



Polymerase Chain Reaction Detection of *Capnocytophaga canimorsus* and *Capnocytophaga cynodegmi* as the Emerging Zoonosis

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Abstract

Background: *Capnocytophaga canimorsus* and *C. cynodegmi*, as gram-negative rods, are commensal in the oral cavities of dogs and cats and *C. canimorsus* can occasionally cause fatal systemic infections in humans. In addition, most human infections, caused by *C. canimorsus*, are associated with dog bites, licking pre-existing wounds, or the other contact with dogs. Therefore, the aim of this study was to evaluate the role of polymerase chain reaction (PCR) in detecting *C. canimorsus* and *C. cynodegmi* in oral swab samples of dogs and cats.

Methods: To this end, oral swabs were taken from 125 dogs and 35 cats enriched in the anaerobic atmosphere and then were used for DNA isolation and evaluated by PCR.

Results: In 125 dog oral swabs, DNA of *Capnocytophaga* genus was prespecified in 32% of these samples (n=40). Further, 20 and 36 samples were positive for DNA of *C. canimorsus* and *C. cynodegmi*, respectively, when PCR was performed for the DNA of *Capnocytophaga* spp. Similarly, 23 out of 35 oral swabs of the cats were positive for the presence of the DNA of *Capnocytophaga* genus. Finally, all the samples were positive for the DNA of *C. cynodegmi* when they were analyzed by the primer specified *Capnocytophaga* species while only 15 samples were positive for the presence of DNA of *C. canimorsus*.

Conclusions: Overall, the risk of infection with *C. canimorsus* is high because of the presence of the bacteria in the mouth of cats and dogs and severe consequences of infection for humans. Therefore, the owners of cats and dogs should be informed about this risk, especially if the owners belong to specific risk groups like young children, pregnant women, elderly people, and immunocompromised patients.

Keywords: *Capnocytophaga*, *C. canimorsus*, *C. cynodegmi*, PCR, Iran

Background

The members of genus *Capnocytophaga* are fastidious, Gram-negative, rod-shaped bacteria which belong to the family of *Flavobacteriaceae* (1) and reside in the oral cavities of humans and domestic animals. Eight species of bacteria including *C. ochracea*, *C. sputigena*, *C. gingivalis*, *C. granulosa*, *C. leadbetteri*, *C. haemolytica*, *C. canimorsus*, and *C. cynodegmi* (2) are the members of this genus. All species except for *C. canimorsus* and *C. cynodegmi* reside in the human oral cavity (3,4) and may cause periodontal diseases and severe infections in immunocompromised patients (3,5). According to several studies (1,2,6-8), these two bacteria reside in the oral cavities of dogs and cats and may be transmitted to humans and infect local open wounds causing systemic infection in humans sometimes through bites (54% of cases), scratches (8.5% of cases), or even by licks (27% of cases). Recently, Butler isolated a new species, named *C. canis*, from the oral cavity of healthy dogs and found that this genus has low pathogenicity for humans (6). *C. canimorsus* is the causative agent of wound

infections and may cause disseminated infections, followed by sepsis, meningitis, and endocarditis (3-5,7). On the other hand, systemic infection is rarely caused by *C. cynodegmi* while it is mainly detected in wound infections (2,5,7,9). According to different reports, this organism is susceptible to penicillin. However, the prognosis of *C. canimorsus* infection is poor in chronic alcohol users, as well as asplenic and immunocompromised patients and thus can cause an overall mortality of 30% (4-7,10). For this reason, rapid identification to species level with the accurate and rapid initiation of treatment with appropriate antimicrobial agents is very important (3).

However, except for a few cases that occur following the cat bites and scratches, most of the infections in humans caused by *C. canimorsus* are related to dog bites, dogs licking pre-existing wounds, or other contacts with dogs (9,11,12). Nevertheless, it is possible that close animal contact or a superficial abrasion may provoke the event. The history of a dog bite is elicited in only 43% to 57% of the cases and exposure to dogs without bites or scratches are reported in

12% to 27% of the cases (9,10). Local wound infections rapidly lead to life-threatening septicemias correlated with intravascular coagulation, generalized purpura, and severe hemorrhages in adrenal glands, and finally, failure in internal organs (13).

After prolonged incubation (48 to 72 hours), colonies appear and rapidly growing bacteria are probably overgrown because the organisms are relatively fastidious and of slow-growing type. Meanwhile, many strains produce a yellow-brown pigment (14-16). The results of oxidase, catalase, ONPG (O-nitrophenyl-b-D-galactoside), and arginine dihydrolase are positive for *Capnocytophaga* spp. while they are negative for urease, nitrate, indole, DNase, gelatin, lysine, and ornithine (9,17). On the other hand, biochemical tests are difficult to perform due to the slow growth of bacteria.

Likewise, the identification of *C. canimorsus* and *C. cynodegmi* is very difficult owing to the similarities in genetic properties and physiological activity between these two species. Therefore, there is a need for the development of more acceptable and specific molecular techniques for identifying the *Capnocytophaga* spp. (18). In the current study, a polymerase chain reaction (PCR) system, described by Suzuki et al, was used to differentiate between the above-mentioned species (19).

Considering the importance of canine and feline reservoir hypothesis, obtaining more information about the epidemiology and genetics of *Capnocytophaga* spp. from the dogs and cats help to control the transmission and treatment of *Capnocytophaga* infections and to improve the overall public health. Thus, the present study aimed to investigate the role of the traditional method and PCR in identifying *Capnocytophaga* spp. The results (backed up by *16S rRNA* gene sequencing) showed that this method reliably identifies *C. canimorsus* and *C. cynodegmi* to species level.

Methods

Specimens Collection

Oral swabs were obtained from 125 dogs and 35 cats of the same age, sex, and breed during October 2014-June 2015. The animals were routinely admitted to the veterinary teaching hospital or animals were maintained for teaching at the Faculty of Veterinary Medicine, Shiraz University. In an attempt to sample a representative portion of the population, specimens were collected at irregular intervals. After getting the informed consent from the owners, swabs were taken from the animals attending this teaching hospital for various reasons. Cotton-tip sterile swabs were rubbed on the gums and tongue and then suspended in nutrient broth and transferred to the laboratory of microbiology under cold condition. Next, the cotton-tipped applicators were suspended in brain heart infusion broth (Merck, Germany) as enriched cultures for 24-48 hours at 35°C in the atmosphere containing 5% CO₂.

DNA Extractions

A 100 µL of enriched cultured samples were used for DNA extraction and the total DNA was prepared from enrichment cultures using Gram-negative DNA extraction kit (Cinnagen, Iran) according to the instructions of the kit. The extracted DNA were determined to be of good quality and DNA concentration was measured using Nanodrop (10000V 3.52). In addition, DNA concentrations were adjusted to 21.8 ng/µL before PCR amplification. Finally, extracted DNA samples were stored at -20°C for further use.

It should be noted that the primers targeting the *16S rRNA* gene of *C. canimorsus* and *C. cynodegmi* were obtained from the study by Suzuki et al (19).

Polymerase Chain Reaction

The reaction mixture solution was prepared in 25 µL volume for each reaction that contained 11.3 µL dH₂O, 2.5 µL PCR buffer, 1.5 µL (1.5 mM) MgCl₂, 1.5 µL (200 mM) dNTPs, 2 µL (100 nmol) of each oligonucleotide primers, 0.2 µL (5 U/µL) of *Taq* DNA polymerase (Cinnagen, Iran), and 4 µL template DNA. After initial denaturation (at 95°C for 5 minutes), the amplification conditions encompassed denaturation at 95°C for 30 seconds, annealing at 58°C for one minute, and extension at 72°C for one minute. This was repeated for 35 cycles in a block assembly 96G thermocycler (Analytic Jena, Germany) with a final extension of 72°C for 7 minutes.

The purified DNA of *C. canimorsus* (ATCC35979) and *C. cynodegmi* (ATCC49044) strains, kindly donated by Dr. M. Suzuki, and sterile nuclease-free distilled water were used as appropriate positive controls and negative control, respectively. The positive control DNA sample was used to test PCR validity, consistently leading to the expected PCR products. The employed samples were as follows.

- CAL2-AS2 (CAL2: 5'GTAGAGTGCTTCGG-CACTTG3' and AS2: 5'GTGATGCCACCAAA-CAATACTA3') for *Capnocytophaga* genus;
- CAL2-CaR (CAL2: 5'GTAGAGTGCTTCGG-CACTTG3' and CaR: 5'GCCGATGCTTATTCAT-ACA3') for *C. canimorsus*;
- CAL2-CyR (CAL2: 5'GTAGAGTGCTTCGG-CACTTG3' and CyR: 5'GCGGATGCTTATTCG-TATG3') for *C. cynodegmi*.

Electrophoresis was performed to examine the PCR products. These products were evaluated by adding 2 µL of the loading buffer to 8 µL of PCR product and loaded into the wells of a 2% (w/v) agarose gel (Agarose I; Cinnagen, Iran) containing 0.5 µg/mL ethidium bromide. Then, the amplicon size a 100 bp DNA ladder (Cinnagen, Iran) was loaded to estimate PCR. Further, the gel was immersed in Tris-Borate-EDTA (TBE) buffer and subjected to a voltage difference of 100 V in order to separate the fragments. Eventually, visualization was undertaken using an ultraviolet transilluminator (BTS-20, Japan) and the

resulted image was captured by a computer software program (AlphaEase, Alpha Innotech).

Results

The extracted DNAs from all enrichment cultures were used as the template for *16S rRNA* specific primer pairs. Further, three primer sets were used, which amplify the fragments of *16S rDNA* gene of *Capnocytophaga* genus, as well as *C. canimorsus* and *C. cynodegmi* species and should yield the PCR fragments of 124, 427, and 427 bp, respectively (Figures 1-3). Then, the primer set was tested on the purified DNA of *C. canimorsus* (ATCC 35979) and *C. cynodegmi* (ATCC 49044). The presence of *Capnocytophaga* genus, along with *C. canimorsus* and *C. cynodegmi* species DNA in the enrichment cultures was thus indicated by positive PCRs for *16S rDNA* gene. Furthermore, the DNA of *Capnocytophaga* genus was prespecified in 32% (n=40) of the total 125 oral swabs of the dogs. When PCR was conducted for the presence of the DNA of *C. canimorsus* and *C. cynodegmi*, 20 (50%) and 36 samples were found positive for the DNA of *C. canimorsus* and *C. cynodegmi*, respectively. Moreover, from 35 oral swabs of the cats, 23 samples were detected positive for the presence of the DNA of *Capnocytophaga* genus. Based on the results, all the samples analyzed by the primer specified *Capnocytophaga* species were positive for DNA of *C. cynodegmi* although only 15 samples were detected as positive for the presence of the DNA of *C. canimorsus* (Figures 1- 3 and Table 1).

Additionally, primers in different combinations were utilized for determining the specificity of PCR to amplify the *16S rRNA* gene of *C. canimorsus* and *C. cynodegmi*. Only the target sequence in the DNA of *Capnocytophaga* was amplified by using the CAL2 and AS1 primers while no PCR products were obtained from the DNA of other bacterial strains included in this experiment (data not

available). Similarly, only amplicon was achieved from the DNA of *C. canimorsus* when using CAL2 and CaR primer pair. On the other hand, primers CAL2–CyR produced a single specific band of about 427 bp from the DNA of *C. cynodegmi* (Table 1, Figures 2 and 3).

Discussion

The members of the genus *Capnocytophaga* are gram-negative, fastidious, catalase, oxidase negative rods (i.e., *C. canimorsus* and *C. cynodegmi* are catalase and oxidase positive) and are considered as one of the emerging bacterial zoonotic diseases. The organism is a part of the oral microbiota of dogs and cats, which cause no disease in these animals. These agents can cause different infectious diseases when they are transmitted to a human, directly or indirectly. For example, it leads to sudden and acute septicemia with disseminated intravascular coagulation that involves many internal organs. Middle-aged and elderly persons are at a greater risk of disease contraction. In addition, individuals who spend a greater portion of their time with canines and felines such as veterinarians, breeders, pet owners, and lab workers are included in a

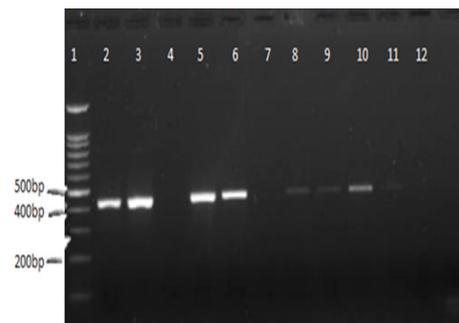


Figure 2. PCR Amplification Profile of *Capnocytophaga canimorsus* From DNA Directly Isolated From Samples With *16S rDNA* Primers
Note. Lane 1: 100 bp DNA ladder; Lane 2: Positive control; Lanes 3, 5, 6, 8, 9, 10, and 11: Some positive samples (amplicon size 427 bp); Lane 12: Negative control.

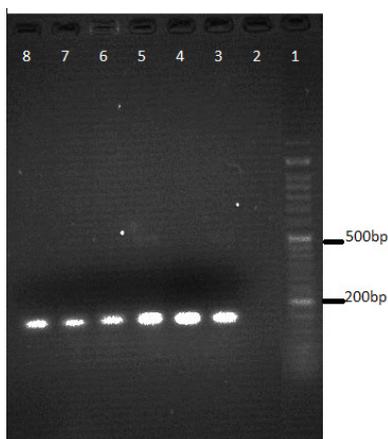


Figure 1. PCR Amplification Profile of *Capnocytophaga* From DNA Directly Isolated From Samples With CaL2–AS1 Primers.
Note. Lane 1: 50 bp DNA ladder; Lane 2: Negative control; Lane 3: Positive control; Lanes 4-8: Some positive samples (amplicon size 124 bp).

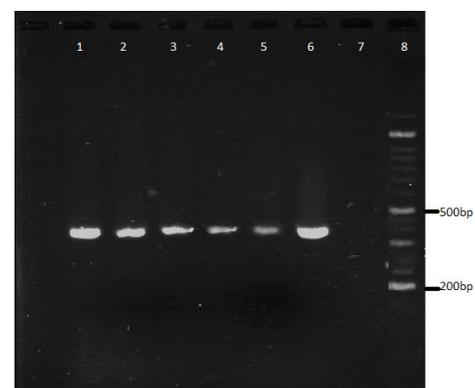


Figure 3. PCR Amplification Profile of *Capnocytophaga cynodegmi* From DNA Directly Isolated From Samples With CAL2–CyR Primers Set
Note. Lanes 1-5: Some positive samples (amplicon size 427 bp); Lane 6: Positive control; Lane 7: Negative control; Lane 8: 50 bp DNA ladder.

Table 1. PCR Results for the Presence of *Capnocytophaga* DNA in Different Samples

Sample Source	Number of Samples	Positive for <i>Capnocytophaga</i>	Positive for <i>C. canimorsus</i>	Positive for <i>C. cynodegmi</i>	Positive for Both Species
Swabs of the dog	125	40 (32%)	20 (50%)	36 (90%)	16 (40%)
Swabs of the cat	35	23 (65.7%)	15 (62.2%)	23 (100%)	8 (22.86%)
Total	160	63	35	59	24

Abbreviation: PCR, polymerase chain reaction.

higher risk category, especially immunocompromised individuals. Further, the chance of infection varies between 3% and 20% after dog bites and as regards the bites of the cats, it may be even more. Nevertheless, it is conceivable that close animal contact or a trivial lesion may be the inciting event. Likewise, a history of a dog bite is obtained only from 43% to 57% of the cases and exposure to dogs without bites or scratches is reported in 12% to 27% of the cases. Finally, no report is available regarding man to man transmission (20).

Although biochemical methods are still useful for identifying rapidly growing bacterial strains in clinical microbiology laboratories, molecular methods are becoming more common for detecting slowly growing strains. Several molecular methods are currently available for the identification and differentiation of bacterial species. Indeed, *16S rRNA* gene sequencing is regarded as the “gold standard” for bacterial identification.

Generally, many studies were conducted regarding the isolation and identification of *Capnocytophaga* spp. using different methods (1,18,19,21-24).

According to different studies, the prevalence of *C. canimorsus* in the dog samples was 8, 24, and 25%, respectively, when using the culture (21-23). On the other hand, other researchers such as Gaastra and Lipman (18), Mally et al (1), and van Dam et al (24) found that the prevalence of *C. canimorsus* was 41%, 57%, and 73%, respectively, in dog samples by using the PCR. These data indicate that the sensitivity of PCR for the detection of *C. canimorsus* is more than that of traditional microbiological methods such as culture and phenotypical traits.

Suzuki et al established a specific PCR which could identify and distinguish *C. canimorsus* from *C. cynodegmi* (19). Using this method, they determined the prevalence of *Capnocytophaga* spp. in dogs and cats. Based on their finding, *C. canimorsus* was detected in 74% of dogs and 57% of cats and *C. cynodegmi* was found in 86% of dogs and 84% of cats. The prevalence of *Capnocytophaga* spp. obtained in this study is somewhat higher than those previously reported where the bacterial isolation method was used for identification. This is probably because PCR detection is more sensitive compared to the bacterial culture concerning detecting *C. canimorsus* and *C. cynodegmi* in samples taken from dogs and cats.

Similarly, Umeda et al genetically compared *C.*

canimorsus isolates using *16S rRNA* gene sequence analysis and pulsed field gel electrophoresis and indicated that *C. canimorsus* was detected in 69.7% of dogs and 54.8% of cats (25).

The PCR method, established by Suzuki et al (19), can be applied to identify and distinguish *C. canimorsus* from *C. cynodegmi* since it is rapid and sufficiently sensitive. In our research, the prevalence of *Capnocytophaga* spp. in dogs and cats was determined using the PCR method. The *16S rRNA* gene of *Capnocytophaga* spp. was observed in 32% of dog and 65.7% of cat samples. According to our finding, the *16S rRNA* gene of *C. canimorsus* and/or *C. cynodegmi* was detected in 20% and 90% of dogs and 62.5% and 100% of cats, respectively. This finding demonstrated that 16 dog samples and 8 cat samples contained both *Capnocytophaga* spp. The prevalence of *C. canimorsus* was lower in dogs compared to the cats, but *C. cynodegmi* was detected in roughly a similar proportion in both animal groups. Based on some previous reports, the prevalence of *Capnocytophaga* spp. by bacterial isolation was shown to be 36-60% for dogs and 24% for cats (1,22). However, the detection rate for *C. canimorsus* was 69.7% and 54.8% in dogs and cats, respectively, when using the PCR technique (25).

In the present study, the DNA of *Capnocytophaga* spp. was detected in 32% and 65.7% of samples through the PCR test, respectively.

Furthermore, our finding revealed that the detection of the DNA of *C. canimorsus* in 50% and 62.2% of dog and cat samples, respectively. Conversely, these findings were 90% and 100% in dogs and cats, respectively, respecting the DNA of *C. cynodegmi*. The findings of the current study are roughly in line with the findings of a study by Suzuki et al in Japan.

Conclusions

To the best of our knowledge, our finding is the first report about the presence of *Capnocytophaga* spp. in the swab samples of dog and cat in Iran. The risk of infection with *C. canimorsus* is high owing to the high percentage of *Capnocytophaga* in the mouth of the cats and dogs and the severe consequences of infection for humans. Accordingly, cat and dog owners should be notified about this risk, especially if they belong to specific risk groups like elderly people, pregnant women, young children, and immune

deficient patients.

Ethical Approval

Not applicable.

Conflict of Interest Disclosures

On behalf of all authors, the corresponding author declares that there is no conflict of interests.

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