Original Article

Comparison of culture and polymerase chain reaction–restriction fragment length polymorphism for identification of various *Capnocytophaga* species from subgingival plaque samples of healthy and periodontally diseased individuals

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Abstract

Introduction: *Capnocytophaga* are facultative anaerobic Gram-negative bacilli and recognized as opportunistic pathogens of various extraoral infections. Only a few studies attempted to identify all the seven species of *Capnocytophaga* phenotypically and genotypically in healthy individuals and patients with chronic periodontitis. Studies to determine the prevalence of *Capnocytophaga* in subgingival plaque samples from healthy individuals, chronic gingivitis and periodontitis among Indian population are lacking.

Aim: The aim of this study was to identify and compare the presence of *Capnocytophaga* species phenotypically through microbial culture and biochemical tests and genotypically through polymerase chain reaction–restriction fragment length polymorphism (PCR-RFLP) in subgingival plaque of healthy individuals and patients with chronic gingivitis and chronic periodontitis.

Materials and Methods: A total of 300 subjects, 100 each with gingivitis, periodontitis and periodontally healthy gingiva subjected, were included. Subgingival plaque was collected and was cultured for phenotypic identification (microbial culture and biochemical test), and for genotypic identification, DNA extraction was done and PCR-RFLP analysis was performed to identify the genus *Capnocytophaga* and also to identify different species of *Capnocytophaga*.

Results: Of 300 individuals, *Capnocytophaga* species were identified from 237 (79%) individuals by PCR and 82 (27.33%) by culture. The prevalence of *Capnocytophaga ochracea* was found to be higher with both the methods followed by *Capnocytophaga gingivalis* and *Capnocytophaga granulosa*. *Capnocytophaga genospecies*, *Capnocytophaga leadbetteri* and *Capnocytophaga Sputigena* were isolated only by culture with very low prevalence that is 1.33%, 1.33% and 0.66%, respectively. We could not get any isolate of *Capnocytophaga haemolytica* by any of the two methods.

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Conclusion: *Capnocytophaga* species could be found in gingival sulci as well as periodontal pockets and can be detected by culture and PCR-RFLP. However, higher prevalence of these species in healthy compared to disease requires further analysis to determine their role in healthy and diseased periodontium.

Keywords: *Capnocytophaga,* chronic periodontitis, gingivitis, polymerase chain reaction–restriction fragment length polymorphism, phenotypic tests

INTRODUCTION

Periodontitis is a chronic infectious disease, the primary agents being Gram-negative anaerobic bacteria that occupy the tooth-associated biofilm in the subgingival plaque.[1,2] It is recognized by increased depth in gingival sulcus leading to periodontal pockets and by loss of periodontal attachment and surrounding alveolar bone.[2] Several Gram-negative bacteria including *Porphyromonas gingivalis*, *Treponema denticola* and *Tannerella forsythia* are frequently isolated from dental plaques in periodontal patients and are initially considered degenerative periodontal pathogens.[3,4] In addition, many other species are considered to be closely associated with this clinical entity such as *Prevotella intermedia, Fusobacterium nucleatum, Capnocytophaga* species*, Parvimonas micra, Selenomonas, Filifactor alocis* and *Campylobacter rectus*. The presence of these microorganisms in the periodontal pocket can be considered a marker in the development of periodontitis or an indicator in the progression of inflammation.[1] Microbes of genus *Capnocytophaga* are Gram-negative bacilli with gliding motility. They are facultative anaerobic bacteria preferring an atmosphere of 5% CO_2.[4]

Seven different species of *Capnocytophaga* are identified from human oral cavity (*Capnocytophaga gingivalis, Capnocytophaga ochracea, Capnocytophaga granulosa, Capnocytophaga sputigena, Capnocytophaga haemolytica, Capnocytophaga genospecies [AHN8471]* and *Capnocytophaga leadbetteri [AHN8855]*).[5] They form part of resident oral flora in children and adults. They are recognized as opportunistic pathogens of various extraoral infections and have been repeatedly recovered from a number of patients with septicemia, osteomyelitis, abscesses and keratitis.[6,7] They have been isolated from human dental plaque retrieved from both healthy and diseased sites. However, the relative isolation frequency of these organisms between studies has been varied considerably.[8] The significance of these individual species in periodontal and extraoral diseases is unclear due to inability of conventional phenotypic tests, failure to identify clinical isolates to species level and lack of other reliable methods for species identification.[9] Previous studies have attempted to distinguish between these species by biochemical tests, DNA probes and 16S rRNA polymerase chain reaction–restriction fragment length polymorphism (PCR-RFLP). Most of these methods other than 16S rRNA PCR-RFLP are labor intensive, costly and time-consuming and therefore not suitable for most microbiology laboratories.[10] There are only a few studies which have attempted to identify all the seven species of *Capnocytophaga* phenotypically as well as genotypically in healthy individuals and patients with chronic periodontitis.[11,12] In the present study, we have made an attempt to examine and compare the prevalence of *Capnocytophaga* species phenotypically using biochemical tests and genotypically with PCR via RFLP in healthy individuals and patients with chronic gingivitis and chronic periodontitis in Indian population.

MATERIALS AND METHODS

A total of 300 subjects, 100 subjects each with chronic gingivitis, chronic periodontitis and periodontally healthy gingiva, were included in the study. The study was approved by the Ethical Committee of the institute. The patients from both genders of age group between 18 and 55 years were enrolled in the study. Clinical examination and collection of sample were done at the Outpatient Department of the institute.

Inclusion criteria for subjects in the healthy group were absence of any clinical sign of gingival inflammation, probing depth ≤3 mm and no clinical attachment loss. Inclusion criteria for patients with gingivitis were generalized presence of clinical signs of gingival inflammation, probing depth ≤3 mm and no clinical attachment loss. Criteria for inclusion of patients with periodontitis were generalized presence of clinical signs of gingival inflammation, generalized probing depth ≥5 mm and generalized clinical attachment loss of ≥3 mm. Exclusion criteria for all the three groups were patients with any systemic disease, smokers, pregnant or lactating women, cervical or subgingival caries or restorations and periodontal or antimicrobial therapy within 3 months before sampling.

After clinical examination, subjects who full filled the inclusion and exclusion criteria were enrolled in the

study. Written informed consent was obtained from each individual, following which subgingival plaque sample was collected using sterile Gracey curette. Pooled subgingival plaque was collected after stripping away the supragingival plaque. The collected material was immediately transferred to reduced transport fluid (RTF) and sent to the laboratory for processing. The samples were processed at the Microbiology Department of the institute. The received RTF sample was vortexed to break the plaque and release the organisms in broth. It was then cultured on blood agar, Dentaid media and Trypticase soy agar with bacitracin and polymyxin B (TBBP), which is a selective medium for *Capnocytophaga*. The culture plates were incubated in anaerobic jar with 5%–10% CO_2 for 72 h. After 72 h, the plates were removed from the jar and examined for colony characters typical of *Capnocytophaga*. The characteristic gliding motility on blood agar with or without hemolysis [Figure 1] and/or yellow-orange- or beige-colored flat thin colonies on selective media [Figure 2] was used to provisionally identify the species of *Capnocytophaga*. Gram staining of suspected colony was performed to see the presence of Gram-negative fusiform bacilli [Figure 3]; simultaneously, catalase and oxidase tests were also performed. Provisionally identified *Capnocytophaga* colonies were further subjected to phenotypic identification by performing biochemical tests that included fermentation of glucose, lactose, sucrose, maltose, mannose, fructose, amygdalin, cellobiose, salicin, mannitol, sorbitol, melibiose, inulin and raffinose. In addition, nitrate reduction test and hydrolysis of aesculin, urea, starch and gelatin were also performed. The procedures used by earlier investigators were followed for carrying out the biochemical reactions.[7,13] Differentiation among seven species was done by adopting the criteria used by Frandsen *et al*.[7]

Simultaneously after culture procedure, the remaining sample was taken for DNA and stored at −20°C for PCR-RFLP analysis.

Procedure for DNA extraction

DNA extraction was carried out by modified proteinase-K method. The samples were first transferred to the tube containing TE buffer and then it was homogenized by vortexing for few seconds. The samples were centrifuged at 5000 rpm for 5 min and the supernatant was removed and again washed with fresh TE buffer. The supernatant was discarded and 50 µl lysis buffer I (1M Tris buffer: 500 µl, Triton X-100: 500 µl, 0.5M ethylenediaminetetraacetic acid: 100 µl and distilled water: made to 50 ml) was added. This was vortexed and kept for 5 min at room temperature and followed by addition of 50 µl lysis buffer II (Tris HCL: 50 mM [pH 8.0], KCL: 50 mM, $MgCl_2$: 2.5 mM, Tween 20: 0.45% and Nonidet P-40: 0.45%) and proteinase-K (10 mg/ml). Tubes were incubated at 60°C for 2 h, followed by enzyme deactivation by keeping in boiling water bath for 10 min. Samples were centrifuged

Figure 1: *Capnocytophaga* colonies on blood agar showing gliding motility

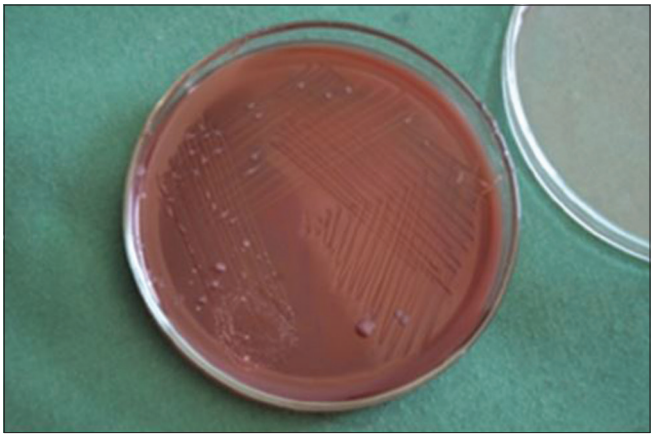

Figure 2: *Capnocytophaga* colonies on selective media (TBBP) showing brown color colonies

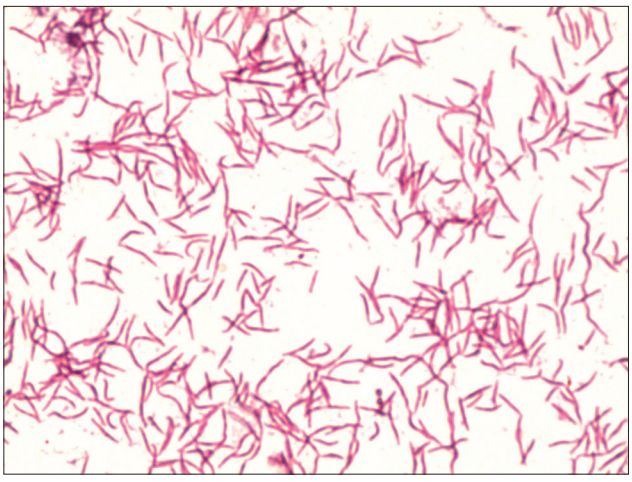

Figure 3: Photomicrograph showing Gram-negative fusiform bacilli of *Capnocytophaga* (Gram stain, ×100)

at 5000 rpm for 5 min and the supernatant containing DNA was collected in fresh tube and stored at −20°C till further analysis.[14]

Polymerase chain reaction–restriction fragment length polymorphism procedure

PCR was carried out in 25 µl total volume. Ampliqon red master mix was used which contained Tris-HCL pH 8.5, $(NH4)_2SO_4$, 3 mM $MgCl_2$, 0.2% Tween 20, 0.4 mM of each dNTP and 0.2 units/µl Amplicon Taq DNA Polymerase.

Primers targeting 16S rRNA conserved region of *Capnocytophaga* species were used: forward primer 27f (5' AGAGTTTGATCMTGGCTCAG 3') and reverse primer 1492r (5' TACGGYTACCTTGTTACGACTT 3'). 15 pmole of each primer and approximately 100 µg/ml of DNA were added to the mixture. Thermal cycling conditions were performed in verity thermal cycler (Applied Biosystems, USA) as follows: 95°C for 5 min, followed by 30 cycles at 94°C for 1 min, 54°C for 1 min and 72°C for 1 min. Final extension was done at 72°C for 5 min. Amplified product of 1500 bp was detected on 2% agarose gel electrophoresis. PCR-amplified samples were digested with restriction enzyme Hhal (Thermo Fischer Scientific, Massachusetts, USA). Ten microliters of PCR amplicons were digested with 1U of FastDigest restriction enzyme Hhal and incubated at 37°C for 5 min. The mixture was then loaded on 2.5% agarose for electrophoresis at 80 v for 1 h. The gel was stained with 0.5 µg/ml of ethidium bromide. The gel was viewed and captured using gel documentation system (Major Science, Saratoga, USA). The analysis of different DNA banding patterns was done by using total laboratory software (Newcastle-Upon-tyne. England)[10] [Figure 4].

Statistical analysis

Statistical analysis was performed using SPSS version 18.0 (SPSS Inc., Chicago, IL, USA). The prevalence of *Capnocytophaga* species by culture and PCR was statistically analyzed by Fisher's exact test. Colony-forming units between healthy, gingivitis and periodontitis were analyzed by Mann–Whitney *U*-test. The difference was regarded as significant when $P < 0.05$ (significant), $P < 0.01$ (moderately significant) and $P < 0.001$ (highly significant).

RESULTS

Totally 300 subgingival plaque samples, 100 from each group healthy, gingivitis and periodontitis, were analyzed for presence and comparison of various *Capnocytophaga* species by culture and PCR-RFLP. We found that the data were statistically significant by Fisher's exact test.

In the present study, *Capnocytophaga* species were identified from 237 (79%) subjects by PCR and 82 (27.33%) subjects by culture. This difference was statistically significant [Table 1]. We also compared median colony-forming units of *Capnocytophaga* among different groups. The colony count was significantly higher in periodontitis patients than in healthy individuals. Furthermore, it was significantly higher in periodontitis than in the gingivitis group. There was no statistically significant difference between the healthy and gingivitis groups [Table 2].

Out of total seven species, six species (*C. ochracea, C. gingivalis, C. granulosa, C. genospecies, C. leadbetteri and C. sputigena*) were detected by culture and only three species (*C. ochracea*, *C. gingivalis* and *C. granulosa*) were detected by PCR-RFLP method. The prevalence of *C. ochracea* was found to be higher with both the methods followed by *C. gingivalis* and *C. granulosa*. When compared by both the methods, these three species (*C. ochracea, C. gingivalis* and *C. granulosa*) were detected in more number of samples by PCR-RFLP method than by culture. The difference was statistically significant for *C. ochracea* and *C. granulosa* [Table 3]. In our study, C. *genospecies, C. leadbetteri* and *C. sputigena* were isolated only by culture with very low prevalence that is 1.33%, 1.33% and 0.66%, respectively.

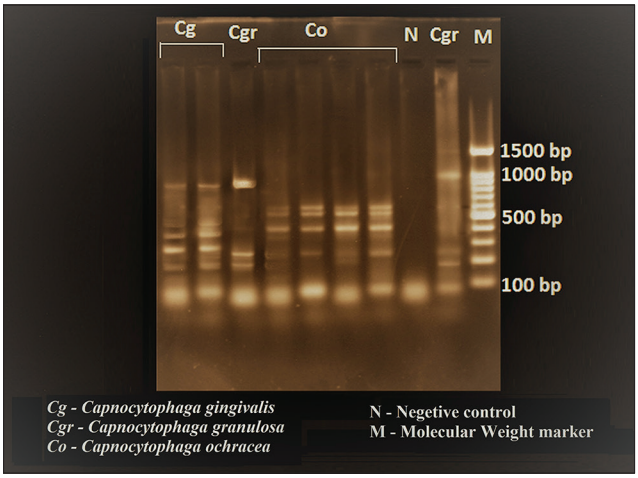

Figure 4: polymerase chain reaction–restriction fragment length polymorphism showing agarose gel electrophoresis with different banding patterns for *Capnocytophaga* species

Table 1: Prevalence of genus *Capnocytophaga* identified by polymerase chain reaction and culture

Capnocytophaga species	Positive	Negative	Total	Fisher's exact test (*P*)
By culture (%)	27.33	72.66	300	<0.0001 (S)
By PCR (%)	79	21	300	
Total	53.16	46.83	600	

S: Significant, PCR: Polymerase chain reaction

We could not get any isolate of *Capnocytophaga haemolytica* by any of the two methods [Table 3].

When the prevalence of overall *Capnocytophaga* species among healthy, gingivitis and periodontitis was studied, we observed that these species were more prevalent in healthy individuals than gingivitis and periodontitis by both the methods (32% by culture and 87% by PCR-RFLP). The detection rate of these species in all the study groups was found to be higher by PCR-RFLP method as compared to culture. This difference was statistically significant [Table 4].

DISCUSSION

A wide range of anaerobic and facultative bacteria can be isolated and identified using standard anaerobic culture techniques, however, only about 50% of bacteria in oral cavity are cultivable.[15] The bacterial diversity in periodontic infections is probably still underestimated. Various studies confirmed the clinical significance to a limited number of key pathogens in acute or chronic periodontitis. Besides culture methods, molecular techniques such as PCR have also been introduced for routine diagnosis of periodontal pathogens. These methods have enabled the detection of bacterial species that are difficult or even impossible to culture.[16] There are no studies conducted on the prevalence of all seven species of *Capnocytophaga* in Indian population. In the present study, an attempt was made to find out and compare the prevalence of all seven *Capnocytophaga* species by culture and PCR in Indian population.

In our study, the prevalence of *Capnocytophaga* genus was 79% by PCR-RFLP and 27.33% by culture which was statistically significant. In earlier studies using culture methods, the isolation frequencies of *Capnocytophaga* genus have been

Table 2: Comparison of colony-forming units for genus *Capnocytophaga* between each group

Group	*n*	Mean	SD	Minimum	Maximum	Percentiles			Mann-Whitney test (*P*)
						25th	50th	75th	
Comparison of median CFU between healthy and CP group									
CP	100	26,370	21,985	12,000	98,000	15,000	18,000	28,000	<0.0330 (S)
Healthy	100	16,313	7921	3000	32,000	9750	15,500	22,000	
Comparison of median CFU between healthy and gingivitis									
Gingivitis	100	15,783	11,453	3000	48,000	9000	12,000	16,000	0.1389 (NS)
Healthy	100	16,313	7921	3000	32,000	9750	15,500	22,000	
Comparison of median CFU between CP and gingivitis									
CP	100	26,000	22,214	8000	98,000	15,000	18,000	28,000	<0.0010 (S)
Gingivitis	100	15,783	11,453	3000	48,000	9000	12,000	16,000	

S: Significant, NS: Not significant, SD: Standard deviation, CFU: Colony-forming unit, CP: Cerebral palsy

Table 3: Comparison of prevalence of different *Capnocytophaga* species identified through culture and polymerase chain reaction-restriction fragment length polymorphism

Capnocytophaga species	Culture (%)		Total	PCR (%)		Total	Fisher's exact test (*P*)
	Positive	Negative		Positive	Negative		
C. ochracea	9.66	90.33	300	36.33	63.66	300	<0.0001 (S)
C. gingivalis	5	95	300	10	90	300	<0.28 (NS)
C. granulosa	9.33	90.66	300	33.66	67.33	300	<0.0001 (S)
C. genospecies	1.33	98.66	300	-	-	300	
C. leadbetteri	1.33	98.66	300	-	-	300	
C. sputigena	0.66	99.33	300	-	-	300	
C. haemolytica	-	-	300	-	-	300	

S: Significant, NS: Not significant, PCR: Polymerase chain reaction, *C. ochracea: Capnocytophaga ochracea, C. gingivalis: Capnocytophaga gingivalis, C. granulosa: Capnocytophaga granulosa, C. genospecies: Capnocytophaga genospecies, C. leadbetteri: Capnocytophaga leadbetteri, C. sputigena: Capnocytophaga sputigena, C. haemolytica: Capnocytophaga haemolytica*

Table 4: *Capnocytophaga* species isolated by culture and identified by polymerase chain reaction-restriction fragment length polymorphism in healthy individuals, chronic gingivitis and chronic periodontitis

Group	Number of samples	*Capnocytophaga* species isolated by culture (%)		*Capnocytophaga* species identified by PCR (%)		Fisher's exact test (*P*)
		Positive	Negative	Positive	Negative	
Healthy individuals	100	32	68	87	13	<0.0001 (S)
Gingivitis	100	23	77	77	23	<0.0001 (S)
Periodontitis	100	27	73	73	27	<0.0001 (S)
Total	300	27.33	72.66	79	21	

S: Significant, PCR: Polymerase chain reaction

reported to range from 20% to 67% in samples from gingival pockets,[17,18] while PCR methods have shown the range of these species from 89% to 100%.[16,17] Even though culture method is known as a gold standard to identify the major putative pathogens, molecular identification is vastly superior to conventional identification. When the prevalence of individual *Capnocytophaga* species was compared with both the methods, *C. ochracea, C. granulosa and C. gingivalis* were highly detected by PCR as compared to culture. The prevalence of *C. ochracea* was higher by both the methods as compared to other species. There are findings which have shown *C. ochracea* to be in higher proportion compared to other species.[7,19] Some studies have shown a 50%–100% prevalence for *C. ochracea* by PCR,[20,21] while some have shown a 40% prevalence for *C. ochracea* by phenotypic tests.[22]

In our study, the prevalence of *C. gingivalis* was found to be more by PCR than culture. Some authors found *C. gingivalis* to be 96% in healthy Japanese children by PCR.[17] Some researchers reported *C. gingivalis* to be 55.8% by conventional phenotypic tests.[22] *C. granulosa* was found to be in higher proportion by PCR than culture. In some studies, the prevalence of *C. granulosa* was found to be 51% from subgingival plaque samples by PCR.[8] The high prevalence of these three species (*C. ochracea, C. gingivalis* and *C. granulosa*) by both PCR and culture suggests that these three species are commonly detected in oral cavity.

C. genospecies and *C. leadbetteri* were found to be 1.33% by culture. These two species could not be detected by PCR-RFLP in our study. Previous articles have mentioned 16S rRNA gene sequences for only five human oral species (*C. ochracea, C. gingivalis, C. granulosa, C. sputigena* and *C. haemolytica*)[10,23] for PCR-RFLP, and these two species were identified later. We were unable to get the gene sequences for *C. genospecies* and *C. leadbetteri* since data related to their sequence are sparse and hence these two species could not be detected by PCR-RFLP. Some studies showed that *C. genospecies* and *C. leadbetteri* were identified from subgingival plaque of children 2–3 years of age.[7] There are no reports of these two species to be identified by culture. Thus, there is a need of further study for these two species by PCR and culture with larger sample size.

C. sputigena was found to be 0.66% by culture, while no isolates of *C. sputigena* were detected by PCR in our study. Some studies have shown *C. sputigena* to be 50% by PCR from saliva samples in Japanese children of age group 2–10 years.[21,24] In contrast, no isolates of *C. sputigena* were detected using PCR method.[25] Using culture method, some investigators have shown the prevalence of *C. sputigena* to be 44.4%.[26] Some studies have shown *C. sputigena* to be less or moderately prevalent.[17,20,21] We observed that there is considerable variation in detection rate of *C. sputigena* from several studies. This variation may be due to racial and geographical variations.

We were not able to get any isolate of *C. haemolytica* in any group by both the methods. Some investigators found a 10% prevalence of *C. haemolytica* from periodontitis patients.[8] Maybe due to its low prevalence, we were unable to get the isolates of *C. haemolytica* by both the methods in all the three study groups or it may be suggestive of geographic variation.[8,13]

We found the prevalence of *Capnocytophaga* species to be significantly higher in samples from the healthy group as compared to those of the gingivitis and periodontitis groups. Similar findings have been reported by several other investigators.[19,20] In contrast to this, some workers have reported a higher frequency of *Capnocytophaga* species in patients with gingivitis and periodontitis than in healthy individuals.[19,27-29] Since PCR-RFLP may not be able to detect all species of *Capnocytophaga,* there is a need for optimizing a different molecular technique for identification of these bacteria.

CONCLUSION

We conclude that the members of *Capnocytophaga* species could be found in gingival sulci as well as periodontal pockets associated with different clinical forms of periodontal disease and can be detected by culture and PCR. However, higher prevalence of these species in healthy compared to disease requires further analysis to determine their role in health and diseased periodontium.

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Nil.

Conflicts of interest

There are no conflicts of interest.

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