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RESEARCH ARTICLE

Detection of *Capnocytophaga canimorsus* and *Capnocytophaga cynodegmi* by Cultural and Molecular Methods in Dogs in Western Turkey [1]

Volkan ÖZAVCI [1] Şükrü KIRKAN [1]

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[1] Department of Microbiology, Faculty of Veterinary Medicine, Adnan Menderes University, TR-09016 Aydin - TURKEY

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Summary

The aim of this study is to exhibit the presence of *Capnocytophaga* species in Turkey and to determine the regional risks of these bacteria for the people in dogs. Totally 200 oral swab samples were taken from owned dogs which have had dental plaque problems. The diagnosis of *Capnocytophaga* infections were done by PCR using *CaL2, AS1, CaR* and *CyR* gene sequence primers. The first PCR was carried out to samples using by *CaL2* and *AS1* primers and results were estimated as *Capnocytophaga* species. The second PCR was applied to samples using *CaR* and *CyR* reverse primers and results were also interpreted as being *Capnocytophaga canimorsus* and *Capnocytophaga cynodegmi*. At the end of the first PCR, *Capnocytophaga* spp. was detected from 11 (5.5%) out of 200 samples. *CaR* and *CyR* gene were investigated in samples which were detected as *Capnocytophaga* spp. It was determined that 2 (18.2%) of the samples were positive for *CaR* gene which were identified as *C. canimorsus* and 9 (81.8%) of the samples were positive for both *CaR* and *CyR* gene which were identified as *C. canimorsus* and *C. cynodegmi*. *C. canimorsus* and *C. cynodegmi* species are concluded for generating risk for human health and due to the lacking of information about the disease it was also difficult to diagnosis these agents.

Keywords: *Capnocytophaga canimorsus, Capnocytophaga cynodegmi, Dog, Identification, PCR*

Batı Türkiye'de Köpeklerde *Capnocytophaga canimorsus* ve *Capnocytophaga cynodegmi* Türlerinin Kültürel ve Moleküler Yöntemlerle Araştırılması

Özet

Bu çalışma ile *Capnocytophaga* türlerinin Türkiye'deki varlığının ortaya çıkarılması ve köpeklerde bulunan bu bakterilerin insanlar için bölgesel risklerinin belirlenmesi amaçlanmıştır. Çalışmada dıştaşı olan sahipli köpeklerden 200 adet oral svap örneği toplandı. *Capnocytophaga* infeksiyonlarının tanısı için *CaL2, AS1, CaR* ve *CyR* gen sekans primerleri kullanılarak PCR uygulandı. İlk PCR işlemi *CaL2* ve *AS1* primerleri kullanılarak yapıldı ve sonuçta *Capnocytophaga* spp. identifikasyonları gerçekleştirildi. İkinci PCR işleminde *CaR* ve *CyR* reverse primerleri kullanıldı ve sonucunda *Capnocytophaga canimorsus* ve *Capnocytophaga cynodegmi* identifikasyonları gerçekleştirildi. İlk PCR işleminden sonra, 200 örneğin 11 (%5.5)'inden *Capnocytophaga* spp. identifiye edildi. *Capnocytophaga* spp. olarak identifiye edilen örneklerde *CaR* ve *CyR* gen bölgeleri araştırıldı. Bu örneklerin 2 (%18.2)'sinde *CaR* geni pozitif bulundu ve örnekler *C. canimorsus* olarak identifiye edildi. Örneklerin 9 (%81.8)'unda *CaR* ve *CyR* genleri pozitif bulundu ve bu örnekler *C. canimorsus* ve *C. cynodegmi* olarak identifiye edildi. *C. canimorsus* ve *C. cynodegmi* türleri insan sağlığı için risk oluşturmaktadır. Bu infeksiyonlar hakkındaki yetersiz bilgi, infeksiyonların tanısını ve bu bakterilerin identifikasyonlarını zorlaştırmaktadır.

Anahtar sözcükler: *Capnocytophaga canimorsus, Capnocytophaga cynodegmi, Köpek, İdentifikasyon, PCR*

INTRODUCTION

Millions of people are somehow bitten by animals every year in the world. Bite wounds cause complications ranging from mild injuries to serious infections. These infections may be related to the mouth flora of the animal

İletişim (Correspondence)
+90 256 2470700
ozavcivolkan@hotmail.com

and/or pathogens available on the skin flora of bitten person. The most frequently seen bacterial zoonotic agents, transmitted to the humans by animal bite, are *Pasteurella multocida, Bartonella henselae, Spirillum minus, Streptobacillus moniliformis, Francisella tularensis* and *Capnocytophaga canimorsus (C. canimorsus)* [1,2].

C. canimorsus, formerly designated Dysgonic fermenter 2 (DF-2) was first described in 1976; it is a commensal bacterium of dogs and cats saliva, which can be transmitted to man by bite (54% of cases), scratch (8.5%), or mere exposure to animals (27%) [3]. The infection distribution with dogs and cats are 24% and 10%, respectively [4]. *Capnocytophaga gingivalis, Capnocytophaga ochracea* and *Capnocytophaga sputigena* species caused localized juvenile periodontitis on the immunocompetent hosts and could be isolated from other regions as part of polymicrobial infections [3].

C. canimorsus and *C. cynodegmi* have regularly been isolated from the oral cavities of dogs. These two species are very similar in morphology and in small subunit ribosomal RNA gene (16S rRNA) sequence with a 97% similarity [5].

C. canimorsus infections were found to occur worldwide and have been reported from the United States, Canada, Europe, Australia and South Africa [6]. Australia has a canine population of about 3.75-4 million. It is estimated that around 63% of Australian households own some type of pet, with 53% owning a cat or a dog. It has been projected that each year more than 100.000 Australians are attacked by dogs, causing injuries of varying degrees of severity. The Emergency Department of Australia's public hospitals treat between 12.000 and 14.000 people for dog bite injuries and almost 1400 of those have injuries that are serious enough to warrant hospitalization. Between 4% and 25% of dog bite wounds become infected. Bacteria also caused to cellulitis, fatal sepsis, organ failure, meningitis and endocarditis [7].

Dilegge et al.[8] showed that 49.2% of canine samples (59 positive out of the total 120) carried a species of *Capnocytophaga* spp. Of the total number of canines sampled, 21.7% of which (26 positive) carried *C. canimorsus*, and 11.7% (14 positive) carried *C. cynodegmi*. Four canines carried *C. ochracea*, one canine carried *C. haemolytica*, and one carried an isolate that was either *C. gingivalis* or *C. granulose, C. canimorsus* was characterized by very mild symptoms or lead to fatal infections such as sepsis with showing severe symptoms. *C. cynodegmi* has a low possibility of creating systemic infection. Therefore most of the *Capnocytophaga* infections that have been reported were contiguous with the oropharynx, including periodontal diseases, ophthalmic lesions, respiratory tract infections, traumatic pericarditis, mediastinal or cervical abscesses and local wound infections [9,10].

Genetic and biochemical similarities between *C. canimorsus* and *C. cynodegmi* make it rather difficult to identify these species and thus there is a need for specific and enhanced molecular methods for both species identification. Reports about the presence of *C. canimorsus* strains showed that this bacteri is hardly distinguished from *C. cynodegmi* by the comparison of the 16S rRNA sequences. Therefore, to develop more convenient and specific PCR systems to identify the *Capnocytophaga* spp. is required [11].

The aim of this study is to exhibit the presence of *Capnocytophaga* species in Western of Turkey and to point out a risk by these bacteria for the human population in the region.

MATERIAL and METHODS

Isolation of Capnocytophaga spp.

A number of 200 oral swabs were taken by the convenient technique from domestic dogs having tartar in Muğla provinces in Western of Turkey the dates between January and April 2012. Samples were delivered to Adnan Menderes University Veterinary Faculty of Microbiology Department in cold chain. Of the 200 swabs samples, 77 (38.5%) were from male dogs and 123 (61.5%) were from female dogs. This study was conducted according to the Ethical Committee regulations of Adnan Menderes University (document ID 2009/54). The distribution of the samples with year and sex were shown on the *Table 1*.

It was aimed to obtain pure culture of *Capnocytophaga* spp. from oral swab samples arrived to the laboratory. For the purpose preventing the other flora bacteria growth, oral swab samples were inoculated onto sheep blood agar including gentamicin. The cultivated agar plates were incubated in 5% CO_2 atmosphere for 5 days. After the incubation Gram staining method were applied to suspected colonies. Biochemical tests were applied to the strains for the identification of *Capnocytophaga* spp.[12].

Extraction of DNA from Oral Swabs

Samples were collected using sterile swabs from 200 dogs gingival tissues (BD BBL culture swab plus) and these samples were suspended with Brain-Heart Infusion Broth and were incubated at 5% CO_2 microaerophilic atmosphere

Table 1. *The distribution of swab samples taken from dogs by year and sex*

Tablo 1. *Köpeklerden alınan svap örneklerinin yaş ve cinsiyete göre dağılımları*

Dogs Groups by Years	Number of Male Dogs	Number of Female Dogs	Total Number of Dogs
0-5	63	107	170
6-9	13	15	28
10 and over	1	1	2
Total	77	123	200

for 24 h at 35°C. Bacterial cells were harvested from broth culture and passed to DNA extraction application. DNAs were extracted from swab samples with Ultraclean Microbial DNA Isolation Kit® (MO BIO Laboratories, Inc.) as recommended by the manufacturer. DNA extracts were stored in cryo-tubes at -20°C until PCR studies [13].

Primers and PCR

CaL2, AS1, CaR, and *CyR* primers were designed for the determination for *Capnocytophaga* spp which informed as Suzuki et al.[12].

In order to obtain positive control DNAs for the PCR assays, two strains of *C. canimorsus* and *C. cynodegmi* were kindly provided by Michio SUZUKI (1-23-1 Toyama Shinjuku-ku Tokyo, Japan).

In the first PCR protocol we used, total volume of 50 µl for a sample in PCR amplification were adjusted as follows for identification of *Capnocytophaga* spp.; ViBuffer A (Vivantis®) 10x enzyme buffer solution 1x, magnesium chloride ($MgCl_2$) 50 mM, 10x dNTP, primer (for *CaL2-AS1* primer pair) 10 pmol, Pfu DNA polymerase 5U (Vivantis®) [12].

The second PCR amplifications were performed to in the same conditions using by *CaL2-CaR* and *CaL2-CyR* primers to identify *C. canimorsus* and *C. cynodegmi* [12].

PCR conditions were as follows; an initial denaturation at 95°C for 5 min, followed by 35 cycles of denaturation at 95°C for 30 s, annealing at 58°C for 1 min, extension at 72°C for 1 min, and a final extension at 72°C for 7 min [12]. PCR was performed in an Eppendorf Master Cycler with a capacity of 25 samples thermal cycling.

Detection of the Amplified Products

The 10 µl amplified products were detected by staining with 0.5 µg/ml ethidium bromide after electrophoresis at 80 V for 40 min in 2% agarose gels. PCR products of 124 bp for *Capnocytophaga* spp. and 427 bp for *C. canimorsus* and *C. cynodegmi* were considered evidence for identification.

RESULTS

Isolation of Capnocytophaga spp.

The *Capnocytophaga* spp. was not identified from all oral samples by conventional and biochemical tests.

PCR

By this work as a part of a postgraduate thesis, the diagnosis of *Capnocytophaga* infections which could not be made as routinely, were shown to be done by using *CaL2*, *AS1, CaR* and *CyR* gene sequence primers. In this study, 200 oral swab samples were taken from owned dogs which have dental plaque and at the end of first PCR, *CaL2* and *AS1* gene were detected from 11 (5.5%) out of 200 samples. So, these samples were evaluated as *Capnocytophaga* spp. After the DNAs of the agent identified as *Capnocytophaga* spp. they were evaluated for being positive for *C. canimorsus* or *C. cynodegmi* using *CaR* and *CyR* gene nucleotide sequences at second PCR. It was also determined that 2 (18.2%) of the samples were positive for *CaR* gene which were identified as *C. canimorsus* and 9 (81.8%) of the samples were positive for both *CaR* and *CyR* gene which were identified as *C. canimorsus* and *C. cynodegmi*. Apmlified PCR products were shown at *Fig. 1* and *2* which were positive for *Capnocytophaga* spp. and *C. canimorsus* and *C. cynodegmi*, respectively.

As the result of this study, 2 (22.2%) positive samples were detected as *C. canimorsus* and *C. cynodegmi* from male dogs and 7 (78.2%) from females. Only 2 positive samples were detected as *C. canimorsus* (100.0%) from female dogs totally. Rates for *C. canimorsus* and *C. cynodegmi* positive according to year were shown on the *Table 2*.

The distribution of positivity by year is examined and the results are as follows; out of the 11 dogs were detected as *Capnocytophaga* spp. one (9.1%) was from 6-9 year group and . ten (90.9%) were from 0-5 year group dogs. Out of nine that found as *C. canimorsus* and *C. cynodegmi* eight were (88.8%) from 0-5 year group and1 (11.2%) was

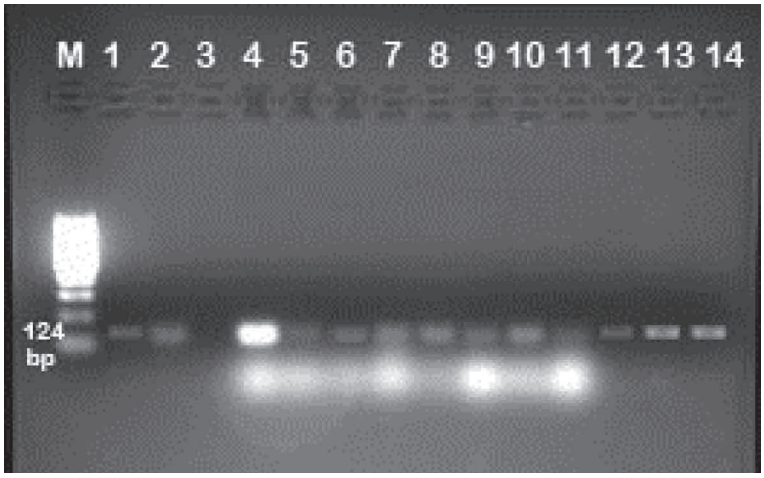

Fig 1. *Capnocytophaga* spp. PCR results (using *CaL2-AS1* primers), **M:** 100bp DNA ladder, **1:** *Capnocytophaga canimorsus* Positive Control, **2:** *Capnocytophaga cynodegmi* Positive Control **3:** Negative Control, **4-14:** *Capnocytophaga* spp. PCR Positive Samples

Şekil 1. *Capnocytophaga* spp. *CaL2-AS1* primerleri kullanılarak elde edilen PCR sonuçları, **M:** 100bp DNA işaretleyicisi, **1:** *Capnocytophaga canimorsus* Pozitif Kontrol, **2:** *Capnocytophaga cynodegmi* Pozitif Kontrol **3:** Negatif Kontrol, **4-14:** *Capnocytophaga* spp. PCR Pozitif Örnekler

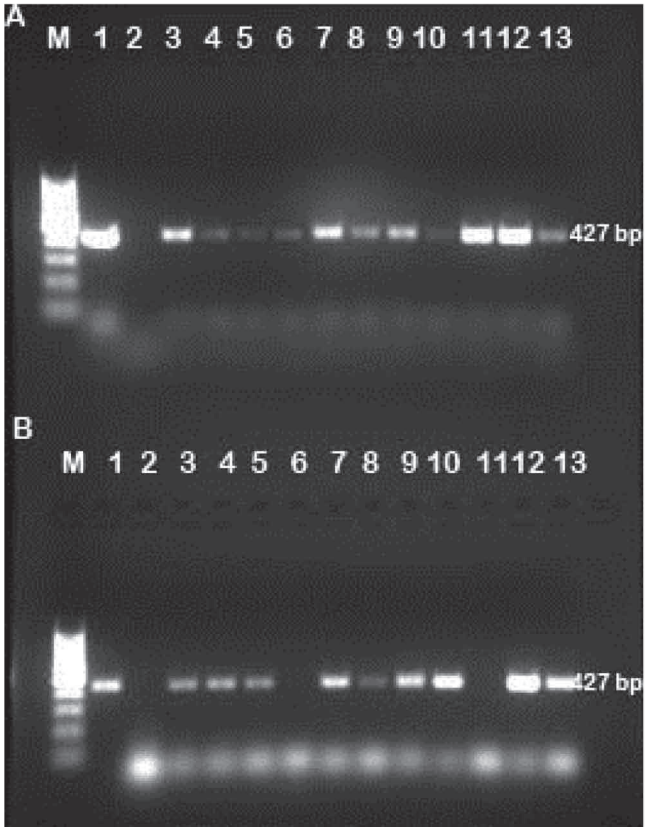

Fig 2. *Capnocytophaga canimorsus* and *Capnocytophaga cynodegmi* PCR results

A) *Capnocytophaga canimorsus* PCR results using *CaL2-CaR* primers, **M:** 100bp DNA ladder, **1:** *Capnocytophaga canimorsus* positive control, **2:** Negative Control **3-13:** *Capnocytophaga canimorsus* PCR positive samples **B)** *Capnocytophaga cynodegmi* PCR results using *CaL2-CyR* primers, **M:** 100bp DNA ladder, **1:** *Capnocytophaga cynodegmi* positive control, **2:** Negative Control **3-5:** *Capnocytophaga cynodegmi* PCR positive samples **6:** *Capnocytophaga cynodegmi* PCR negative samples **7-10:** *Capnocytophaga cynodegmi* PCR positive samples **11:** *Capnocytophaga cynodegmi* PCR negative sample **12-13:** *Capnocytophaga cynodegmi* PCR positive samples

Şekil 2. *Capnocytophaga canimorsus* ve *Capnocytophaga cynodegmi* PCR sonuçları

A) *Capnocytophaga canimorsus CaL2-CaR* primerleri kullanılarak elde edilen PCR sonuçları, **M:** 100bp DNA işaretleyicisi, **1:** *Capnocytophaga canimorsus* Pozitif Kontrol, **2:** Negatif Kontrol **3-13:** *Capnocytophaga canimorsus* PCR pozitif örnekler **B)** *Capnocytophaga cynodegmi CaL2-CyR* primerleri kullanılarak elde edilen PCR sonuçları, **M:** 100bp DNA işaretleyicisi, **1:** *Capnocytophaga cynodegmi* Pozitif Kontrol, **2:** Negatif Kontrol **3-5:** *Capnocytophaga cynodegmi* PCR pozitif örnekler **6:** *Capnocytophaga cynodegmi* PCR negative örnek **7-10:** *Capnocytophaga cynodegmi* PCR pozitif örnekler **11:** *Capnocytophaga cynodegmi* PCR negative örnek **12-13:** *Capnocytophaga cynodegmi* PCR pozitif örnekler

Table 2. *C. canimorsus and C. cynodegmi positive rates by year*
Tablo 2. *Yaşlara göre C. canimorsus ve C. cynodegmi pozitiflik oranl/arı*

PCR Positive Samples	0-5 Years	6-9 Years	10 Year and over	Total Number of Positive Samples
C. canimorsus	2	-	-	2
C. cynodegmi	-	-	-	0
C. canimorsus and *C. cynodegmi*	8	1	-	9

from a 6 years old dog. Two samples were detected as *C. canimorsus* from the 0-5 year group dogs.

DISCUSSION

In 1976, *C. canimorsus* was reported as a commensal bacterium in the oral flora of dogs and isolated from the blood and spinal fluid of a patient caused by an unidentified Gram negative bacillus, after a recent dog bite was first described [14]. Five *Capnocytophaga* species *(C. gingivalis, C. ochracea, C. sputigena, C. granulosa* and *C. haemolytica)* have been reported to be found in the human oral cavity and been associated with peridontitis. It is now clear that *C. cynodegmi* and *C. canimorsus* are part of the commensal oral microbiota of canines and more rarely of cats [6,15,16].

In the first study for *C. canimorsus* [17], the researchers examined 50 dogs samples and found *C. canimorsus* prevalence at 8%. In the latter reports *C. canimorsus* infections are associated with dog bites or close animal contact such as licking of human wounds. In a study done with the conventional culture method *C. canimorsus and C. cynodegmi* were detected in 74% and in 86% of dogs, respectively [6].

In another report including the culture and PCR results of *C. canimorsus* detection, 26% of the dogs tested were positive by both culture and PCR [18].

Currently, PCR based detection system is described which discriminates between *C. canimorsus* and *C. cynodegmi* and using this method the prevalence of both bacteria was determined in the dogs and cats [12].

The same study reported that on the basis of purely molecular screening of oral swabs using PCR amplification of a fragment of the small subunit ribosomal RNA gene (16S rRNA), that 74% of canines sampled (out of 325 canines) harbored *C. canimorsus*. The researcher determined the specificity and sensitivity of the PCR performed with different combinations of primers for discriminatory amplification of the 16S rRNA gene of *C. canimorsus* and *C. cynodegmi*. The *CaL2–AS1* primer pair could amplify the target sequences from the DNA derived from both *C. canimorsus* and *C. cynodegmi*. Specific amplification of *C. canimorsus* DNA but not *C. cynodegmi* DNA was achieved by the *CaL2–CaR*, whereas the DNA fragment of *C. cynodegmi* alone was amplified by the PCR using the *CaL2-CyR* primer pair [12].

The samples of the dogs and cats were, therefore, examined by PCR with these primers for the presence of specific sequence of *C. canimorsus* and *C. cynodegmi*. 240 of 325 (74%) dogs and 66 of 115 (57%) cats were tested positive for *C. canimorsus*, while *C. cynodegmi* was detected in 279 of 325 (86%) dogs and 97 of 115 (84%) cats. Both of these species were detected in 219 (67%) of 325 dogs and 64 (56%) of 115 cats [12].

Isolation of *C. canimorsus* (DF-2) from sheep and cattle (25-30% of the animals tested) but not from pigs was reported [19].

In this study, 200 oral swap samples which were taken from owned dogs which have had dental plaque, were examined and at the end of first PCR, *CaL2* and *AS1* gene were detected from 11 (5.5%) out of samples. Therefore, these samples were evaluated as *Capnocytophaga* spp. positive. After identifying the DNAs of the agent as *Capnocytophaga* spp. the strains were verified for being positive of *C. canimorsus* and *C. cynodegmi* using *CaR* and *CyR* gene nucleotide sequences by a second PCR. After the second PCR using the specific *CaR* and *CyR* primers, *CaR* and *CyR* gene regions were investigated in samples already detected as *Capnocytophaga* spp. positive. It was determined that 2 (18.2%) of the samples were positive for *CaR* gene which were identified as *C. canimorsus* and 9 (81.8%) of the samples were positive for both *CaR* and *CyR* gene which were identified as *C. canimorsus* and *C. cynodegmi*. As a result of this study, there were no positive samples detected for only *CyR* gene sequence.

Lavy et al.[20] reported that, *Capnocytophaga* species were found in 3 out of 17 dogs which were under 6 months of year. This year range is of a particular concern since puppies begin to lose their first teeth around 4 months of year and the teeth are completely replaced by their adult canines by 6 months.

In another study, the researcher reported that *Capnocytophaga* species were carried 72.7% in toy dog breeds. These results must be interpreted with caution that transmitted from dogs to human [8].

In this study when the result of positivity range is analyzed by year, 10 (90.9%) of the samples were detected in 0-5 years animals, and 1 (9.1%) of the sample were detected in 6 years animal out of 11 *Capnocytophaga* spp. positive samples. Eight (88.8%) of the samples were detected in 0-5 years animals and 1 (11.2%) of the sample was detected in 6 years animal out of 9 *C. canimorsus* and *C. cynodegmi* positive samples. Two (100%) samples which were only *C. canimorsus* positive, were detected in 0-5 years animals. In males, 2 (22.2%) *C. canimorsus*/*C. cynodegmi* positivity were detected while in females the corresponding figure was 7 (78.2%). Only 2 (100%) samples were detected as *C. canimorsus* positive in female animals. According to our data, female dogs were sensitive *Capnocytophaga* spp. infections.

C. canimorsus and *C. cynodegmi* species are considered for generating risk for human health and these agents hard to determine. The difficulty in diagnosis also causes the ignorance of these infections. These strains isolated from oral flora of dogs, the most common pet animal of our country, can be transmitted from dogs to human. Therefore, dogs can take an important part of *Capnocytophaga spp.* infections dissemination to human via oral excrections, such as saliva, and direct contact with mouth and tongue. Beside *Capnocytophaga* infections', these bacteria are associated with serious diseases such as meningitis, acute organ failures, intravascular coagulopathy, etc. in humans especially in immunodeficient patients. *Capnocytophaga* infections can likely be transmitted from dogs to humans by means of especially oral ways and even by kissing.

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