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UTILITY OF SILVER NITRATE STAINING IN DETECTION OF SPIROCHETES FROM ORAL CAVITY OF PERIODONTALLY HEALTHY AND DISEASED INDIVIDUALS

VARSHA CHOUDHARY [1] M. SC, PREETI INGALAGI [2] M.SC, DR.CHETANA BOGAR [2] BDS, DR.SUNIL RAO [4] MD AND DR. KISHORE BHAT [3*] MD

[1] Research Officer Maratha Mandal's NGH Institute of Dental Sciences & Research Centre, Department of Microbiology Belgaum-590010 Karnataka
[2] Lecturer Maratha Mandal's NGH Institute of Dental Sciences & Research Centre, Department of Microbiology Belgaum-590010 Karnataka
[3] Prof and Head Maratha Mandal's NGH Institute of Dental Sciences & Research Centre, Department of Microbiology Belgaum-590010 Karnataka
[4] Prof and Head Yenepoya Medical College, Yenepoya University, Mangalore

ABSTRACT

Background: Oral spirochetes play a definitive role in the etiology of periodontitis. But their study is hampered due to difficulty in cultivating them and molecular methods are expensive. Hence the study explores the possibility of using staining as a screening procedure for the study of oral spirochetes. Experimental approach: The study included 100 patients with chronic periodontitis and 100 controls. Subgingival plaques were collected and silver nitrate staining was performed. The positive slides were screened for density of organisms and an attempt was also made to determine the relative size of spirochetes and their predominance.Findings: There was a significant difference in the positivity rate between the patients and controls. Higher number of patients had greater density of spirochetes. The presence of large spirochetes in controls was significantly higher.Conclusions: Silver nitrate staining is a useful screening method for detection of spirochetes. It can also be used to assess the cell size, density and post therapy evaluation.

KEYWORDS: Spirochetes, Silver Nitrate Staining, Periodontitis, Microscopy, Cell Density.

DR. KISHORE BHAT MD
Prof and Head Maratha Mandal's NGH Institute of Dental Sciences & Research Centre, Department of Microbiology Belgaum-590010 Karnataka

INTRODUCTION

Oral microbial flora is a highly complex community comprising nearly 700 different bacterial species[1]. Spirochetes belonging to the genus Treponema make up a very important component of this microbial population. Even though more than 60 different phylotypes of oral spirochetes are known to exist, only ten have been cultivated and speciated so far[2]. A large body of evidence accumulated over the last three decades has shown definite association between oral spirochetes and various periodontal and endodontic infections[3-7].

In spite of so much work being done, cultivation and identification of oral spirochetes remains one of the most challenging and difficult tasks for a microbiology laboratory. This could be due to the fastidiousness and stringent requirements for anaerobic atmosphere and complex nutritional supplements of these organisms[8]. As a result, the laboratory identification of oral spirochetes is largely dependent on either various microscopic techniques or different molecular methods such as Polymerase Chain Reaction and Hybridization[9].

The microscopic methods used for detection of spirochetes from dental plaque include wet preparation techniques such as dark ground or phase contrast microscopy or silver nitrate staining using Fontana's method. The oral spirochetes can be provisionally classified as small, intermediate and large forms based on their size by using these procedures[9,10]. In the present study, an attempt has been made to detect spirochetes from plaque specimens of patients with chronic periodontitis and also from healthy individuals.

MATERIALS AND METHODS

The study was performed in the department of Microbiology, Maratha Mandal's NGH Institute of Dental Sciences, Belgaum. A total of one hundred patients with chronic periodontitis and an equal number of periodontally healthy individuals were selected for the study. Informed consent was obtained from each participant before enrolling in the study. The study subjects were adults in an age range of 25 to 50 years and belonged to both the sexes. The inclusion criteria for patients with chronic periodontitis were presence of periodontal pockets of ≥ 4 mm, bleeding on probing, clinical attachment loss of ≥ 3 mm[2] and radiologic evidence of bone loss. Subjects with a probing depth of 2-3mm and without any evidence of inflammation were selected as controls. Pregnant women and lactating mothers, those with debilitating medical conditions, those who had received systemic antibiotics and/or had undergone periodontal treatment during the last six months were excluded from the study.

Subgingival plaque samples were collected by means of curetting taking appropriate precautions not to contaminate the specimen with material from surrounding areas. The silver nitrate staining was done according to Fontana's method[11]. Briefly, the film prepared from subgingival plaque was allowed to air dry and then fixed by treating with a fixative (acetic acid 1%, formalin 0.8%) three times, 30 seconds each time. After washing the fixative, smear was treated with alcohol for 3 minutes, followed by mordant (phenol 1%, tannic acid 5%) for 30 seconds. Finally the smear was exposed to ammoniated silver nitrate solution and the slide was heated till the steam was seen and left for 30 seconds before washing in distilled water. The smear was dried, mounted in Canada balsam and seen under the oil immersion field of bright field microscope. The spirochetes are stained brownish black with a light brown background (Fig 1).

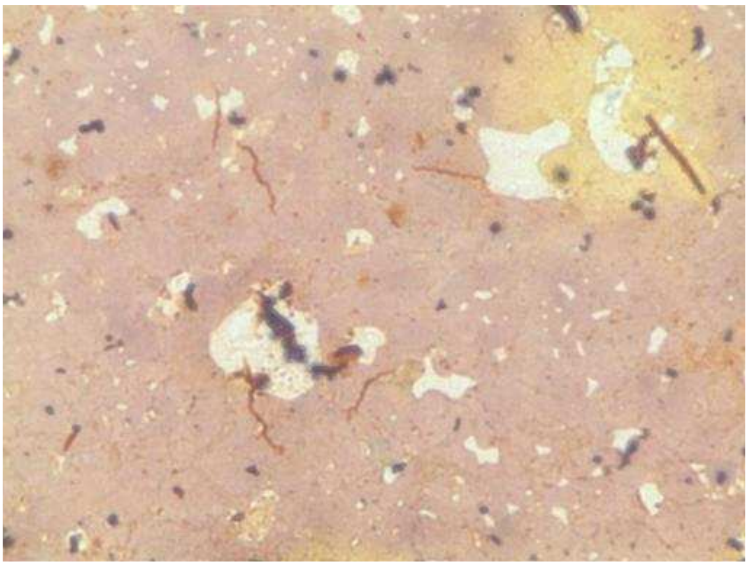
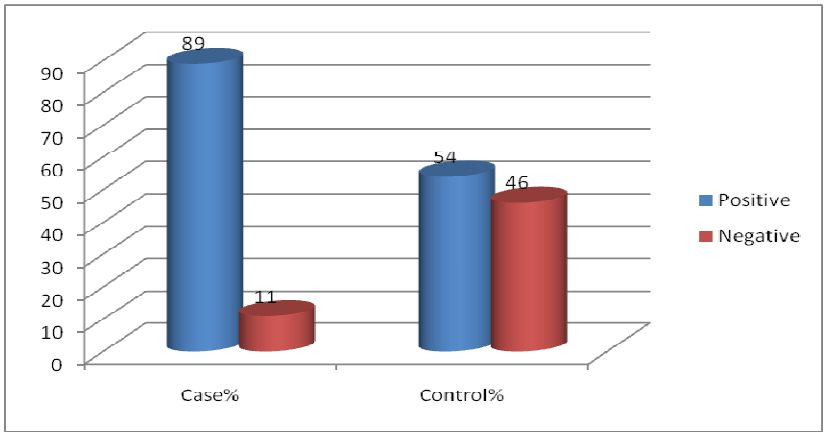


Figure 1
Fonatana's Silver Nitrate Staining for Spirochetes

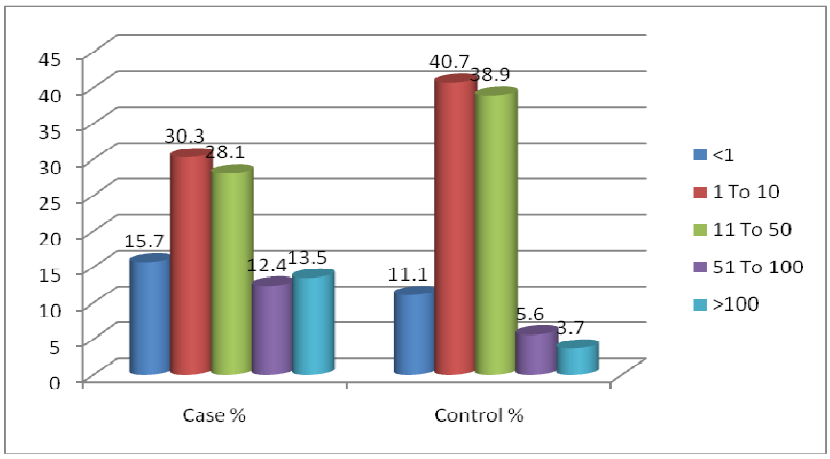
RESULTS

Among one hundred patients with chronic periodontitis, 89% were positive for the presence of spirochetes. On the other hand, spirochetes could be demonstrated in the oral cavity of only 54% of healthy controls. This difference was highly significant with a p value of <0.001 (Graph 1).



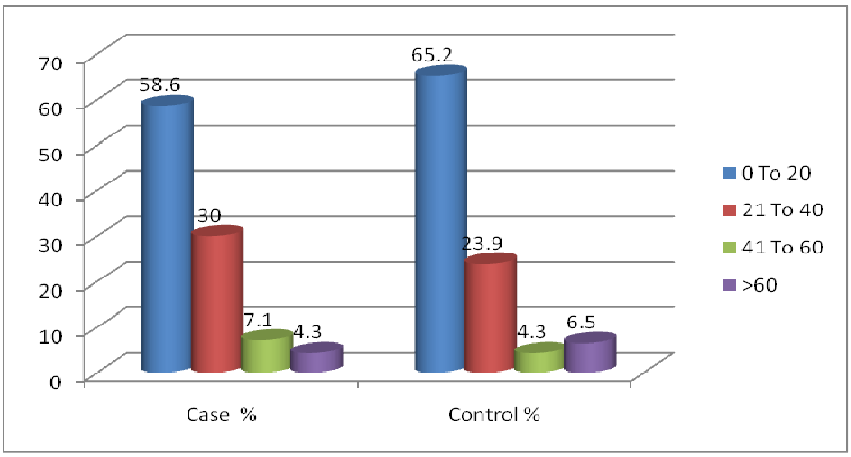
Graph 1
showing differences in positivity among patients and controls

All the positive results were expressed as number of spirochetes / oil immersion field (OIF). An average of at least 50 fields was scanned and the average was noted. For the sake of comparison, the total spirochete counts were arbitrarily categorized into 5 groups as <1, 1-10, 11-50, 51-100 and >100 /OIF. It could be seen that more number of patients expressed higher spirochete counts in comparison to control; 25.9% of patients had counts of >50 spirochetes /OIF, in contrast to 9.3% of control subjects. However, the overall difference among different groups between patients and controls was statistically not significant (Graph 2).

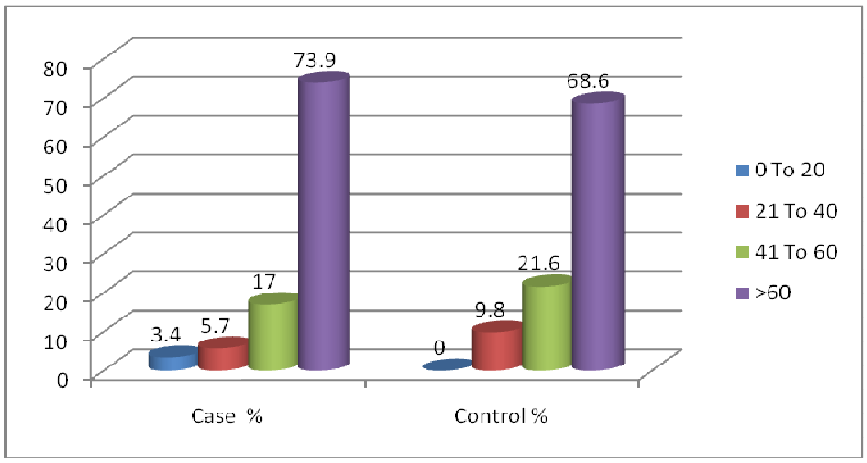


Graph 2
showing spirochete density in patients and controls

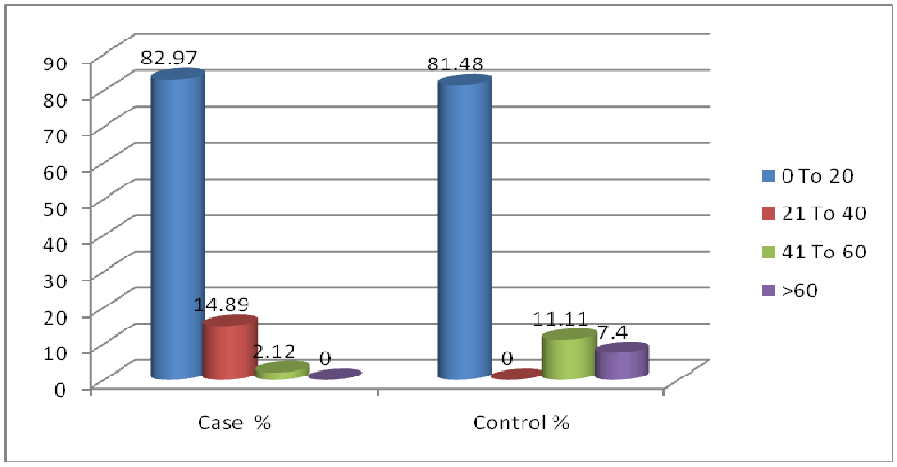
We further classified the spirochetes provisionally as small, intermediate and large based on the size as mentioned previously. Approximate density of each type in all positive samples was noted. An attempt was made to analyze the differences in the presence of each type between patients and healthy subjects. There was a predominance of intermediate forms followed by small and large types in both the groups studied. The density of each type of spirochete from every positive sample was further arbitrarily divided into 4 groups as 1-20, 20-40, 41-60 and >60 for the sake of comparison (Graphs 3,4.5). A significant difference could be seen only in the presence of large forms between the study subjects and controls (p=0.002, Graph 5).



Graph 3
Showing distribution of small spirochetes among patients and controls



Graph 4
Showing distribution of intermediate spirochetes in patients and controls



Graph 5
Showing distribution of large spirochetes in patients and controls

DISCUSSION

Spirochetes comprise a highly heterogenous and complex population of the oral cavity. Even though they were described more than 200 years ago, they remained an enigma for a long time. This was mainly due to difficulty in culturing because of their requirement for strict anaerobic conditions, complex nutritional needs and variability in their morphology[9,12]. As a result, the early studies on the role of spirochetes in the etiology of periodontal diseases was assessed mainly by microscopic methods, and they are still the most sought after techniques especially in places where facilities for cultivation and molecular techniques such as PCR are not available.

Among the various microscopic techniques available, dark ground and phase contrast microscopy are used for immediate detection of motile spirochetes in clinical specimens. Here, the preparations have to be screened within half an hour of collection, specialized microscopes are needed and it will not be possible to maintain any permanent record of the sample preparations. On the

other hand, silver nitrate staining method has several advantages: it is simple and economic to perform, does not need any specialized equipment, and permanent records can be maintained, making it easier to evaluate the prognosis after therapy in patients[13] .

We could show a significant difference in the percentage positivity of spirochetes between healthy subjects and patients with chronic periodontitis by using silver nitrate stain. Similar observations have been made by several other workers [5-7,14]. Moreover, the density of spirochetes was much higher in patients with periodontitis, once again reconfirming the importance of these organisms in periodontitis. Apart from T. denticola, the most studied oral treponeme, several other species of treponemes are also known to play a role in the etiology of periodontitis. All of these are either small or intermediate in size [1,2]. Even though electron microscopy is the best method to determine the size of oral spirochetes [15], one can have a reasonable assessment by using silver nitrate stain especially with an experienced eye. We could differentiate between the three morphotypes by looking at the thickness. As expected, intermediate and small forms predominated in both healthy subjects and patients with periodontitis. An interested finding was that the positivity of large forms was significantly higher in healthy subjects. It is also a proven fact that so far large forms have not been cultivated and their pathogenic potential is not known. Several workers have also shown that the microscopic methods can be used to assess the efficacy of treatment, making them a simple and rapid method to be used in clinical setting[16,17].

One must not forget that silver nitrate staining methods also have several disadvantages. The final outcome is heavily dependent on the quality of reagents used and the experience of the personnel involved in staining and microscopic analysis. Moreover, it would be difficult to quantitate the organisms by microscopy and there would be a high amount of variability among different microscopists reporting the same slide.

In conclusion, microscopic method by silver nitrate staining can be a very useful screening method for evaluation of oral spirochetes in a clinical setting if used judiciously keeping in mind the variables that can affect the results. The method also can be used to assess the size and density of spirochetes in oral cavity to a reasonable extent.

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