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RESEARCH ARTICLE

STUDY TO EVALUATE ORAL MICROBIAL FLORA IN CVD (CARDIO VASCULAR DISEASE) PATIENTS FROM GUNTUR DISTRICT OF ANDHRA PRADESH

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ABSTRACT

Over the decades our understanding of the pathogenesis of Cardiovascular Disease(CVD) has increased, and infections, including those caused by oral bacteria, are more likely involved in CVD progression than previously thought. The two disorders share several common risk factors, including cigarette smoking, age, and diabetes mellitus. The focus of this study is to assess whether available data support a causative relationship in systemic inflammation and endothelial dysfunction. Oral samples were collected from 19 patients with cardiovascular disease. The microbial flora was evaluated by drawing swab samples from the oral cavity by using sterile swabs and on Grams staining, both Gram Negative and positive organisms were found. Most of the patients had mostly gram positive organisms, only few patients showed both gram negative and positive organisms. Two cultures 3 and 5 showed coagulase and catalase positive. Biochemical tests of both 3 and 5 culture were performed and Culture 3 biochemical characteristics were little different from the normal *Staphylococcal* biochemical tests. Whereas Culture 5 showed similarities with the normal *Staphylococca*l biochemical tests. Culture 5 was taken and antibiotic sensitivity test was performed. Culture 5 was sensitive to Gentamicin followed by Linezolid, Penicillin G and Furoxone. This matches with the literature data wherein most of the CVD patients show Staph.non aureus organisms, which are responsible for them developing the CVD conditions.

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INTRODUCTION

Abnormal vascular flow around valves causes clotting deposits, and bacteria which enter the bloodstream and become attached to clots then grow by forming biofilm (Xiaojing Li *et al*., 2000). According to the most recent surveillance data staphylococcuse aureus has also been identified as a leading cause of death cases (Alshammary *et al*., 2008; Yoshinaga *et al*., 2008, Niwa *et al*., 2005; Di Filippo *et al*., 2006). The identification of bacteria or bacterial products in the circulation and in cardiovascular tissues is major and crucial evidence for establishing oral infections as causal for CVDs (Harlan, 1983; Valtonen, 1991). Bacteria have shown invasive properties into cardiovascular structures such as arterial walls, aortic aneurysms, and heart valves (Mattila, 1989; Mattila, 1995). Periodontal disease in its various forms is one of the most common disorders affecting mankind. The signs include inflammation, pocket formation, resorption of alveolar bone, and ultimately tooth loss. It is believed that the quantity and virulence of the plaque microorganisms, as well as the intensity of the host inflammatory response, will determine the progression and severity of the disease. The clinical course of periodontal disease can be modified by a number of factors, including diabetes mellitus, cigarette smoking, hormonal influences, and certain genetic disorders. Periodontal disease has risk factors in common with CAD, including smoking, diabetes mellitus, and low socioeconomic status. (Joshipura, 1997) also recognized the connection between dental disease and cardiovascular disease. Among the environmental risk factors and indicators shared by periodontitis and systemic diseases, such as cardiovascular disease, are tobacco smoking, stress, aging, race or ethnicity, and male gender. Sub gingivalbiofilm constitute an enormous and continuing bacterial load. They present continually renewing reservoirs of LPS and other gram-negative bacteria with ready access to the periodontal tissues and the circulation. Systemic challenge with gram-negative bacteria or LPS induces major vascular responses, including an inflammatory cell infiltrate in the vessel walls, vascular smooth muscle proliferation, vascular

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fatty degeneration, and intravascular coagulation (Fedele, 2011). Infective endocarditis is a bacterial infection of the heart valves or the endothelium of the heart. It occurs when bacteria in the bloodstream lodge on abnormal heart valves or damaged heart tissue. Endocarditis occurs rarely in people with normal hearts. However, people who have certain preexisting heart defects are at risk for developing endocarditis when a bacteremia occurs. Infective endocarditis is a serious and often fatal systemic disease that has been associated with dental diseases and treatment. There are over 1,000 case reports associating dental procedures or disease with the onset of endocarditis (Loesche, 1998). Three controlled studies have recently been conducted, all showing an association of dental procedures with bacterial endocarditis (Mendez, 2012; Dahlen, 2012). In addition, multiple animal models (rats, rabbits, and pigs) have shown that oral bacteria and even dental extraction can create histologic evidence of endocarditis under experimental conditions (Lund, 2008). It appears that dental procedures, especially extractions and possibly scaling, meet currently accepted epidemiological criteria for causation of endocarditis, (Chukkapalli, 2014).

MATERIALS AND METHODS

Collection of Sample

Material from oral cavity was also collected through oral rinses, during 1min, in 10mL of sterile solution (PBS 0.1M/pH 7.2) previously distributed in sterile universal containers. The samples were maintained in ice until the process in the laboratory of Microbiology. The maximum period of 3 hours between collection and processing was respected. Microbiological analysis of the samples was performed in the laboratory of Microbiology.

Processing of sample for isolation of Staphylococcus sp

Each sample was centrifuged by 10 min (8000 Xg) .The pellet was resuspended in 2.5 mL and 0.6mL PBS respectively, obtaining the final suspension. From each sample 0.1 mL aliquots were plated in duplicate copy onto Baird-Parker agar (Difco) supplemented with egg yolk (12.5 egg yolks in 25ml saline solution o.85%) and potassium tellurite (0.1g of potassium tellurite in 10 mL of distilled water) and were incubated by 24 to 72 hours at 37ºC.

Differentiation of Hemolytic and non hemolytic activity 0n Blood Agar Medium

Nutrient agar medium can be enriched with 5-10 % blood and inoculated with the Staphylococcal sp isolated.

Identification

After the incubation period, the identification of *Staphylococcus* isolates was based on colonial morphology and Gram stain characteristics.

Grams staining

Grams' staining was performed according to the standard protocol given by Aneja. The slides were viewed under light microscope for shape and arrangement.

Biochemical tests to identify Staphylococcal sp

Coagulase test

Colony is treated with a drop of citrated plasma and mixed well with a needle. The distilled water serves as control. The control suspension serves to rule out false positivity due to auto agglutination. Clumping of cocci within 5-10 seconds is taken as positive. Some strains of S.aureus may not produce bound coagulase, and such strains must be identified by tube coagulase test

Catalase test

A clean glass slide was divided into two sections with grease pencil. One should be labeled as "test" and the other as "control". A small drop of normal saline was placed on each area. With a sterilized and cooled inoculating loop, a small amount of the culture was picked from the nutrient agar slant or Petri plate. One or two colonies on each drop was emulsified to make a smooth suspension. The smear should be about the size of a pea. With a Pasteur pipette, one drop of hydrogen peroxide was placed over the test smear. The other drop that serves as control. The fluid over the smears was observed for the appearance of gas bubbles. the slide is discarded in a jar of disinfectant.

Biochemical characterization tests

Indole Test, Carbohydrate Fermentation, Citrate Utilization Test, Glucuronidase test, ONPG Test, Methyl Red Test, Voges-Proskauer Test ,Nitrate reduction test, Lysine utilization test was performed by biochemical Kits (Himedia)

Kirby-Bauer Disk Diffusion Susceptibility and agar well diffusion Test Protocol

Using an aseptic technique, a sterile swab was placed into the broth culture and the plate was streaked. Antibiotic Disc Dispenser was used to dispense discs containing specific antibiotics onto the plate. Plates were incubated overnight at 37 °C (98.6 °F). (Haheim 2004) However incubating it at this temperature can create microbes that are harmful to humans, so do so with caution. The same protocol is followed as above instead of discs 10 ug/ml antibiotics were prepared and loaded into agar wells that are already made in the agar plate.

RESULTS

Sterile swabs were drawn from the oral cavities of the 19 CVD patients from Clinics and hospitals of Guntur town in Andhra Pradesh after informing the patients of the details of the study. From the above table it is clear that the microbial flora contained is mostly mixed cultures of Streptococcus, Staph. aureus in only. Gram negative were non gas formers and micro aerobic in nature. Majority of the patients showed mixed cultures of both Gram negative and positive organisms. Some of them have shown only gram positive organisms also. The total numbers were enumerated by tube dilution method followed by plating 0.1ml of the broth on to plate count agar/TSA, and the colonies were counted.

19 cultures were isolated which were analyzed for hemolytic or non hemolytic activity.

Fig.1. Hemolytic activity of Gram Positive colonies from the total plate count

Table 1. Details of the Grams staining and Total count r of the oral microbial flora of CVD patients

Details of the results of the oral microbial flora of CVD patients (enumeration)						
S.no	Accession Number	Age	Sex	Smoking	Total Count	Gram Stain
1	SLS01	62	M	Y	10^6	Mixed culture
2	SLS02	55	M	Y	10^5	Mixed culture
3	SLS03	57	F	N	10^6	Positive
4	SLS04	63	M	Y	10^5	Mixed culture
5	SLS05	54	M	Y	10^5	Positive
6	SLS06	77	M	Y	10^6	Positive
7	SLS07	19	M	N	10^6	Mixed culture
8	SLS08	80	M	N	10^4	Positive
9	SLS09	65	M	Y	10^4	Positive
10	SLS10	70	F	N	10^5	Mixed culture
11	SLS11	65	M	Y	10^5	Positive
12	SLS12	48	M	N	10^6	Mixed culture
13	SLS13	70	M	Y	10^6	Positive
14	SLS14	50	F	N	10^5	Mixed culture
15	SLS15	57	M	Y	10^5	Mixed culture
16	SLS16	56	M	Y	10^6	Mixed culture
17	SLS17	48	F	N	10^5	Positive
18	SLS18	48	M	Y	10^5	Mixed culture
19	SLS19	46	M	Y	10^5	Mixed culture

Table 2. Biochemical & Serological tests

S.No	Accession Number	Fermentation of sugars		Catalase test	Coagulase
		Mannitol	Sorbitol		
1	SLS01	Positive	Positive	Negative	Negative
2	SLS02	Positive	Positive	Negative	Negative
3	SLS03	Positive	Positive	Positive	Positive
4	SLS04	Positive	Positive	Negative	Negative
5	SLS05	Positive	Positive	Positive	Positive
6	SLS06	Positive	Positive	Negative	Negative
7	SLS07	Positive	Positive	Negative	Negative
8	SLS08	Positive	Positive	Negative	Negative
9	SLS09	Positive	Positive	Negative	Negative
10	SLS10	Positive	Positive	Negative	Negative
11	SLS11	Positive	Positive	Negative	Negative
12	SLS12	Positive	Positive	Negative	Negative
13	SLS13	Positive	Positive	Negative	Negative
14	SLS14	Positive	Positive	Negative	Negative
15	SLS15	Positive	Positive	Negative	Negative
16	SLS16	Positive	Positive	Negative	Negative
17	SLS17	Positive	Positive	Negative	Negative
18	SLS18	Positive	Positive	Negative	Negative
19	SLS19	Positive	Positive	Negative	Negative

Then the cultures were separately evaluated for biochemical and serological characterization. The results are shown in Table 9.

From the above table it is clear that the microbial flora consists of Streptococcus sps and *Staphylococcus aureus*in 2 samples (culture 3, culture 5).

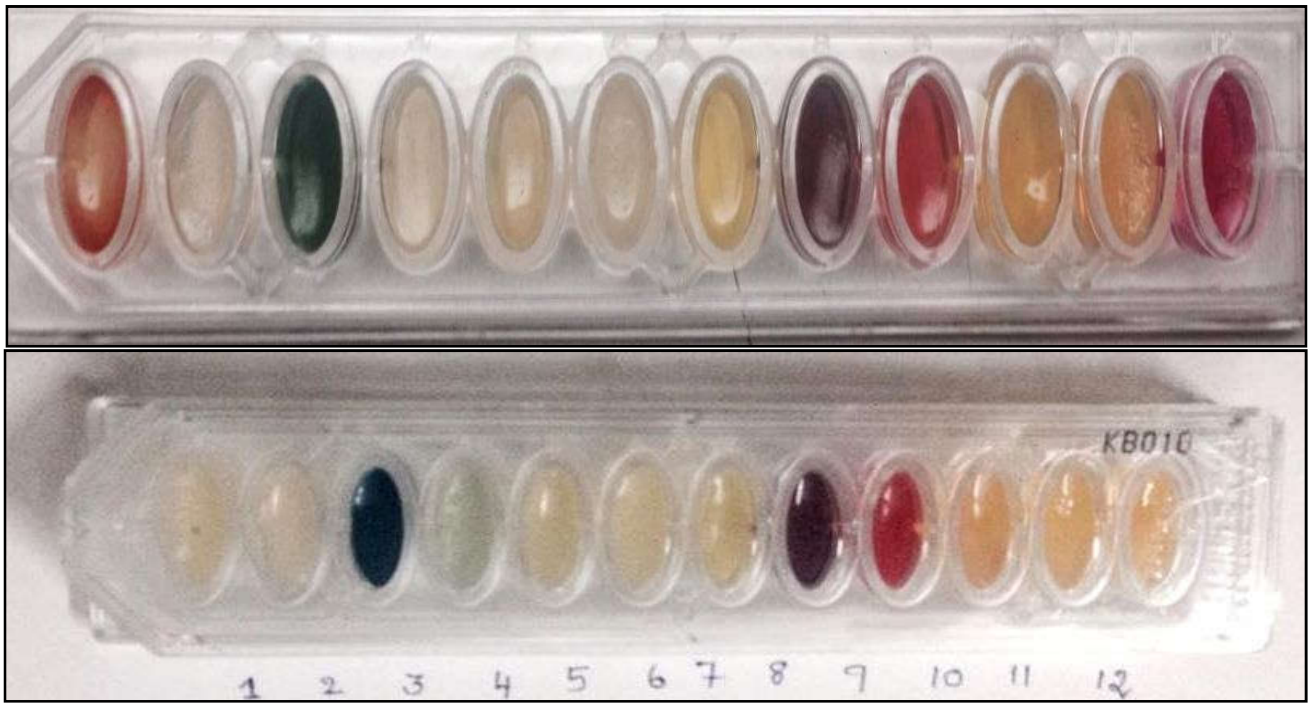
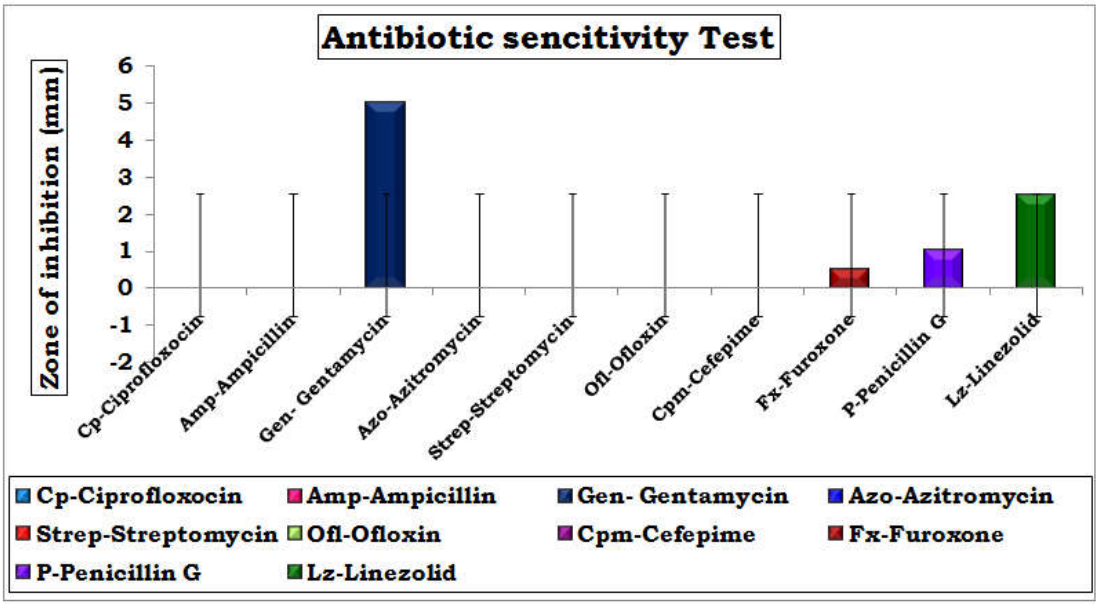


Fig. 2. Biochemical test using Kits –Culture-3 and Culture-5

Table 3. Test Biochemical kit test for Culture 3 and Culture 5

1.	TEST NAME	CULTURE 3	CULTURE-5
2.	Methyl red reduction test	Positive	Negative
3.	Voges proskaures test	Negative	Negative
4.	Citrate utilization test	Negative	Positive
5.	Indole production test	Negative	Negative
6.	Glucuronidase test	Negative	Negative
7.	Nitrate reduction test	Negative	Negative
8.	ONPG test	Postive	Positive
9.	Lysine utilization test	Negative	Negative
10.	Lactose fermentation test	Negative	Negative
11.	Glucose fermentation test	Positive	Positive
12.	Sucrose fermentation test	Positive	Positive
13.	Sorbitol fermentation test	Negative	Positive



Culture 5 was sensitive to Gentamicin followed by Linezolid, Penicillin G and Furoxone.

Fig. 3. Graphical presentation of Antibiotic sensitivity of different antibiotic on culture 5

As shown in the table most of the patients have shown the presence of Streptococcal sps. Followed by staph.non aureus, and only two patients showed the presence of *Staphylococcus aureus* colonies. Biochemical tests of both 3 and 5 culture were performed .although both cultures showed coagulase and catalase positive .Culture 3 biochemical characteristics were little different from the normal *Staphylococca*l biochemical tests. Whereas Culture 5 showed similarities with the normal *Staphylococca*l biochemical tests. Culture 5 was taken and antibiotic sensitivity test was performed.

DISCUSSION

After individual data collection and clinical examination, samples of sub gingival dental biofilm were obtained in those individuals suffering with CVD nineteen bacterial isolates were isolated and Haemolytic and non hemolytic activity was performed using blood agar medium. After hemolytic activity all the bacterial samples were morphologically identified by Grams staining .Both Gram positive, Gram negative and mixed cultures were observed. To confirm the identification of Staphylococcal sp Coagulase test and Catalase test were performed. Among 19 bacterial culture only 2 cultures showed positive culture 3 and culture 5. For both culture 3 and 5 different biochemical tests like Indole Test, Carbohydrate Fermentation, Citrate Utilization Test, D. Glucuronidase test, ONPG Test, Methyl Red Test, Voges-Proskauer Test, Nitrate reduction test, Lysine utilization test. Comparing the results of culture 3 and 5. Culture 3 showed minor difference in biochemical tests of *Staphylococcus* species. Culture 5 showed similarity of standard biochemical reports of Staphylococcus aureus.

Antibiotic sensitivity test was done using 10 (Ciprofloxocin, Ampicillin, Gentamicin, Azitromycin, Streptomycin, Ofloxin, Cefepime, Furoxone, Penicillin G, Linezolid) antibiotics for Culture 5 using Kirby-Bauer Disk Diffusion Susceptibility and agar well diffusion test. Culture 5 was sensitive to Gentamycin, Linezolid, Penicillin G and Linezolid. It is fair to inform patients that evidence suggests a relationship, and that periodontal therapy will help maintain the dentition in health and comfort, but it is premature to claim a cardiovascular-protective effect of treatment. It is important to remember that not all studies have been supportive. Howell *et al* (Noack, 2001) suggested that self-reported periodontal disease is not an independent predictor of subsequent cardiovascular disease in middle-aged to elderly men. However, (Wick, 1996) did not find convincing evidence of a causal association between periodontal disease and coronary heart disease risk. Larkin also disputed this association.

The studies reported a measure of association between periodontal disease and atherosclerosis, In prospective studies on incident and mortality on CVD oral infection has been categorized according to tooth brushing or tooth extractions (Michalowicz, 2013), being either self-reported or clinically confirmed periodontitis or gingivitis genetic associations 85, or simultaneous oral disease factors of dental plaque, calculus, gingival inflammation and number of missing molars (Fedele, 2011).

Conclusion

A study was carried out to evaluate the microbial flora of the oral cavity of CVD patients from Guntur District of Andhra Pradesh. The microbial flora was evaluated by drawing swab samples from the oral cavity by using sterile swabs. The swabs were cultured in Trypticase soy broth for 48 hrs and later 0.1ml of the culture was plated onto different media to evaluate the characteristics of the organisms. Both Gram Negative and positive organisms were found. Most of the patients had mostly gram positive organisms, only few patients showed both gram negative and positive organisms. Among the gram positive organisms, *streptococci, Staph. aureus* and Staph. non aureus were found. Very few patients showed *Staphylococcus aureus* coagulase positive, most of the patients showed *Staphylococcus*non *aureus* organisms. Two cultures 3 and 5 showed coagulase and catalase positive. Biochemical tests of both 3 and 5 culture were performed and Culture 3 biochemical characteristics were little different from the normal *Staphylococca*l biochemical tests. Whereas Culture 5 showed similarities with the normal *Staphylococca*l biochemical tests. Culture 5 was taken and antibiotic sensitivity test was performed. Culture 5 was sensitive to Gentamicin followed by Linezolid, Penicillin G and Furoxone. This matches with the literature data wherein most of the CVD patients show Staph.non aureus organisms, which are responsible for them developing the CVD conditions.

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