

Some Rapid Methods for Oral Treponema Detection

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ABSTRACT

The present study investigates the presence of four oral Treponemal species using rapid molecular methods. Subgingival fluid samples were obtained before and after one week of scaling and the nucleic acid was liberated from the bacterial cells to be used as a template for PCR. Specific primers for each Treponemal species targeted to 16SrDNA sequence was depended. 89% of samples were positive for *Treponema* sp., 88% of them were positive to the presence of more than one type. *T. amylovorum*, *T. medium*, *T. socranskii*, and *T. vincentii* were detected in 72%, 87%, 76 % , and 28% respectively with higher percentage at pocket depths >5 mm. Molecular method was able to detect Treponemal species even after scaling, however, in lower percentage than before it.

Keywords: Oral Treponema, 16S rRNA, PCR.

	Treponema							
scaling								
	.PCR							
		%89			.Treponema		16SrDNA	
<i>T. amylovorum</i>		.				%88	<i>Treponema</i> sp.	
	%28	%76	%87	%72	<i>T. vincentii</i>	<i>T. socranskii</i>	<i>T. medium</i>	
scaling						.	5<	
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INTRODUCTION

Several methods have been employed in dentistry to better understand and diagnose oral infectious agents and consequently achieve the patient risk assessment for caries,

periodontal, and other oral disease. These include culture methods, microscopy, immunofluorescent assays, ELISA, and DNA probes (Santos *et al.*, 2004). Although many putative periodontal pathogens involving anaerobic, microaerophilic, and aerobic bacteria have been extensively evaluated e.g. *Prevotella intermedia*, *Actinobacillus actinomycesomittans*, *Porphyromonas gingivalis*, *Tannerella forsythia*, *Treponema denticola* etc. (Boyanova *et al.*, 2009), detection and identification of large numbers of anaerobes implicated in oral polymicrobial infections are hampered by the complexity of oral biofilm (Bayingana *et al.* , 2010).

Because the analysis of the complexity and genetic diversity of microbial communities have been limited by conventional culture – depended methods , and in spite of close attention to specimen collection and processing on selective and non- selective agars under appropriate atmospheric conditions has improved the routine diagnosis of oral infections, it is severely biased and yet many oral bacteria remain uncultured and uncharacterized from many oral diseases (Sakamoto *et al.*, 2003; Robertson and Smith, 2009). Therefore, application of molecular methods to the analysis of bacterial diversity in the oral cavity opens the door for culture- independent analysis and classification of previously unknown members of many different microbial communities and allows for construction of evolutionary trees for bacteria to the species level (He and Shi, 2009).

Broadly speaking, the molecular analysis may take one of the following approaches. First, the use of molecular cloning and sequencing techniques to identify uncultivable microorganisms using 16S rRNA or rDNA leading to identification of several novel species. The second approach utilizes PCR or DNA-DNA hybridization checkerboard techniques and more recently 16S rRNA gene sequencing and species- specific primers searching for the presence of specific microbes which demonstrated the higher prevalence of more fastidious organisms such as *Treponema* sp. (Robertson and Smith, 2009).

The role of Treponemal species in oral infections established based on molecular- depended techniques. For instance, recent studies using species- specific primer for PCR approaches revealed a high prevalence of oral treponemes in endodontic infections of which *T. denticola*, *T. socranskii*, and *T. amylovorum* recorded in 36%, 19%, and 2% respectively, while in the past, their occurrence was obscured by difficulties in culturing these bacteria (Siqueira and Rôças, 2005 ; Siqueira and Rôças, 2009). Their etiologic role in periodontitis have been postulated based on their high frequency in periodontal lesions of chronic or aggressive infections and less often in healthy subjects. The majority of them seems to be rather opportunistic and necessary for maintaining the disease, also potentially being a major player in subgingival biofilm architecture (Moter *et al.*, 2006).

Treponemes were noted to make 20-50% of the total microscopic count in subgingival plaque of periodontitis patients and the more prevalently detected to be associated with periodontitis are *T.denticola*, *T. socranskii*, and *T. lecithinolicum*. However the diversity of spirochetes in the oral cavity has been reported to be far greater than expected (Siqueira and Rôças, 2005; Jun *et al.*, 2008).

Most of previous studies concerned with the cultivable *T.denticola* and study its pathogenicity and virulence properties in full-dress as a convenient model for understanding the putative virulence determinants of oral treponemes while little is known about the physiology and virulence of other oral treponemes (Correia *et al.*, 2003 ; Kuramitsu, 2003) and since PCR is not routinely used in Iraq for the identification of periodontal pathogens, the current study aims to explore other types of oral *Treponema* using rapid accurate

species-specific molecular methods as an alternative of routinely culturing methods which need further hardship and much of nutritional supplements and specialized incubation conditions to meet the growth promoting and identification of these fastidious group of bacteria.

MATERIALS AND METHODS

Samples: Steps outlined by (Sakamoto *et al.*, 2002) were followed to obtain 46 gingival fluid samples from 25 patients with chronic periodontitis (15 male with an average age of 32 years and 10 female with an average age of 35 years) before and after one week of scaling by inserting absorbent paper point into the pockets either buccally, distally, mesially, or lingually after measuring pocket depth (Ximenez- Fyvie *et al.*, 2000) and placed in an eppendorf tube containing 500µl normal saline. The samples were collected in the Teaching Hospital of the Dentistry College in Mosul university at the period from December 2010 to March 2011.

DNA template preparation: Bacterial cells were collected from the sample in the PCR lab. at Biology department in the collage of Sciences by 10000xg centrifugation (Hettich company- Germany) at 4°C and washed twice then the nucleic acid was liberated by three cycles of freezing and thawing (Sakamoto *et al.*, 2003). The lysate was suspended in 25 µl distilled water and stored at -20 °C until use as a template for PCR reaction.

DNA primers preparation: Species- specific primers for each Treponemal species targeted to 16SrDNA sequence was depended (Willis *et al.*, 1999) as shown in Table (1). The primers were purchased from Bioneer Ltd.(South Korea) and prepared according to its recommendations.

Reaction mixture: Go Taq® Green Master Mix,2X solution(Promega ltd.) was used to make up 25µl reaction mixture volume shown in table 2 according to manufacturer instruction, this solution contained Taq DNA polymerase, 400µM dNTP, 3mM MgCl₂, reaction buffer at optimal concentration (pH8.5), and two dyes (blue and yellow) function as loading dyes to allow monitoring of progress during electrophoresis.

Table1: Primer sequence and product size (base pairs) for each DNA type.

Treponemal species	Primer sequence	Position(bp)
<i>T. amylovorum</i> 16S	F AGA GTT TGA TCC TGG CTC AG R CTC ACG CCT TTA TTC CGT GAG	8-211(193)
<i>T. medium</i> 16S	F AGA GTT TGA TCC TGG CTC AG R CCT TAT GAA GCA CTG AGT GTA TTC	8-200(192)
<i>T. socranskii</i> 16S	F GAT CAC TGT ATA CGG AAG GTA GAC A R TAC ACT TAT TCC TCG GAC AG	179- 468(285)
<i>T. vincentii</i> 16S	F AGA GTT TGA TCC TGG CTC AG R AAT ACT TCT TAT GAA CAT TGA GAC	8-201(193)

Table 2: PCR reaction mixture.

Component	Volume/sample	Final concentration
Go Taq® Green Master Mix, 2X	12.5 µl	1X
Upstream primer, 10 µM	2.5 µl	1.0 µM
Downstream primer, 10 µM	2.5 µl	1.0 µM
DNA template	5 µl	<250ng
Nuclease- Free water	To be 25 µl	N.A.

PCR (Thermocycler) reaction: The thermocycler equipage (Eppendorf, Germany) was programmed to three stages :

1. Initial denaturation at 97 °C for 1 min.
2. 26 cycles of repeated denaturation at 97 °C for 45 sec. ; annealing steps for 45 sec at 54 °C for *T. amylovorum* and *T. medium* , 53°C for *T. socranskii* , and 56°C for *T. vincentii* ; and extension at 72 °C for 1 min.
3. Final extension at 72 °C for 4 min.

Amplicon detection: PCR product was tested for presence the amplification of appropriate size by subjecting the PCR product to electrophoresis in 1% agarose gel (containing 100 µl ethidium bromide to be bounded with the double helix)with 0.5X TAE buffer at 100V for 1hr.. Then the DNA bands were visualized by UV transilluminator and photographed.

RESULTS AND DISCUSSION

89% of samples were positive for *Treponema sp.* by denotation of the amplified DNA bands appearance of the target organism, 12% of the positive cases were positive to the presence of one DNA species while 88% of them were dominated by more than one type (Fig.1). Image (1) show the specific DNA bands of each Treponemal species and Fig. (2) expresses the percentages of each of the four Treponemal DNA prevalence in 46 subgingival samples.

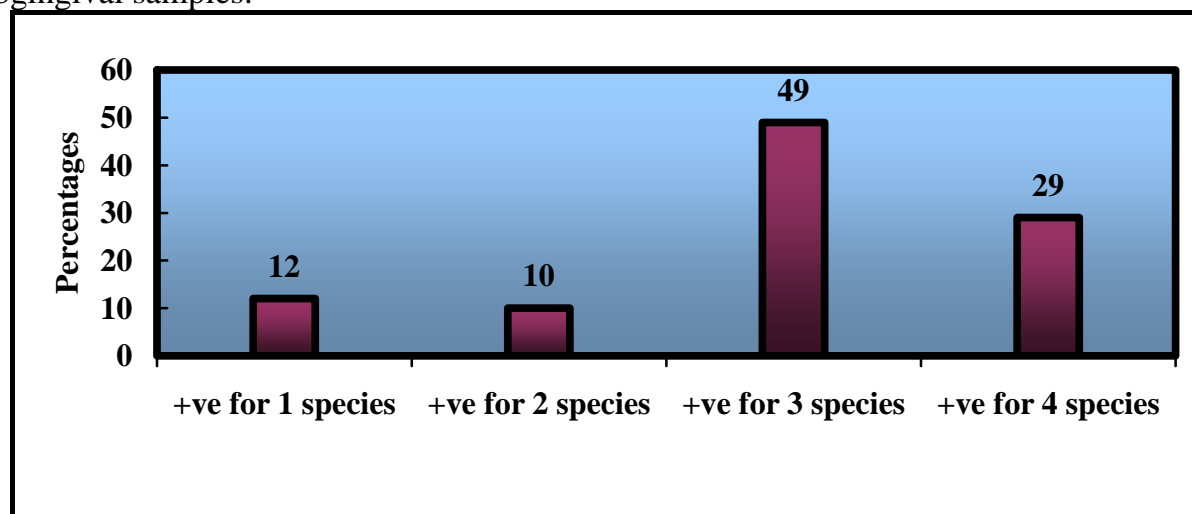


Fig. 1: the detection rates of *Treponema species* in the same sample.

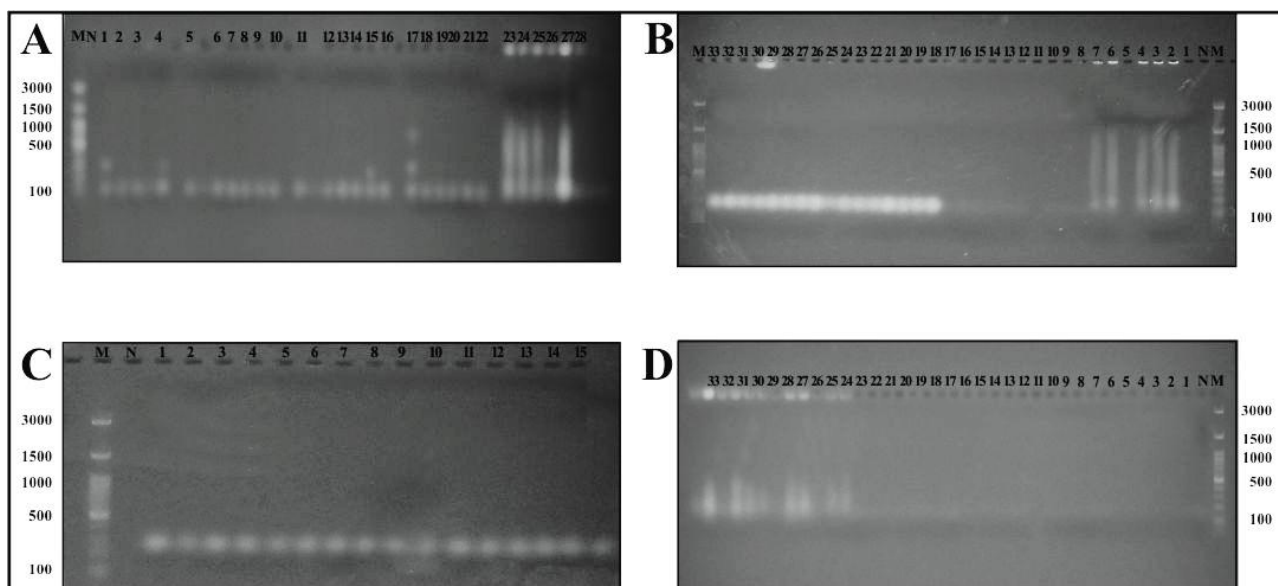


Image 1: DNA bands of *T. amylovorum* (A), *T. medium* (B), *T. socranskii* (C), *T. vincentii* (D), M: master DNA, N: negative control, the numbers represent the samples.

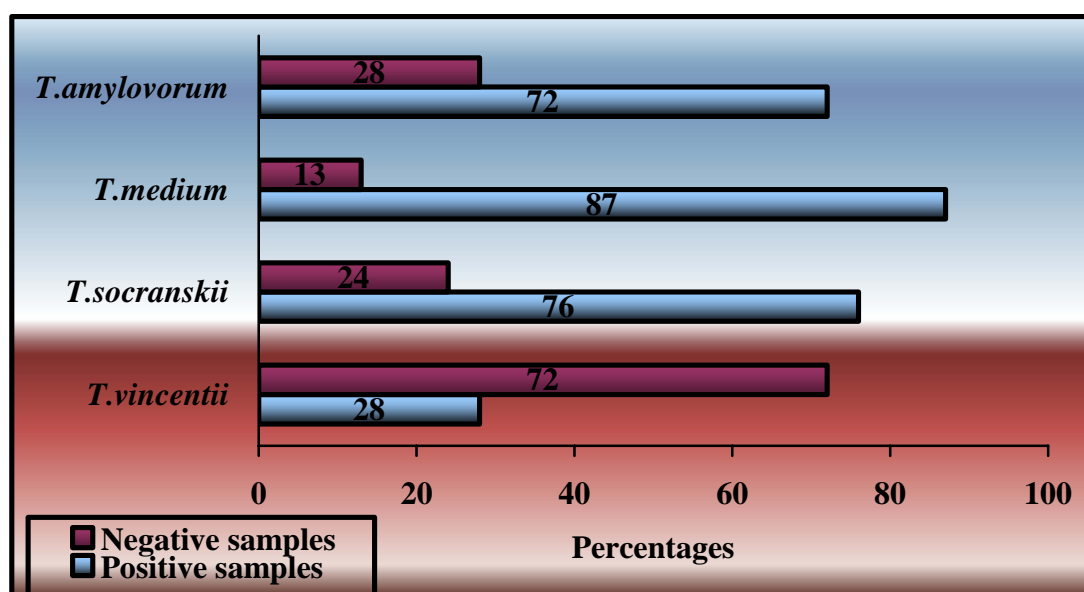


Fig. 2: Prevalence of four Treponemal DNA in 46 subgingival samples.

In Iraq, PCR application is limited to identify some viral infections, while rare attention is paid in respect to investigate the periodontal biofilm community as a mixed population. In this study, a culture-independent PCR method was used to find out the prevalence of some oral treponemes species since this currently available accurate molecular methods for

bacterial identification from subgingival specimens can rapidly detect microbial species in mixed population (Nishiyama *et al.*, 2007). Depending on this technique, the current study could detect the presence of oral *Treponema* in 89% of subgingival samples as these methodologies, particularly PCR are more specific, sensitive and rapid than culture methods to detect the uncultivable fastidious microorganisms. PCR also allows more precise identification of cultivable bacterial strains with a phylotypically divergent behavior because it does not depend on cell viability which is of a particular advantageous in studying strictly anaerobic infectious where cell death can occur during sampling and transportation. These methodologies can detect as little as $10-10^2$ bacteria per sample, resulting in a large number of positive cases which is particularly important when monitoring the prevalence of such pathogens in oral samples (Siqueira *et al.*, 2002; Santos *et al.*, 2004).

Based on these concepts the use of molecular techniques lead to a better comprehension of microbial profile of oral infections and expanded the list of oral putative pathogens by inclusions of some fastidious or even uncultivated bacteria that have never been previously discovered by cultivation procedures, hence over 700 different species of bacteria have been selected in human oral cavity and about 50% of these bacteria are known only by 16S rRNA gene sequence, including several *Treponema sp.*, *Provetella tanneriae*, *Filifactor alocis* and *Dialister pneumonia* (Siqueira and Rôças, 2005 ; Sassone *et al.*, 2008). For instance these methods allowed the detection of *T. denticola*, *T. medium*, *provetetella tanneriae* for the first time in infected root canals in higher prevalence. Other fastidious bacterial species such as *Slakia exigua*, *Mogibacterium timidum*, and *Eubacterium saphenum* have been reported in endodontic infections in high prevalence values when molecular methods were used (Siqueira *et al.*, 2002). It was also successfully used for direct identification and elucidating the role of specific bacteria in periodontal disease with no evidence for the obvious risk for losing the sensitivity of this procedure in recovering the species in concern even if dead (Avila-Campos, 2003). So we call for the importance of using this technique in the identification program of anaerobic oral species that are possible to lose during conventional culturing methods.

Treponema sp. are examples of fastidious bacteria that are difficult to culture, differentiate and maintain although some progress has been made in isolation. There are nearly 60 treponemes species- level phlotypes with phenotypically divergent behavior and approximately 80% of which remain uncultivable, so a comprehensive analysis of the diversity of spirochetes associated with oral disease is necessary and requires the utilization of culture- independent approaches. Dark field microscope used to differentiate their motility depends on living bacteria and, however, unable to differentiate between species. Here, PCR is superior as successful DNA analysis does not require living organisms (Strube *et al.*, 2010; Sakamoto *et al.*, 2009).

In the present study a specific primer for each *Treponemal* species targeted to specific region on 16S rDNA was used to be accurate in the detection as the use of a universal bacterial primers may underestimate spirochetal population especially if they are in low numbers (Sakamoto *et al.*, 2009), thereby it was able to detect *T. amylovorum* in 72%, *T. medium* in 87%, *T. socranskii* in 76%, and *T. vincentii* in 28% from subgingival samples. In addition to, 88% of the positive samples harbored more than one *Treponemal* species while 12% of them were positive for one species, also it was possible to detect *Treponemal* species at the fourth site of teeth. The higher prevalence values obtained with PCR are more

likely to be explained by the detection rate of this method (Siqueira *et al.*, 2002). The high detection percentage of *T. socranskii*, *T. medium*, *T. lecithenolyticum*, and *T. denticola* in 95%, 88%, 36% and 62% of chronic periodontitis samples respectively and 59%, 53%, 17%, and 17% of healthy samples respectively was recorded by (Kumar *et al.*, 2003) when 16S rDNA-based PCR was applied.

Whenever dealing with a variety of closely related and not-yet-cultured organisms, the specificity is favored rather than sensitivity, so the use of specific primer represents an appropriate accurate far needful tool to investigate the diversity of cultivable and non-cultivable periodontal pathogens from mixed bacterial population and provide direct sequence information (Sakamoto *et al.*, 2003 ; Avila-Campos, 2003). 16S rDNA sequences are relatively short highly informed and often conserved sequences but varied enough to differentiate bacteria at species level, hence, this approach can provide us with the microbial composition within the community and demonstrate the relationship of pathogens (He and Shi, 2009; Santos *et al.*, 2004).

Subgingival environment is a diverse polymicrobial community that opportunistically cooperate causing periodontitis. In the present study most specimens from the same patient and the same site were positive for the presence of more than one species, herein; the importance of using 16S rDNA is vouched in substituting traditional culture methods which may be unable to detect all these species in the same sample. This is because of the impossibility to offer all nutrients required for each species in the same culture media, or certain species may be so vulnerable that may die during transport or experimental process, or even the biochemical diagnosis is unable to distinguish between the closely correlated species especially in respect with the enormous diversity of oral treponemes even within a single patient which has been a roadblock to the study of their common mechanisms of pathogenicity (Bayingana *et al.*, 2010; Jun *et al.*, 2008). Oral bacteria in particular have evolved in a mixed community in a biofilm that one species can obtain essential substances from other species in the biofilm, therefore, it will be unable to grow independently *in vitro*; or because of disruption of the bacterial cytokine networks (bacterial-bacterial signaling) which is particularly important in coordinating the growth of component organisms in bacterial biofilm (Wade, 2002). In comparison, it was possible to distinguish between these species by using species- specific 16S rDNA genes sequencing techniques which demonstrate that significant number of species present in subgingival plaque have yet to be characterized and several bacterial taxa simultaneously may play an important role in periodontitis, also correlate the presence or absence of those organisms with disease (Kuramitsu, 2003).

Moter *et al.*, (2006) used oligonucleotide probes to detect seven major phylogenetic groups and several Treponemal species in periodontal infections. They found that it is possible to find all oral Treponemal types in samples of chronic, aggressive infections, and healthy subjects(except for *T. pectinovorum*)in at least one sample that strengthens the view that in periodontitis as a mixed bacterial infection not a single species is responsible for severity of disease alone and aggressive patients were more likely to harbor certain phylotypes in every subgingival pocket compared to chronic patients which harbor enormous types. In their subjects, Treponemal phylogenetic group II to IV, *T. socranskii*, *T. denticola*, and *T. maltophilum* were more detected than *T. medium* , *T. vincentii*, and *T. amylovorum* which were less prevalent.

The pie chart in Fig. (3) showed that the percentage of detection of the all tested Treponemal species was higher at pockets greater than 5 mm. The current results coincide with other findings done by Kumar *et al.*, 2003 ; Asia *et al.*, 2005 ; Tamai *et al.*, 2007 where *T. medium* and *T. socranskii* have been discovered in subgingival plaque of patients with adult and chronic periodontitis by PCR techniques in increasing numbers with deep periodontal pockets. This is because the mean counts of this bacteria are generally high at sites with greater pocket depth, hence, more bacterial mass is taken by paper point and treponemes would be easier above the detectable level. In another word the higher percentage of Treponemal species would just reflect the higher number of deeper pocket because these organisms are obligate anaerobes and tend to exhibit far away from oxygen in deep pockets (Ximenez-Fyvie *et al.*, 2000 ; Moter *et al.*, 2006).

Fig. (4) showed the ability of molecular methods to detect treponemal species in subgingival fluid in patients even after scaling, however in lower percentage than before it. As bacterial bioburden is swilled after scaling process, the detection rate of the four species was lowered especially with *T.vincentii* which is never detected after scaling while the ability to detect the other three species even after scaling may be discussed by the surpassing capacity of this PCR technique to disclosure the presence of even low DNA level.

In spite of subject rarity of the current study, it represents the first application of a rapid, accurate, sensitive, and specific technique to determine the prevalence of some periodontitis associated *Treponema* sp.. The present results were qualitative (presence/absence species) and referred to the importance of outfitting molecular techniques for further identification of other treponemal species and as yet uncultured organisms and investigate the phylogenetic relationship between these species by exploitation these techniques.

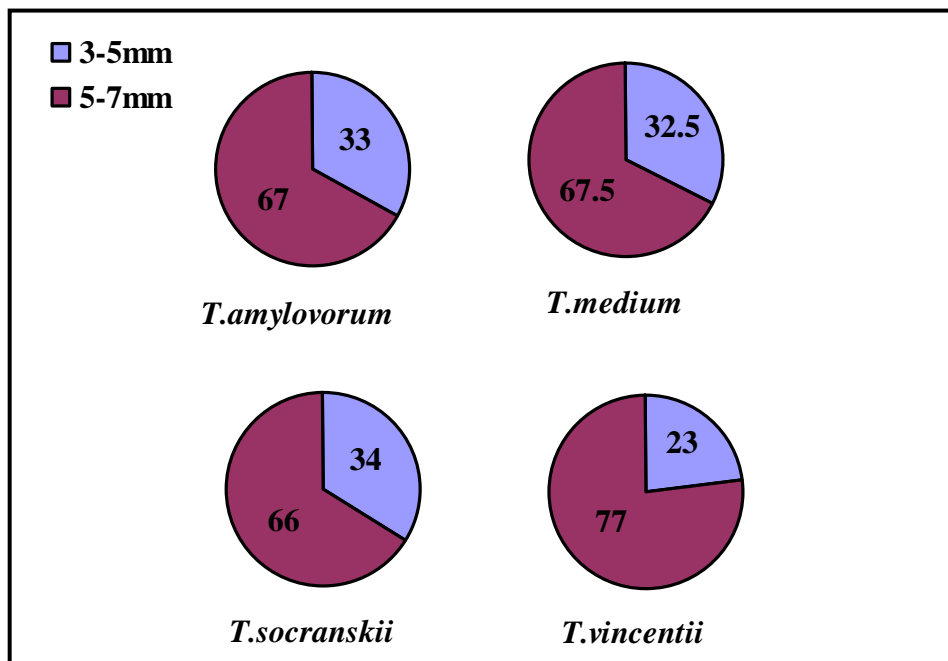


Fig.3: Pie chart of the prevalence of the four Treponemal species in respect to pocket depth

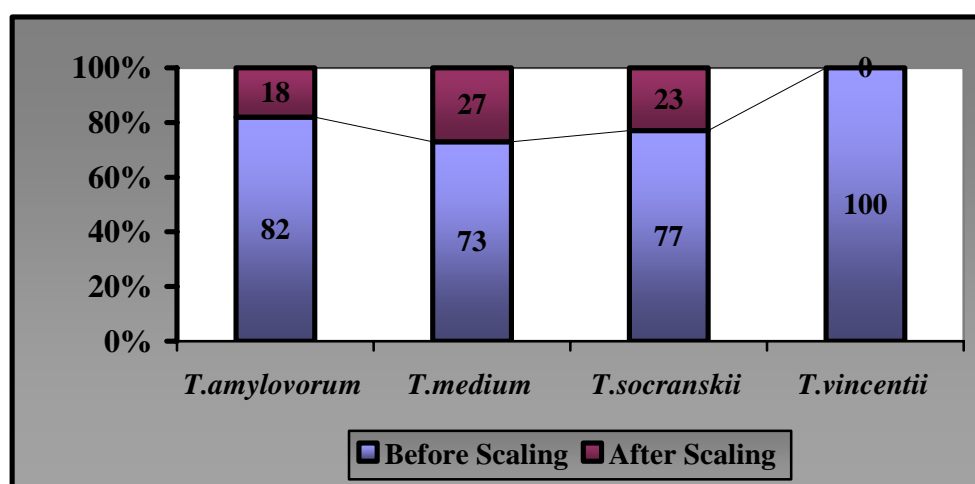


Fig. 4: The percentages of the four Treponemal species detection before and after scaling.

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