmi*. Both dogs

TABLE 3. Occurrence of *C. canimorsus* and *C. cynodegmi* DNA in dogs, as assessed by species-specific PCR[a]

Specimen type	*C. canimorsus*			*C. cynodegmi*		
	+	+/−	−	+	+/−	−
Tongue	38	1	14	49		4
Gums	34	2	17	49		4
Any	39	2	12	51		2

[a] +, positive; +/−, weakly positive; −, negative.

that showed no detectable *C. cynodegmi* DNA also showed no detectable *C. canimorsus* DNA. No relationship between *C. canimorsus* positivity and breed, age, gender, or clinical status could be detected. For instance, most of the infection-negative dogs were young (1 year or younger), but some very young dogs also appeared to be positive. On the other hand, some of the older dogs appeared to be negative in the PCR. Also, the condition of the gums was not related to the presence or absence of *C. canimorsus* as detected by PCR.

C. canimorsus was grown from only 1 (5%) dog of the 21 dogs from which samples were cultured. *C. cynodegmi* was cultured from 6/14 dogs that were screened for the presence of this species. In the corresponding samples, DNA from *C. canimorsus* and *C. cynodegmi*, respectively, also was detectable.

DISCUSSION

Whereas biochemical methods still are useful to identify rapidly growing strains in the clinical microbiology laboratory, molecular methods are becoming more common to identify slowly growing strains. In addition, molecular methods are of use in identifying the strains in the case of inconclusive phenotypical data. We used *rrs* gene sequencing, the amplification of a specific target in the *rpoB* gene, and the PCR-RFLP of the *rpoB* gene as tools to characterize oxidase-positive *Capnocytophaga* species strains of human, canine, and feline origin.

The *C. canimorsus* strains from patients used in our study already had been characterized by *rrs* gene analysis and were found to be highly related to the type strain ATCC 35979. The *rrs* genes of the type strains of *C. canimorsus* and *C. cynodegmi* differ in only 19 nucleotides, resulting in a homology of 98.6%.

The four strains with heterogeneous sequences in the first 500 nt of the *rrs* genes had the same genetic distance to both *C. cynodegmi* and *C. canimorsus.* Ambiguous nucleotides in 16S rRNA gene analysis can be explained by the existence of multiple different gene copies in a strain. The *rrs* sequences of clones 1 and 4 from strain 24231 (AY643077 and AY643078) support this, since the consensus sequence of both clones results in the same ambiguous nucleotides as those of the four patient strains (Fig. 2). Sequencing the entire gene introduced more copy variation. The ambiguous nucleotides of strain 03-351 (Fig. 3) are in favor of the multicopy theory.

The sequences of the single-copy genes *rpoB* (AY643071) and *gyrA* (AY643074) of strain 24231 have a similarity of 98 to 99% to the *C. canimorsus* type strain. Therefore, we conclude that the *rpoB* gene is a better tool than the analysis of the *rrs* gene to distinguish between *C. canimorsus* and *C. cynodegmi.* Using the *rpoB*-based techniques, we conclude that the strains 01-351, 02-39, 02-195, and 98-196 belong to the species *C. canimorsus*.

C. canimorsus is a serious pathogen causing sepsis, meningitis, and occasionally other infections, such as keratitis and endocarditis, in healthy and immunocompromised patients. Infection usually is associated with contact with dogs. A few cases of infections have been described after contact with cats or without any animal contact. Whereas dog bites are very common, infections with *C. canimorsus* are rare. One explanation is that the prevalence of *C. canimorsus* in dogs is low. Our PCR study, however, showed that this is not the case, and that 74% of routinely tested dogs had *C. canimorsus* DNA in their mouths. *C. cynodegmi* DNA was found even more frequently, being present in 96% of the dogs. The higher frequency of *C. cynodegmi* is in contrast with the results of Westwell et al., who found *C. canimorsus* in 24% and *C. cynodegmi* in 11% of dogs after the culturing and biochemical identification of isolates. For culturing, we used sheep-blood agar plates containing gentamicin, whereas Westwell et al. used horse blood agar plates supplemented with cysteine, kanamycin, and vancomycin. It is possible that this accounts for the discrepancy. We were much less successful with the culture of *C. canimorsus*: we could culture a strain from only 2 out of 22 dog samples, and it turned out that both of these samples originated from the same dog, so our culture rate was only 1/21 (5%). We looked only for *C. cynodegmi* strains in the first 14 samples from different dogs and cultured this species from six dogs (43%). Our combined sensitivity for both species therefore can be calculated as 7/14 (50%). Our finding that *C. cynodegmi* was more frequently cultured from dogs than *C. canimorsus* is in agreement with our PCR findings. A much higher sensitivity for PCR compared to that of culture also has been shown for other slowly growing microorganisms, such as *Bordetella pertussis* (6). Apparently, many strains do not grow so well on the routinely used culture media. Unfortunately, a gold standard is lacking. Using our specific real-time PCRs, a panel of strains, including all so-far-described *Capnocytophaga* species, were not reactive, and two newly described *Capnocytophaga* species described in the present paper did not react in our PCRs. Therefore, we are confident that specific *C. canimorsus* and *C. cynodegmi* DNA was amplified from the samples. Also, the frequency of other difficult-to-culture pathogens in dogs, such as *Mycoplasma* (10), *Ehrlichia,* and *Babesia* (16), also have been approximated by PCR.

In a recent paper, the presence of *C. canimorsus* in dogs by culture was estimated at 60% (15). However, these authors used *rrs* gene sequencing and subsequent cluster analysis to discriminate between *C. canimorsus* and *C. cynodegmi*. The analysis of their 16S rRNA sequences (GQ167551 to GQ167622) shows that the 22 strains of a *C. canimorsus* subcluster had a maximal homology of only 95% with the type strains of *C. canimorsus* and *C. cynodegmi*. As indicated above, the identity of the other *C. cynodegmi* and *C. canimorsus* strains should be confirmed by other methods. The authors suggested that they could differentiate *C. canimorsus* and *C. cynodegmi* on colony morphology; this is not supported by the original colony description (3) and is not our experience. Mally et al. (15) found that the combined yield for both species, after correction for the subcluster, is 41%, which is comparable to our combined yield of 50%.

Whereas we could confirm that *C. canimorsus* is present in the mouths of most dogs, we could not confirm that these dogs harbored the same genotype. With PCR-RFLP, we could distinguish three different PCR-RFLP types. Although our two dog strains were in the least common group, more dog strains need to be cultured and typed to see whether all *C. canimorsus*-positive dogs can infect patients with this pathogen. A toxin produced by some strains could be a virulence factor (7); however, we could not confirm its presence (data not shown), and in a recent publication a toxin was not found (21). Recently, some potential virulence mechanisms for *C. canimorsus* have been revealed. It was shown that human macrophages incubated with *C. canimorsus* failed to produce tumor necrosis factor alpha, interleukin-1α (IL-1α), IL-6, Il-8, and gamma interferon; that TLR-4 could not be activated by the reference strain; and that even tumor necrosis factor alpha release by another pathogen, *Yersinia enterocolitica*, was impaired by live *C. canimorsus* (21). The resistance to phagocytosis of *C. canimorsus* by macrophages and the blocking of the ability of macrophages to kill other bacteria also were demonstrated by the same group of investigators (17), as well as its resistance to killing by complement and polymorphonuclear leukocytes (22). However, *C. canimorsus* sepsis is characterized by extensive signs of inflammation, and alternative virulence mechanisms should be present. It would be highly interesting to look at these features in a number of genetically well-characterized *C. canimorsus* strains obtained from dogs.

In addition to *C. canimorsus* and *C. cynodegmi*, we also obtained evidence for the existence of other, so-far-uncultured oxidase-positive *Capnocytophaga* spp. in dogs' and cats' mouths. The virulence potential of such species still is unknown. A case report describing a cat with sinusitis and rhinitis (8) showed the presence of DNA in a supernatant on a cell culture vial that was identical to that of strains LUMC-CatG1 and LUMC-CatE1; the authors, however, were not able to culture the strain. It is not clear whether this strain was a pathogen. The catalase-negative, oxidase-positive strain LUMC-DogNido1 is a novel type for which pathogenic properties still are unknown.

The rarity of *C. canimorsus* infections in humans in spite of their abundance in dog mouths as well as the almost complete absence of infections with other canine *Capnocytophaga* species remain unaccounted for. The further analysis of strains and patients should reveal specific host and bacterial factors contributing to severe sepsis in selected cases.

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