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Biochemical Identification of *Aggregatibacter actinomycetemcomitans* in an Indian Sample with Aggressive Periodontitis

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Authors' contributions

This work was carried out in collaboration between all authors. Author RS designed the study, performed the statistical analysis, wrote the protocol and the first draft of the manuscript. Authors BM, GBB and Rajasekar managed the analyses of the study. Authors IN and AA managed the literature searches. All authors read and approved the final manuscript.

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ABSTRACT

Background: The role of microorganisms and the etiology of periodontal disease is well established. Despite the fact that periodontal diseases are caused by dental plaque there existed some controversies regarding the role of specific organisms in the pathogenesis of the periodontal disease. This problem was overcome by the specific plaque hypothesis. Generally, the periodontal pathogen *Aggregatibacter actinomycetemcomitans* (Aa) is identified employing anaerobic culture and in recent years through polymerized chain reaction. Very few attempts have been done to establish the confirmation of Aa through biochemical reactions. Hence this study was under taken to confirm Aa from the plaque sample by various biochemical tests.

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Aim of the Study: Biochemical identification of *Aggregatibacter actinomycetemcomitans*, a specific periodontal pathogen from the plaque samples of aggressive periodontitis patients.
Materials and Methods: A total of forty patients 24 males and 16 females in the age range of 18-25 yrs. Diagnosed as aggressive periodontitis were included in this study. Plaque samples were collected from the periodontal pockets of aggressive periodontitis patients and were subjected to various biochemical tests.
Results: Biochemical tests confirmed that the periodontal pathogen collected from the plaque samples were *Aggregatibacter actinomycetemcomitans*.
Conclusion: Biochemical tests can be used as a viable economic alternative for the confirmation of the periodontal pathogen Aa.

Keywords: Aggregatibacter actinomycetemcomitans; periodontal pathogen; aggressive periodontitis; biochemical tests.

ABBREVIATIONS

Aa : *Aggregatibacter actinomycetemcomitans*,
AgP : Aggressive periodontitis.

1. INTRODUCTION

1.1 Background

Periodontitis is a multifactorial disease caused primarily by the interaction between periodontal microbes and the host response. Periodontal pathogens mainly reside in the sub gingival plaque and occurrence of these pathogens may vary among different individuals depending on ethnicity or social status. *Aggregatibacter actinomycetemcomitans* (Aa) is an important periodontal pathogen known for its strong virulence characteristics that cause periodontal disease. Detection of Aa facilitates the development of a better treatment plan for patients with aggressive periodontitis (AgP).

1.2 Taxonomy

Klinger (1912) coined the term 'Bacterium actinomycetemcomitans' to a coccobacillary bacteria that was isolated along with Actinomyces from actinomycetemic lesions of cervicofacial actinomycosis. Hence the species name actinomycetemcomitans (together with Actinomyces). Lieske (1921) renamed it as Bacterium comitans. It is a coccobacillary organism (bacillus) and has a star shaped internal morphology (actino) hence the genus name Actinobacillus. Tropley and Wilson (1929) reclassified and termed it as *Actinobacillus actinomycetemcomitans*. Potts et al (1985) termed it as *Haemophilus actinomycetemcomitans*.

Actinobacillus actinomycetemcomitans was found to be more closely related to Haemophilus based on the phylogenic similarity than to the genus Actinobacillus. Hence it underwent another taxonomical classification. Norskov Lauristen and Kilian (2007) renamed and reclassified it to the current term Aggregatibacter [1].

This species was of significant clinical interest due to its association with localized aggressive periodontitis [1]. Serologically it is classified into six serotypes as a-f and has a defined structural and antigenic o-polysaccharide component with their respective lipopolysaccharide molecule [2,3]. Aa is a capnophilic, microaerophilic facultative anaerobe that can be cultured *in vitro*.

1.3 Virulent Factors

A variety of virulence factors can be produced by Aa that plays a role in the pathogenesis of periodontal disease. These factors have the potential to destroy the periodontal tissues either directly or indirectly. Factors that can have a direct effect on the periodontal tissues are the bacterial collagenase that destroys the gingival connective tissues [4]. Epithelotoxin a factor produced by this organism facilitates bacterial penetration of junctional epithelium and pocket lining [5]. Another factor is fibroblast inhibiting factor which impede the repair of the tissuses [6]. Virulence factors from Aa that can indirectly affect the periodontal tissues are those which essentially act on the host's immune response. Two such factors are the leukotoxin which destroys the polymorphonuclear neutrophils (PMN) which leads to the release of lyzozymal enzymes that potentiates further tissue destruction [7]. and a chemotactic inhibiting factor which impairs the chemotactic response of PMN [8]. Both factors would deplete the protective role of PMN in the periodontal tissues. Moreover, Aa can activate T suppressor cells, which in turn suppresses both B cell and T cell

responses [9]. An additional feature of the bacteria is to invade the underlying connective tissues that facilitates tissue breakdown [10,11]. Tissue invasion by Aa may in part explain the poor response of aggressive periodontitis to scaling and root planing and augurs for a planned treatment protocol.

Though advanced diagnostic techniques and polymerized chain reactions are used to identify the organism, culture has been long known as the gold standard for identifying bacteria. Also biochemical tests can be used as an economic alternative for confirmation of Aa. Hence the present study was designed to confirm the presence of Aa in AgP using various biochemical tests.

2. MATERIALS AND METHODS

Informed consent of the patients was obtained. The study was conducted among South-Indian population. A pilot study was done and based on the results the sample size was decided. Probability sampling technique using simple random sampling was employed.

2.1 Inclusion Criteria

Both male and female patients were included for the study. A total of 40 patients, 24 male and 16 female patients diagnosed as aggressive periodontitis in the age group of 15-25 years were selected for the study. Patients chosen were free from any systemic diseases, have not undergone oral prophylaxis or any other periodontal treatment and not taken antibiotics 6 months prior to the study.

According to the American Academy of Periodontology 1999 Aggressive periodontitis should have the following characteristics.

a. Circumpubertal onset of disease
b. Localized first molar or incisor disease with proximal attachment loss on at least two permanent teeth, one of which is a first molar.
c. Otherwise healthy patient
d. Rapid attachment loss and bone destruction.
e. Amount of microbial deposits inconsistent with disease severity.
f. Familial aggregation of diseased individuals.

A detailed clinical history was taken. A thorough intra oral examination was done to check oral hygiene status of the individual. Radiographic examination revealed an arc shaped intra bony defect extending from the mesial aspect of lower second premolar to the distal aspect of first molar.

2.2 Exclusion Criteria

Pregnant and lactating women, smokers, patients with immune modulatory therapy, and systemic diseases were excluded from the study.

2.3 Sample Collection

A baseline microbiology sampling was done for each subject and the sample sites chosen were first molar and central incisors. The selected teeth were isolated with sterile cotton rolls. The loosely adherent plaque in direct proximity to the sample site was carefully removed using sterile cotton gauze. A sterile paper point was introduced into the mesiobuccal interproximal pocket until resistance was met. It was kept in place for 10 seconds and the collected sample was transferred into a screw capped test tube containing sterile saline and dispersed in a vortex mixture for 60 seconds. The test tube is taken to the laboratory within 30-60 minutes. In the laboratory, the plaque sample was cultured anaerobically in Trypticase Soy Bacitracin Vancomycin medium. Mc Intosh Field's anaerobic jar was used to cultivate the organism. Chemicals such as citric acid 3mg and sodium borohydrate 700 mg were used to provide the anaerobic environment. These chemicals liberate hydrogen and carbon dioxide on addition of 10 ml of water. The plaque sample was inoculated in the above mentioned medium and kept in the anaerobic jar and incubated at 37℃ for 48-72 Hrs. After this period the jar was opened and the colony morphology examined. The strains of the culture revealed a star shaped colony morphology, the colonies were rounded with an irregular edge and dome shaped and colourless in appearance which suggested the strain to be Aa. It is a facultative anaerobic gram negative capnophilic non motile coccobacilli. In order to confirm whether the organism cultivated was Aa it was subjected to various biochemical tests. [12,13,14]

2.4 Biochemical Tests

2.4.1 Oxidase test

The cytochrome oxidase enzyme is able to oxidase the substrate tetramethyl-p-phenylene

diamine dihydrochloride by forming a coloured end product, indophenol [15] The dark purple end product will be visible when placed on a substrate impregnated filter paper when a small amount of growth from a strain that produces the oxidase enzyme is placed over it. It was a positive reaction as the substrate kovac's reagent, on the filter paper changed from a colourless to a dark purple colour after the organisms (Aa) from the colony was streaked on the filter paper with a glass rod.

2.4.2 Catalase test

The breakdown of hydrogen peroxide into oxygen and water is mediated by the enzyme catalase. When a small amount of organism that produces catalase is introduced into the hydrogen peroxide, rapid elaboration of bubbles of oxygen, the gaseous product of the enzyme will be actively produced [16]. A similar reaction was observed, when the organism Aa was introduced into hydrogen peroxide, due to the breakdown of hydrogen peroxide suggesting the reaction to be positive.

2.4.3 Nitrate test

Organism that possesses nitrate reductase can reduce nitrate to nitrite. Nitrite combines with an acidified napthylamine substrate to form a red coloured product. If the organism has further reduced nitrite to nitrate gas, the test for nitric acid will yield a negative (colourless) result. An additional test for the presence of unreacted nitrate must be performed to validate such a colourless result. Metallic zinc catalyzes the reduction of nitrate to nitrite; thus, with the addition of zinc a negative test will yield a red colour, indicating the presence of unreacted nitrate. It was a positive reaction, as the organism Aa reduces nitrate to nitrite, as evidenced by a colour change from brown to red.

2.4.4 Indole test

The end product of the action of tryptophanase on tryptophan can be detected by its ability to combine with certain aldehydes to form a coloured compound. 2 ml of the broth suspension (peptone water) along with 0.5 ml (5 drops) of the kovac's reagent was introduced into the broth kept in the tube and was observed for a purple colour in a ring around the interface between the broth and alcoholic reagent, which rises to the surface. This was a negative reaction as no colour change was observed at the end of the reaction.

2.4.5 Esculin hydrolysis

On bile esculin agar medium the organism hydrolyzes esculin to esculetin, which combines with ferric ions to form a brown black colour. change. This again was a negative reaction as no colour change was observed at the end of the reaction.

2.4.6 Urease test

Hydrolysis of the urea by the enzyme urease releases the end product ammonia, the alkalinity of which causes the indicator phenol red to change from yellow to pink. This was again a negative reaction, as there was no colour change from yellow to pink at the end of the reaction.

2.4.7 Fermentation of sugars

The sugar solutions were prepared using peptone water and the particular sugars (Maltose, Sucrose, Lactose, Trehalose, Salicin). Indicator Bromothymol Blue was added. Sterile sugar solutions were taken in a test tube and the organism inoculated anaerobically at 37℃ for 48 hours. During the fermentation of sugar, acid is liberated. At the acidic PH the indicator changes from blue to yellow. Of the sugars tested for fermentation only maltose was positive.

2.5 Ethics

Ethics were followed in this study as patient's identity was not displayed to the laboratory and it was not an interventional study as it only assisted in the management of the patients.

2.6 Statistics

No statistical analysis was employed as it was only a confirmatory study using biochemical agents and the results are self-explanatory.

3. RESULTS

The culture did not reveal Aa in all the cases, Out of 40 plaque samples examined only 32 samples showed positive for presence of Aa that is 80% showed Aa positive and 20% showed Aa negative as represented in Fig. 1.

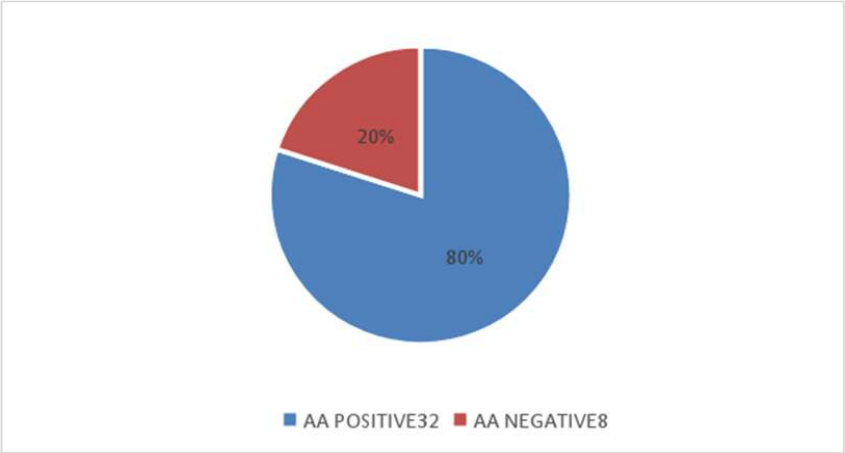

Fig. 1. Pie chart showing patients with aggressive periodontitis with Aa

The intensity of the reaction does not indicate the percentage of microorganisms colour change shows a positive reaction and no colour change indicates a negative reaction

There is no role of intensity its only the positivity and negativity of the reactions.

Biochemical test confirms the results of the culture for all the patients.

4. DISCUSSION

The etiopathogenesis of periodontal disease and the microbial etiology of Aa in aggressive periodontitis is well established and understood.Numerous studies [17,18] have established the correlation of the microorganism to the disease. Many clinical trials have been successfully done to eradicate the organism [19-22]. Only a few biochemical studies have been conducted for the identification of the organism.

Aa is a microaeroplilic, gram negative facultative anaerobic microorganism that had a typical star shaped colony morphology. It underwent several taxonomic classifications and the present term *Aggregatibacter actinomycetemcomitans* was coined in 2007. Earlier it was difficult to isolate and identify this organism from the diseased sites. With the advent of technical advances the organism was successfully isolated and was cultured *in vitro* using various culture mediums. The drawback was the low level of sensitivity of the organism from the culture. Moreover it was laborious and a time consuming procedure. Aa was identified from the culture based on its specific star shaped colony morphology Owing to the complex nature of the organism and its similarity to the species in the same genus in the recent years polymerized chain reactions (PCR) is employed to identify this organism directly from the dental plaque samples. This again was an expensive procedure.

Alternate ways of confirming the organism was thought of based on its biochemical properties. Literature revealed a few studies that were employed to identify the organism by biochemical tests. Studies that have employed biochemical reactions revealed that the organism showed a positive reaction to oxidase and catalase [23]. Our study also revealed a similar observation as in Table 1. In another study [24] the organism reduced nitrate to nitrite and on fermentation tested positive to sucrose, glucose and mannose but in our study we observed the reduction of nitrate to nitrite but the organism fermented only maltose as in Table 2 & 1.

Table 1. Reactions of *Aggregatibacter actinomycetemcomitans* to biochemical tests

Test	Results of the reaction	
OXIDASE TEST	+	
CATALASE TEST	++	
NITRATE TEST	+	
INDOLE TEST		-
ESCULIN HYDROLYSIS		-
UREASE TEST		-

Studies employing various biochemical test procedures revealed the organism to be catalase

positive and of the sugars tested for fermentation lactose was positive [25,26]. The findings of our study showed only a catalase positive reaction but fermented maltose which contradicted the findings of the study as in Table 1 & Table 2. Biochemical studies on Aa showed the various chemical reactions of the organism and it had a positive reaction to oxidase, catalase, and nitrate and also fermented maltose [27]. The results of our study also revealed a positive correlation to the above mentioned findings as in Table 1 & 2.

Table 2. Fermentation of sugars

Sugars	Results of the reaction
● MALTOSE	+
● SUCROSE	-
● LACTOSE	-
● TREHALOSE	-
● SALICIN	-

A review of the biochemical reactions of bacteria has emphasized the need to isolate a periodontal pathogen and subject it various biochemical reactions that augurs well in the management of periodontal diseases [28,29]. This review study employed the fermentation of sugars which showed a positive reaction to glucose and sucrose, while the findings of our study showed only maltose to be positive.

The different results observed between the various studies might be attributed to the differences in the strains and also due to the ethnic and social race.

The organism Aa could not be detected in 8 cases that might be due to the contamination of plaque samples, a delay in transport medium to the laboratory and error in culturing techniques.

An attempt was made by subjecting the organism to other biochemical tests such as indole, urease and esculin hydrolysis which was not mentioned in the literature but all of them revealed a negative result. This was done to further evaluate whether the organism showed any reactions in the Indian population.

A study on the accuracy of real time polymerase chain reaction versus anaerobic culture in Aa concluded that real time polymerase chain reaction showed high diagnostic accuracy in detecting Aa. However, it concluded that choice of microbiologic tests is determined by several factors including diagnostic accuracy, cost effectiveness, and availability of antibiotic susceptibility tests [30].

A similar study on detection of Aa emphasized that quantitative PCR technology may have a major role in near future as adjunctive diagnostic tool in both epidemiological and clinical studies [31]. However, culture techniques still hold some inherent capabilities, which makes this diagnostic tool the current gold standard in periodontal microbiology.

5. CONCLUSION

Within the limitations of the present study, it can be said the biochemical tests serves as a confirmatory method to identify the periodontal pathogen as Aa. Though there are more advanced diagnostic aids and techniques available in the modern era that can precisely identify the organism, the conventional method of isolating and identifying the organism through biochemical means still holds good. It can be attributed to the fact due to its cost effectiveness.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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