Isolation and Identification of Microorganisms Associated With Removable Denture: Prevalence of Non Oral Pathogens

Fatma Alzahraa M. Gomaa and Zeinab H. Helal
Microbiology & Immunology Department, Faculty of Pharmacy for girls Al-Azhar University, Cairo, Egypt

ABSTRACT
The oral cavity may act as a reservoir for several pathogens related to systemic infections. Therefore, the aim of this study was to identify the microorganisms found in dentures and to determine the prevalence of pathogenic isolates. Bacteriological investigations were performed in 30 denture patients. Cultures in aerobic and anaerobic conditions were conducted on solid non-selective and selective media, as well as, media enriched with 5% sheep blood. Standard procedures of bacterial culture and identification were applied. A wide range of potentially pathogenic microorganisms was found in dentures. Streptococci were the dominant species. Potential respiratory pathogens were found to have colonized the denture surfaces in (9%) of the patients. Thus, attention should be paid to the bacterial population in denture as a potential source of oral and systemic diseases.

Keywords: Dentures, microorganisms, aerobic, anaerobic.

INTRODUCTION
Oral health status declines with age and as a result the need for removable prostheses increases. Oral health is a reflection of one's general health, affecting the ability of an individual to eat and speak, and contributes significantly to a sense of confidence and well-being (Daniluk et al., 2006).
Different studies have suggested that oral bacteria may be risk factors for a number of prevalent systemic diseases (Scannapieco, 1998 and Li et al., 2000). Oral bacteria have been implicated in bacterial endocarditis, aspiration pneumonia, gastrointestinal infection and chronic obstructive pulmonary disease, among others, and dentures offer a reservoir for microorganisms associated with these infections (Li et al., 2000).
Wearing removable dental prosthesis causes an alteration in the oral microflora (Girard et al., 1996). For certain individuals, this new environment is responsible for the development of a particular condition: dental prosthetic stomatitis or denture associated stomatitis.
Denture-induced stomatitis is also a recognized clinical challenge. The responsible microorganisms have not been delineated (Glass et al., 2010). Denture stomatitis is characterized by mucosal inflammation and redness underneath a denture (Spratt, 2003). It is caused by the microbial biofilm on the fitting surface of the denture rather than on the mucosal surface of, for example, the palate (Olsen, 1974). Denture associated stomatitis is associated with variety of Candida spp. (Lamfon et al., 2005- Dorko et al., 2001) as well as bacteria from several genera (Koopmans et al., 1988; Spratt, 2003 and Lamfon et al., 2005).
The microbiology of denture has received little attention in comparison with dental microbiology, yet it differs in location and composition. Thus the aim of this study was to evaluate microbial flora found in previously worn denture and to determine the prevalence potentially pathogenic isolates.
MATERIALS AND METHODS

Thirty complete denture wearers had no systemic disease and were wearing their present dentures for around 3 years were included in the study. Swabs were taken from the palatal surface of the upper denture according to a 2 cm x 2 cm template delimiting the area to be swabbed. This was done immediately after removal of the denture.

Samples were placed in tubes containing 4.5 ml of sterile thioglycollate broth. Within half an hour, the samples were mixed by using a vortex mixer for 30 s and ten-fold serial dilutions up to $10^{-5}$ were obtained in saline. Portions (0.1 ml) of dilutions were spread onto blood agar plates consisting of 5% sheep blood agar (Oxoid Ltd.), sabouraud dextrose agar containing 0.005% chloramphenicol and 0.04% cycloheximide (Difco, Detroit, USA) and Colombia blood agar (CBA) plates supplemented with vitamin $K_1$, Hemein and 5% sheep blood. All plates were incubated for 48 hrs at 37 °C except CBA plates were incubated for five days anaerobically at 37°C in Anaerobic Gaspack systems (BBL Becton Dickinson Microbiology System, Cockeysville, MD, USA).

Anaerobic bacteria were isolated by streaking 1 ml of the cell suspension diluted at $10^{-2}$ on the surface of CBA plates supplemented with vitamin $K_1$, Hemein and 5% sheep blood, Neomycine-CBA and Kanamycine-Vancomycin- CBA. Aerobic and facultative bacteria were isolated by streaking 1 ml of the cell suspension diluted at $10^{-3}$ on the surface of non-selective blood agar plates. Enterobacteria were isolated by streaking 1 ml of the cell suspension diluted at $10^{-1}$ on the surface of MacConkey agar plates (Difco, Detroit, USA).

Yeasts were isolated by spreading 1 ml of the cell-suspension diluted at $10^{-1}$ on the surface of Sabouraud dextrose agar containing 0.005% chloramphenicol and 0.04% cycloheximide. All the plates were incubated for 48 hrs at 37 °C. CBA plates were incubated anaerobically as mentioned before.

Identification of isolates:

Facultative aerobic bacteria were identified by standard conventional biochemical tests.

Identification of Gram-positive aerobic bacteria

Gram-positive cocci were identified according to Baird (1996), this include catalase test which differentiate *Staphylococci* (positive catalase) and *Streptococci* (catalase-negative). Coagulase, DNAase, detection of $\alpha$-haemolysis on blood agar and mannitol fermentation test were used for differentiation of *Staphylococci*. *Streptococci* were differentiated by first detecting type of haemolysis on blood agar. A Microscan Positive Identification (PID) panel type 20 (Dade Behring, West Sacramento, USA) were used to confirm the identification of Gram positive facultative cocci (*Streptococci* and *Staphylococci*). PID type 20 is an in vitro diagnostic product that uses fluorescence technology to detect bacterial growth or metabolic activity, and thus can automatically identify Gram-positive facultative cocci to species level. The system is based on reactions achieved with 29 pre-dosed substrates that are incorporated into the test media to determine bacterial activity. The panel was reconstituted using a prompt inoculation system.

Biochemical tests included in PID

In each Microscan PID type 20, several biochemical tests were performed. These included carbohydrate fermentation tests using rafinose, menhalose, sorbitol, arabinose, inulin, mannose, and lactose. Also, susceptibilities to optochin, crystal violet, bacitracin, and novobiocin were tested. In addition, fluorogenic substrate tests
were performed for the detection of various bacterial enzymes. In these tests, different substrates linked chemically to fluorophores were used (e.g., indoxyl phosphatase, phosphatase, pyrrolidase, urease, lactamase, and glycosidase). Other specific tests were also used, such as NaCl 6.5% tolerance, Voges Proskauer, nitrate reduction, and hemolysis of blood.

**Identification of Gram negative bacteria**

Gram negative bacteria were identified according to Finegold (1996), this include oxidase test, indole production test, methyl red and voges-Proskauer test, citrate utilization test, urease test, Triple sugar iron agar, ornithine decarboxylase test, phenyl alanine deaminase test and detection of motility on semi-solid agar. Also a Microscan Negative Identification panel Type 2 (NEG ID Type 2) (Dade Behring, West Sacramento, USA) was used to confirm the identification of gram-negative facultative bacteria to species level. The system is based on reactions obtained with 34 pre-dosed dried substrates which are incorporated into the test media in order to determine bacterial activity.

**Biochemical tests included in NEG ID Type 2**

In each Microscan NEG ID Type 2 kit, several biochemical tests were performed. These included carbohydrate fermentation tests, carbon utilization tests, and specific tests such as Voges Proskauer (VP), Nitrate reduction (NIT), Indole test, Esculin hydrolysis, Urease test, Hydrogen Sulphide production test, Tryptophan deaminase test, Oxidation-Fermentation test, and Oxidase test.

**Identification of anaerobes:**

Anaerobic bacteria were identified conventionally according to Sutter et al., (1980). The following medium were used, kanamycin-vancomycin lacked Blood Agar (KVLBA), neomycin blood agar, Bacteriod’s bile esculin agar, egg yolk agar (for testing lipase and lecithinase production), proteose peptone-yeast extract broth (for carbohydrate fermentation tests), thioglycolate gelatin medium (for gelatin liquefaction test), cooked meat broth (for indole production).

Identification included two levels, first a presumptive identification depending on information obtained from gram stain, colony morphology of a pure isolate and haemolysis on BAP. Second level include group of testes for identification of anaerobic isolates to genus level and in some cases to species level using antibiotic identification test, growth in presence of 20% oxygall, indole production, urease activity, sugar fermentation, lecithinase/lipase reaction, esculin hydrolysis, gelatin Liquefaction and catalase production. Anaerobes were carried to final identification to species level using RAID panel (DADE BEHRING, West Sacramento, USA). RAID is an invitro kit for identification of anaerobes isolated from clinical specimens. It consists of 24 dehydrated substrates. It is rehydrated and inoculated with the bacterial suspension (3-5 McFarland) and incubated aerobically for 4 hrs.

**Identification of yeast:**

Candida was identified using germ tube test, chlamydospore formation on corn-meal agar according to Davise (1995). API 20C AUX (BioMerieux Vitek, Étoile, France) system was also used to confirm identification.

**RESULTS AND DISCUSSION**

In the present study 30 (21 females and 9 males; age range: 48 to 71 years; mean age: 60.8 ± 11 years) with complete denture were tested. Most patients wore their dentures for extended periods without sanitization.

The presence of a denture on the oral mucosa alters the local environmental conditions due to the inaccessibility of saliva and lack of
mechanical cleaning by the tongue. Hence, dentures act as reservoirs that harbor a mixed species of bacterial biofilm (Daniluk et al., 2006). According to Nikawa et al. (1998), denture related stomatitis (inflammation of palatal mucosa) induced by wearing the denture or by plaque on the denture (Nikawa et al., 1998).

The cultivable flora of the denture showed a complex bacterial community. As would be expected the highest bacterial load was consist of normal oral flora but an unexpected spectrum of both pathogenic and opportunistic microorganisms was also found in the dentures examined, including a wide range of gram-negative bacteria, gram-positive bacteria, and yeasts. A total of 206 isolates were carried to final speciation and 56.8% of these were aerobic bacteria, 36.9% were anaerobic bacteria and 13% were yeasts (Table, 1). Gram-positive cocci (Staphylococcus spp. and Streptococcus spp.) more often isolated than Gram-negative cocci (45.1% and 13.6%, respectively).

Table 1: Microorganisms isolated from denture

<table>
<thead>
<tr>
<th>Isolated microorganism</th>
<th>No (%)</th>
<th>Isolated microorganism</th>
<th>No (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gram-positive Cocci</td>
<td>93 (45)</td>
<td>Gram-negative Cocci</td>
<td>28(13.5)</td>
</tr>
<tr>
<td>Streptococcus species</td>
<td>86</td>
<td>Veillonella parvula</td>
<td>23</td>
</tr>
<tr>
<td>S. salivarius</td>
<td>19</td>
<td>Nisseria spp.</td>
<td>5</td>
</tr>
<tr>
<td>S. mitis</td>
<td>16</td>
<td>Gram-negative Rods</td>
<td>34 (16.5)</td>
</tr>
<tr>
<td>S. sanguis</td>
<td>29</td>
<td>Bacteroides spp.</td>
<td>14</td>
</tr>
<tr>
<td>S.intermedius</td>
<td>13</td>
<td>B. denticola</td>
<td>9</td>
</tr>
<tr>
<td>S. mutans</td>
<td>9</td>
<td>B. melaninogenicus</td>
<td>5</td>
</tr>
<tr>
<td>S. aureus</td>
<td>7</td>
<td>Fusobacterium spp.</td>
<td>7</td>
</tr>
<tr>
<td>Gram-positive Rods</td>
<td>38(18.4)</td>
<td>F. necrophorum</td>
<td>5</td>
</tr>
<tr>
<td>Actinomyces species</td>
<td>17</td>
<td>F. mortiferum</td>
<td>2</td>
</tr>
<tr>
<td>A. odontolyticus</td>
<td>14</td>
<td>E.coli</td>
<td>3</td>
</tr>
<tr>
<td>A. israelii</td>
<td>3</td>
<td>K.pneumonia</td>
<td>3</td>
</tr>
<tr>
<td>Lactobacillus spp.</td>
<td>15</td>
<td>E.aerogenes</td>
<td>2</td>
</tr>
<tr>
<td>L. fermentum</td>
<td>11</td>
<td>Acinetobactr freundi</td>
<td>1</td>
</tr>
<tr>
<td>L. acidophilus</td>
<td>4</td>
<td>Ps.fluorescence</td>
<td>1</td>
</tr>
<tr>
<td>Not identified</td>
<td>6</td>
<td>Haemophylus spp.</td>
<td>3</td>
</tr>
<tr>
<td>Candida</td>
<td>13 (6.3)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Candida albicans</td>
<td>8</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Candida glabrata</td>
<td>3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Candida tropicalis</td>
<td>1</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

The most predominant organism was Streptococcus species (41.7%). Among Streptococcus spp. S. sanguis, S. salivarius, S. mitis, S.intermedius and S. mutans were found on the study group. Many studies also reported that streptococci are associated with denture plaque from those with healthy (Perciva et al., 1991; Marsh, et al., 1992 and Aas et al., 2005) and diseased mouths (Theilade and Budtz-Jürgensen 1988).

Veillonella parvula was the second most common isolate on denture, followed by Actinomyces species and
Isolation and Identification of Microorganisms Associated With Removable Denture

Lactobacillus spp. and Bacteroides spp. was common Gram negative anaerobic isolates. Aas et al. (2005) and Preza et al. (2009) who study the predominant micro flora of young and elderly individuals also reported that Streptococcus species followed by Veillonella spp. were the most common bacteria. Surprisingly in the present study the presence of species often associated with caries, such as Lactobacilli, S. mutans and Actinomyces.

A high carriage rate for cariogenic bacteria was also reported in a study of elderly Swedish individuals (Emilson and Thorselius, 1988) and elderly Americans (Fitzgerald et al., 1983) where lactobacilli was more prevalent in individuals wearing dentures.

Candida albicans was also isolated in 6.3% patients with denture. Other studies have shown Candida incorporation into biofilms covering different biomaterials such as dentures: these biofilms may be an increased risk factor for invasive candidosis when the host immune system is compromised (Ahariz et al., 2010). Also, Marsh and Martin, (2000) reported an increase in yeast colonization as consequences of aging, and he attribute such increase to long-term medication, reduced salivary flow rate, and denture wearing in those patients (Hägg et al., 2004).

Interestingly, co-aggregation studies have shown that C. albicans colonization can be aided by primary colonizers such as streptococci (Jenkinson et al., 1990).

Denture especially denture base acrylic resin is easily colonized by oral endogenous bacteria and Candida spp. and eventually by extra oral species such as Staphylococcus spp. or members of Enterobacteriaceae. This microbial reservoir can be responsible for denture-related stomatitis and aspiration pneumonia, a life threatening infection, especially in geriatric patients (Gornitsky et al., 2002).

Some microorganisms which are unusual in the oral microbiota and considered a potential pathogens had colonized the dentures of the examined patients, the predominant one being Staphylococcus aureus (3.4%).

A variety of potential respiratory pathogens were also isolated from dentures include H. parainfluenzae, E. coli and K. pneumoniae (1.45% each), and En. aerogenes (1%). Goldberg et al. (1997) evaluated the prevalence of Enterobacteriaceae in four different populations and they detected Enterobacteriaceae in 48% of patients with complete dentures and 13% of orthodontic patients. Daniluk et al. (2006) reported that inhalation pneumonia is a common cause of death amongst the elderly debilitated, thus the role of the denture in harboring such potential pathogens may be significant.

Several studies indicate that the oral microflora can change with advancing age, possibly due to impaired immune function and subsequent colonization with non-oral bacterial species such as staphylococci and enterobacteria (Perciva et al., 1991; Nyvad, 1993 and Fure, 1998).

Additional anaerobic species, such as Bacteroides spp., Fusobacterium spp. Actinomyces species were also found. Some species with low prevalence appear to be subject specific as it detected only as a single individual.

Microorganisms that are unusual in the oral microflora have been isolated from dentures were also reported in different studies (Goldberg, et al., 1997, Akpan and Morgan, 2002; Senpuku et al., 2003; Spratt, 2003; Lamfon et al., 2005; Verran, 2005 and Pereira-Cenci et al., 2008).

CONCLUSION

A wide range of microorganisms must be considered when treating either oral or systemic diseases in denture wearers. The dentures of even health individuals must be considered as...
possible sources of pathogenic microorganisms. It might be worthwhile to pay more attention to the bacterial population in denture instead of focusing only on Candida. An effective denture hygiene and decontamination is recommended to control denture microbial biofilm to overcome associated oral and systemic diseases.

ACKNOWLEDGMENT
Thank to Dr. Fatma Sami El-Sayed dentist in medical center affiliated to the ministry of health for her help and cooperation throughout the study.

REFERENCES


ARABIC SUMMARY

عزل وتعريف الكائنات الحية الدقيقة المرتبطة باطقم الأسنان: مدى انتشار العزلات البكتيرية المسببة للأمراض غير متعارف عليها بالفم

فاطمة فاطمة هلال، زينب هلال
قسم الميكروبيولوجي والمناعة - كلية الصيدلة للبنات - جامعة الأزهر - القاهرة - مصر

تجويف الفم قد يكون بمثابة خزان للمسببات الأمراض العديدة التي تتعلق بالالتهابات الجهازية و لذلك استهدف هذا الفم صحة الذين يعانون من الأمراض时限ًا، تمت دراسة عدد المرضى الذين يعانون من هذه الأمراض. تم زرع العينات في ظروف هوائية و لا هوائية وكذلك زرعها على بيئة تحتوي على 5% من الدم. كما تم تطبيق الطرق المعايير للفصل والتجديد على العزلات البكتيرية و قد تم تحديدها على مجموعة واسعة من الكائنات الدقيقة المسببة للأمراض في أطقم الأسنان. كما وجد أن البكتيريا الكروية السجية هي العزلات السائدة في هذه الدراسة. وقد تم تحديد العائلة البكتيرية من سبب الأمراض السجية مستحيلة على سطوح الاطقم في 5% من المرضى. لذلك ينبغي إيلاء الاهتمام البكتيريا المشتركة في أطقم الأسنان كمصدر محتمل لأمراض الفم والأمراض الجهازية.