Original Article

Detection And Quantification Of Treponema Denticola In Subgingival Plaque Of Humans By Polymerase Chain Reaction.

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Abstract:

Introduction: Inflammation of the supporting tissues which is also known as periodontitis contributes to the major etiological factor for tooth loss. Consortium of pathogenic bacteria with elevated levels lead to onset and progression of periodontal disease. Gram negative bacteria, primarily severe anaerobes, are responsible for most of it.

Aims and Objectives: The present study aimed to assess the presence of Treponema denticola in periodontal disease in different subgingival plaque samples collected from the oral cavity of 30 patients diagnosed with chronic periodontitis. The plaque samples were compared with similar plaque samples of 15 healthy patients.

Method: Subgingival dental plaque samples from shallow and deep crevices were examined for the presence of bacterial DNA using the PCR method, which enables the simultaneous identification of multiple bacterial genomes.

Results: PCR results show amplification and quantification of T. denticola in 10 out of 15 patients in group-1, 9 out of 15 patients in group-2, and 4 out of 15 patients in group-3. The statistical analyses of the results show that they are statistically significant.

Conclusion: According to the aforementioned study, PCR microorganism identification is sensitive and specific. For early infection diagnosis and subsequently for the prevention and treatment of periodontal disease, a rapid and accurate assessment of periodonto-pathogens is crucial.

Keywords: Clinical trial(s), Gingivitis, Periodontitis

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Introduction:

The diseases affecting the periodontal tissues are known as periodontal diseases. Among all the periodontal diseases most common is the gingival inflammation.[1] The reason for such diseases mainly accounts to plaque and calculus accumulation.

Periodontitis refers to the disease in more advanced and progressed state.

These illnesses are typically linked to microbial infection as a result of plaque biofilm and calculus buildup. A group of more severe and connected disorders that fall under the general category of

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periodontal disease are referred to as periodontitis. It is described as "an apical extension of gingival inflammation to involve the tissues supporting the tooth, including periodontal ligament and bone".[2] A periodontal pocket develops as a result of the breakdown of the fibre attachment. Armitage[2] has recently revised this broad spectrum of illnesses, and at least 48 distinct periodontitis classifications are now acknowledged. Chronic periodontitis is by far the most prevalent and is the main reason for tooth loss in the adult population. The bacteria that creates the plaque biofilm on the tooth mediates the disease. Adult patients who have chronic periodontitis experience a complex interaction between the host response and mixed polymicrobial infection. Numerous inflammatory mediators are released in the underlying soft tissue as a result of the adhering microorganisms. In actuality, the host tissue is finally destroyed by these activation products.[1,2,3]

A vast variety of complicated subgingival microorganisms, including facultative and anaerobic organisms, perhaps yeasts, and both Gram-positive and Gram-negative bacteria, are linked to human periodontitis. A particularly well-researched microbial habitat, the subgingival crevice, has yielded at least almost 500 bacterial strains.[4,5,6] These strains are categorised as potential opportunistic pathogens and commensals, respectively, for the majority of them.[7]

Spirochetes are a varied genus of bacteria that Obermeier originally identified in 1868 in connection with a human illness (Birkhaug, 1942). There are numerous pathogenic spirochete lifestyles. Like the majority of other Borrelia species, the Lyme disease agent Borrelia burgdorferi typically cycles between ticks and small rodents. Swine dysentery is brought on by the bacteria Brachyspira (formerly Serpulina) hyodysenteriae, which is spread from pig to pig via the oral-fecal pathway (Harris et al., 1972). Together with the other microbial residents of the mouth, Treponema denticola dwells in the intricate human periodontal environment and is linked to periodontal disease. The syphilis agent, Treponema pallidum, is not yet well-established in culture. The acetogenic endosymbionts identified in termite stomachs are spirochetes strongly related to, if not actually belonging to, the genus Treponema (Leadbetter et al). Leptospira is a genus that includes both pathogenic and saprophytic species, with the pathogenic species having the ability to infect both humans and animals with sickness (Vinetz, 1997).[8-11]

One of the major suspects in the development of periodontal disease in humans is Treponema denticola.The main spirochete found in the gingival crevice and forming periodontal pocket of different types of periodontitis, infected root canals, and acute alveolar abscesses is the organism in question.[12-19] According to reports, the bacterium reportedly possesses a number of putative virulence factors, including attachment factors, proteolytic activity, and an immunosuppressive component.[20,21,22]
In the current investigation, human subgingival plaque samples were tested for oral treponemes, and Treponema denticola was identified using species-specific PCR techniques. We also used PCR to count the number of each organism.

Materials and Methods

Patient selection:

This study included 15 people in good health and 30 subjects with periodontitis who visited our office between January 2011 and May 2011 (28 men and 17 women). Briefly, those with periodontal disease had at least 20 teeth, at least eight sites with a pocket depth of at least 4 mm, and at least eight sites with attachment loss of at least 3 mm. In contrast, the healthy subjects had no pockets deeper than 3 mm and no attachment loss greater than 2 mm at any site in the mouth. The patients were subdivided into 3 groups- 15 patients with ≥ 6mm pocket probing depth were denoted as group-1, 15 patients with 4 – 6 mm pocket probing depth were denoted as group-2, and 15 patients with ≤ 3mm pocket probing depth were denoted as group-3. Patients who were systemically compromised were excluded from the study.

Sample Collection:

In the previous three months, no professional cleaning nor antibiotic treatment had been given to the patients. Each participant received information about the study and signed the informed consent form after it was authorised by the I.T.S centre for dental studies and research's ethics committee. Each tooth was isolated with cotton rolls and allowed to air dry before the subgingival plaque sample was taken, and the depth of the pocket was determined by probing. After using sterile cotton to remove supragingival plaque, Gracey curettes were then placed into the periodontal pockets (figure-1). Then these Gracey curettes were removed (figure-2) and placed into 0.5 ml of theoglycollate medium for the storage of plaque sample and DNA extraction (figure-3). In this study, we classified individuals with periodontal disorders as having a shallow pocket if their pocket depth was between 4 and 5 millimetres and a deep pocket if it was larger than 6 millimetres.

DNA Extraction:

DNA extraction from plaque sample was done as follows. A 2 ml tube was filled with a 500 µl sample of plaque. The supernatant was discarded after centrifuging it for 3 to 4 minutes at 10,000 rpm. Tris EDTA buffer in the amount of 500 µl was added. After thoroughly mixing the solution, it was centrifuged for 3–4 minutes at 10,000 rpm. Supernatant was once more discarded. Four times this step was repeated. Add 500 µl of Lysis buffer 1 and centrifuge at 10,000 rpm for 3 to 4 minutes before once more discarding the supernatant. 5 ml of proteinase K and 500 µl of Lysis buffer 2 were added. It was stored at -200 C after spending two hours in a water bath at 750 C and ten minutes in a boiling water bath.

PCR Analysis:

All of the components required for creating new DNA strands were included in the PCR Master Mix. The reagents for the Master Mix included:
1. Water with a 1X buffer (Maintains the master mix's appropriate pH so that the PCR Reaction can occur.),
2. 200 mM deoxynucleotides (Provides the energy and the nucleosides required for DNA synthesis. To avoid base mismatches, equal amounts of each nucleotide (dATP, dTTP, dCTP, and dGTP) were added to the master mixture.
3. Taq DNA polymerase enzyme is activated by the presence of 0.2–1.0 uM primers (short DNA fragments (20–30 bases) bound to the DNA template). 4. 2.5U/100 l Ampli Taq polymeras (A heat stable enzyme that attaches the deoxynucleotides to the DNA template. Both specific and universal primers may be employed.)
5. 0.05 to 1.0 ug of template DNA (the DNA that the PCR process will amplify).
Treponema Denticola probe used was

1. Forward- 5' TAA TAC CGA ATG TGC TCA TTT ACA T 3'
2. Reverse- 5' TCA AAG AAG CAT TCC CTC TTC TTC TTA 3'

Results:

The results so obtained are shown in figure-4, figure-5, and figure-6. In these figures, the wells which showed weak banding pattern were taken as negative. They were given the value $<10^3$, which was then taken as cut off value for all other samples. That means all positive samples had the value $>10^3$ and band intensity was greater than those of negative samples. The position of amplification was from 193bp to 508 bp. The amplicon size was 315 bp. We have used lamba DNA/Hind III as the marker in the study, 8 fragments- Ranges: 125 bp to 23,130 bp.

The results are shown as follows- PCR results show amplification and quantification of T. denticola in 10 out of 15 patients in group-1, 9 out of 15 patients in group-2, and 4 out of 15 patients in group-3. The results are shown in figure-7, figure-8, figure-9, figure-10 and figure-11. The statistical analyses of the results show that they are statistically significant, as shown in figure-12.

Discussion:

Periodontal disorders have been linked to the onset and progression of microbial flora alterations and periodontal bacteria. Among the several bacterial species discovered in subgingival plaque samples, the periodontal pathogens P. gingivalis, A. actinomycetemcomitans, F. nucleatum, and Bacteroides forsythus have been identified.[23]

Additionally, the oral treponemes T. denticola, T. vincentii, and T. medium have been discovered to be present in subgingival plaque.[24,25,26,27] Though the majority of potential periodontopathogenic microbes have been grown in anaerobic environments, they have shown fastidious growth habits, are picky about the media they grow in, and demand lengthy processing times for cultivation of specific organisms. Furthermore, using culture methods to identify oral treponemal species is challenging. In the current work, we used PCR analysis to find T. denticola in subgingival plaque samples.

Dewhirst et al. only partially sequenced the cloned spirochetal 16S rRNA genes that were amplified from the DNA in samples with subgingival plaque.25 demonstrated that different Treponema species could be identified. A nested PCR technique was used by Willis et al.[28] to identify Treponema species in dental plaque.

All of the individuals with periodontal disorders had T. denticola, with group 1 showing the highest prevalence and group 3 the lowest. Statistics show that the outcome is substantial. T. denticola was infrequently found in subgingival plaque samples from healthy patients in group 1 (pocket probing depth, _3 mm) in the current investigation. Three of the total number of healthy subjects had T. denticola. Additionally, T. amylovorum, T. denticola, T. maltophilum, T. medium, and T. socranskii, one or more species, were found in subgingival plaque samples from persons without periodontal disorders (pocket probing depth, _4 mm), according to research by Willis et al.[28].

Through the use of PCR and plaque from people with and without periodontal disease, we also calculated the quantities of oral T. denticola. For the Treponema denticola species, the detection responses were linear and strongly correlated. Lyons et al.[29] used real-time PCR with a universal primer and a fluorescent probe to measure the amounts of total bacteria in plaque samples, despite the fact that it is difficult to estimate the total number of bacteria in plaque samples due to the fact that the PCR amplification reaction differs for each organism in a complex microbial mass. When comparing the abundance of each organism, we found that the method we utilised in the current study, which normalised the number of Treponema species using the concentration of DNA from each plaque sample, was a rather straightforward approach. T. Denticola cells tended to multiply more frequently in deep pockets.

These findings imply that T. denticola multiplies as periodontal disease worsens. With each stage of periodontal disease, different species are involved. The results of the current study shown that using specific primers, a PCR assay may detect oral treponemes, such as T. denticola, quantitatively in subgingival plaque samples.

Conclusion:

This study has found a strong relationship between Treponema denticola and the severity of periodontal disease. These tests support the development of a proper diagnosis based on the most recent assessment of periodontal health and of a sophisticated periodontal treatment based on clinical and paraclinical data. We further conclude that in the field of periodontal microbiology, PCR is a quick and simple test to do. The goal of this study is to motivate physicians to conduct comparable studies for the periodontal disease diagnosis.

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Legends for figures:

Figure-1: Gracey curettes inserted into the periodontal pockets
Figure-2: Supragingival plaque removed
Figure-3: Plaque sample in medium
Figure-4: Banding pattern (group-1)
Figure-5: Banding pattern (group-2)
Figure-6: Banding pattern (group-3)
Figure-7: Amplication of *T denticola*
Figure-8: Amplification of *T denticola* (Graphical representation)
Figure-9: Results of group-1 (tabular form)
Figure-10: Results of group-2 (tabular form)
Figure-11: Results of group-3 (tabular form)
Figure-12: Statistical analysis of results

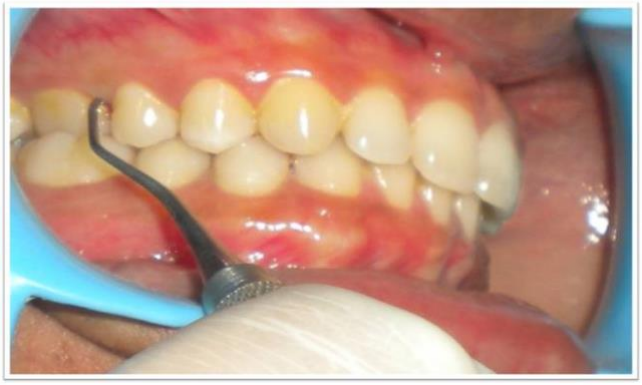

Fig 1

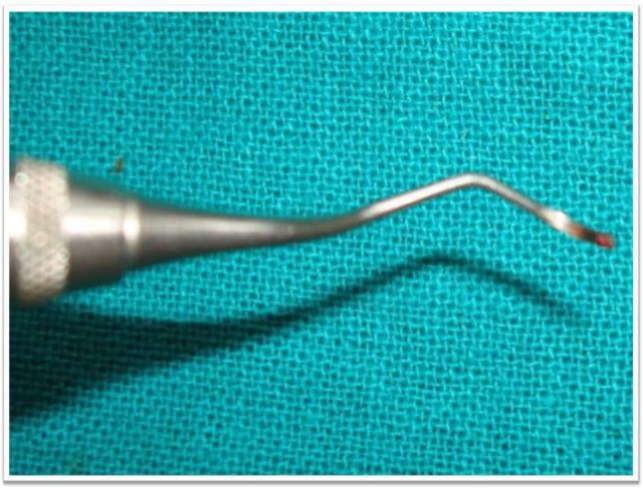

Fig 2

Fig 3

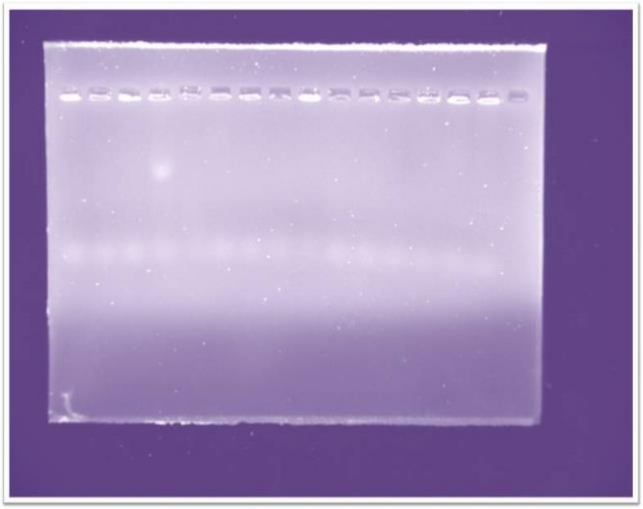

Fig 4

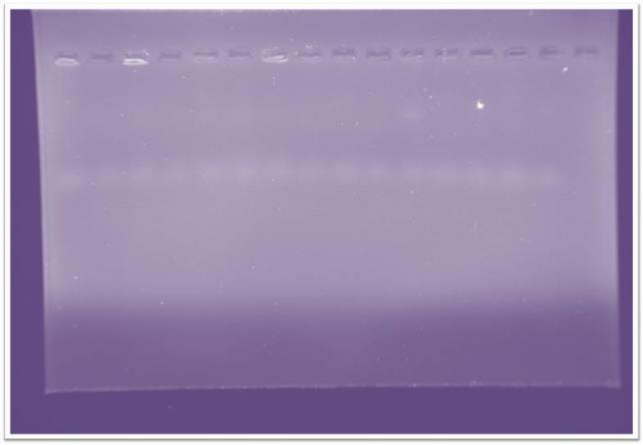

Fig 5

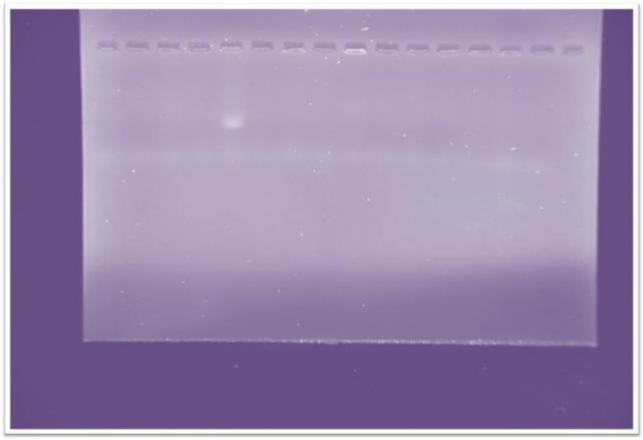

Fig 6

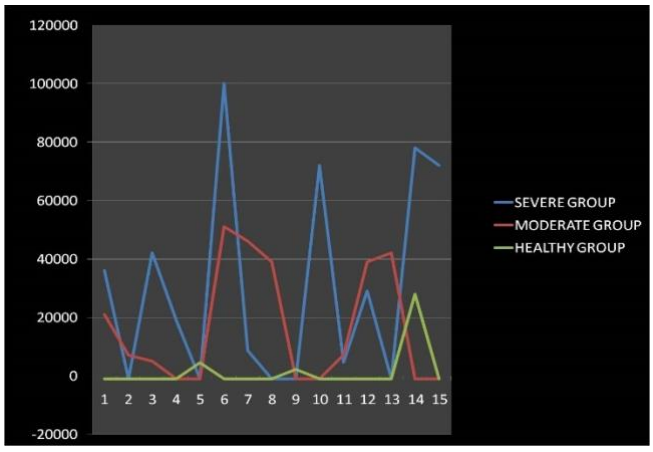

Fig 7

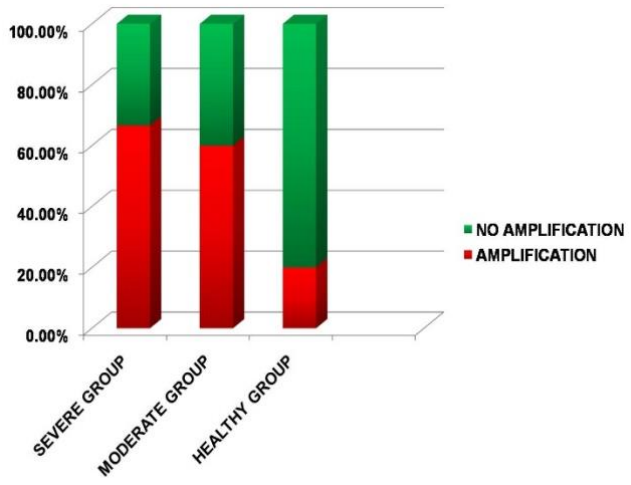

Fig 8

Sl No.	Samples	Results
	Healthy group	
1	H-1	- $<10^3$
2	H-2	- $<10^3$
3	H-3	- $<10^3$
4	H-4	- $<10^3$
5	H-5	+ (4.5 X 10^3)
6	H-6	- $<10^3$
7	H-7	- $<10^3$
8	H-8	- $<10^3$
9	H-9	+ (2.2 X 10^3)
10	H-10	- $<10^3$
11	H-11	- $<10^3$
12	H-12	- $<10^3$
13	H-13	- $<10^3$
14	H-14	+ (2.8 X 10^4)
15	H- 15	- $<10^3$

Fig 11

	Samples	Results
	Chronic Periodontitis (Group I)	SEVERE GROUP
1	CP-1	+ (3.6 X 10^4)
2	CP-2	- $<10^3$
3	CP-3	+ (4.2 X 10^4)
4	CP-4	+ (1.9 X 10^4)
5	CP-5	- $<10^3$
6	CP-6	+ (1 X 10^5)
7	CP-7	+ (8.6 X 10^3)
8	CP-8	- $<10^3$
9	CP-9	- $<10^3$
10	CP-10	+ (7.2 X 10^4)
11	CP-11	+ (4.6 X 10^3)
12	CP-12	+ (2.9 X 10^4)
13	CP-13	- $<10^3$
14	CP-14	+ (7.8 X 10^4)
15	CP-15	+ (7.2 X 10^4)

Fig 9

	GROUPS		
	SEVERE (N %)	MODERATE (N %)	HEALTHY (N %)
AMPLIFICATION	10 (66.67%)	9 (60.00%)	3 (20%)
NO AMPLIFICATION	5(33.33%)	6(40.00%)	12(80%)

Fig 12

Sl No.	Samples	Results
	Chronic Periodontitis (Group II)	MODERATE GROUP
1	CP-1	+ (2.1 X 10^4)
2	CP-2	+ (7.1 X 10^3)
3	CP-3	+ (5 X 10^3)
4	CP-4	- $<10^3$
5	CP-5	- $<10^3$
6	CP-6	+ (5.1 X 10^4)
7	CP-7	+ (4.6 X 10^4)
8	CP-8	+ (3.9 X 10^4)
9	CP-9	- $<10^3$
10	CP- 10	- $<10^3$
11	CP-11	+ (7.2 X 10^3)
12	CP-12	+ (3.9 X 10^4)
13	CP-13	+ (4.2 X 10^4)
14	CP-14	- $<10^3$
15	CP-15	- $<10^3$

Fig 10