Title

Dentures are a Reservoir for ~~Pathogenic~~ Respiratory Pathogens

Running Title

Putative Respiratory Pathogens in Denture Plaque

Authors (First Middle Last)

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ABSTRACT

Purpose: Recent studies have established a relationship between dental plaque and pulmonary infection, particularly in elderly individuals. Given that approximately 1 in 5 adults in the UK currently wears a denture, there remains a gap in our understanding of the direct implications of denture plaque on systemic health. The aim of this study was to undertake a comprehensive evaluation of putative respiratory pathogens residing upon dentures using a targeted quantitative molecular approach.

Materials and Methods: One hundred thirty patients' dentures were sonicated to remove denture plaque biofilm from the surface. DNA was extracted from the samples and was assessed for the presence of respiratory pathogens by qPCR. Ct values were then used to approximate the number of corresponding colony forming equivalents (CFE's) based on standard curves.

Results: Of the dentures, 64.6% were colonized by known respiratory pathogens. Six species were identified: *S. aureus, S. pneumoniae, P. aeruginosa, H. influenzae B, S. pyogenes*, and *M. catarrhalis. P. aeruginosa* was the most abundant species followed by *Streptococcus pneumoniae* and *Staphylococcus aureus* in terms of average CFE and overall proportion of denture plaque. Of the participants, 37% suffered from denture stomatitis; however, there were no significant differences in the prevalence of respiratory pathogens on dentures between healthy and inflamed mouths.

Conclusions: Our findings indicate that dentures can act as a reservoir for potential respiratory pathogens in the oral cavity, thus increasing the theoretical risk of developing aspiration pneumonia. Implementation of routine denture hygiene practices could help to reduce the risk of respiratory infection among the elderly population.

KEYWORDS: Denture plaque; *Staphylococcus aureus; Pseudomonas aeruginosa*; respiratory; biofilm.

Globally, 810 million people are aged 60 years or over, a figure that is expected to reach two billion by 2050 (22% of the entire global population).[1] With this ever-increasing elderly population, there will inevitably be greater challenges for both systemic and oral health care delivery. Clear associations between oral and systemic disease have been reported, for example with rheumatoid arthritis, cardiovascular disease, and respiratory infection.[2,3] Many of these aging individuals will experience a general decline in oral health. Current estimates suggest that around 20% of the US and UK population wear some form of removable denture.[4,5] Dentures have been shown to support the growth of biofilms known as denture plaque, which are complex polymicrobial consortia of bacteria and yeasts. Given a denture's close proximity to the respiratory tract, denture wearers are potentially at an increased risk of aspirating opportunistic pathogens from the denture into their lungs.

Aspiration pneumonia (AP) is a potentially life threatening respiratory infection associated with entry into the bronchial tree of contaminated foreign material such as gastric or oropharyngeal contents, including food debris, dental and/or denture plaque, and saliva. Pneumonia is the leading cause of death attributable to infection in patients aged 65 years and older, costing the NHS in excess of £440 million annually.[6] Moreover, it has been found that aspiration of oropharyngeal contents is a common occurrence in healthy individuals, of whom approximately 45% aspirate material into the lungs during sleep.[7] Risk factors for AP, such as dysphagia and chronic obstructive pulmonary disease COPD, are more common in the elderly, thus putting this sub-population at an increased risk of infection.[8] Poor oral hygiene has been linked to respiratory infection with common pathogens being identified in dental plaque.[9] The microbial composition of dental plaque has been well characterized, and these oral bacteria have been linked with systemic infections.[10] However, a gap remains in our understanding of the direct implications of denture plaque. The aim of this study was to undertake a comprehensive evaluation of putative respiratory pathogens residing upon dentures using a targeted quantitative molecular approach.

MATERIALS AND METHODS

Patient sampling

Samples for the study were obtained from 130 denture wearers attending the Glasgow Dental Hospital and School, UK, for treatment. Written informed consent was

obtained from all participants. Ethical approval for recruitment of study participants was granted by the West of Scotland Research Ethics Service (12/WS/0121). A dentist was responsible for the collection of clinical samples. The presence or absence of DS was assessed by the clinician, and those patients with DS were grouped according to Newton's classification.[11] Demographic data, including age, gender, and oral hygiene habits were recorded. Patients were excluded from the study if they had been receiving antimicrobial/antifungal treatment or using prescription mouthwashes within 6 weeks prior to sampling.

Collection of denture plaque

Dentures were removed from the patient's mouth and placed in sterile bags (Fisher Scientific, Loughborough, UK) filled with 50 ml PBS (Sigma-Aldrich, Dorset, UK) then placed in a sonic bath (Ultrawave, Cardiff, UK) for 5 minutes to remove biofilm from the surface. The denture sonicate was transferred to a 50 ml tube and transported to a category II laboratory where it was centrifuged for 10 minutes at 3700 *g*. The plaque pellet was then re-suspended in 2 ml of RNAlater® (QIAgen, Manchester, UK) and stored at -80^{o}C.

Microbial culture and DNA extraction

For identification of respiratory pathogens in this study we took a targeted approach. Bacterial type strains of nine pathogens known to cause respiratory infection were selected and grown in appropriate media and atmospheric conditions for 24 hours as outlined in Table 1. Bacteria were subsequently washed in PBS and standardized to 1 x 10^{8} CFU/ml, prior to serially diluting in preparation for calibrated standard curve analysis. These samples and denture plaque sonicates were centrifuged for 10 minutes at 10,000 *g*, re-suspended in proteinase K extraction buffer, and incubated at 55^{o}C for 20 minutes. DNA was extracted using the QIAmp mini DNA extraction kit (Qiagen) according to manufacturer's instructions, with a minor modification to include a mechanical disruption step with sterile acid-washed glass beads of 0.5 mm diameter (Thistle Scientific, Glasgow, UK). This was achieved by vortexing for 3 × 30 seconds on a Mini-BeadBeater (Sigma-Aldrich, Gillingham, UK), while intermittently being placed on ice. DNA quality and quantity was then quantified by NanoDrop® (ThermoScientific, Loughborough, UK).

Quantitative PCR

Primers were either taken from published literature or designed using the web-based GenScript real-time PCR primer design software (https://www.genscript.com/ssl-bin/app/primer). Primer sequences were checked for specificity to each target organism using the NIH-BLAST database. PCR amplification efficiencies of all primer sets were optimized prior to gene expression analysis, with efficiencies ranging from 90 to 110%. Details of the oligonucleotide primers (Eurogentec, Southampton, UK) used in this study are listed in Table 2. Two hundred ng of DNA was used in a mastermix containing SYBR® GreenER™ (Life Technologies, Paisley, UK), UV-treated RNase-free water, and forward/reverse primers (10 μM), following manufacturers' instructions. qPCR was carried out using the step one plus real-time PCR unit (Applied Biosciences, Paisley, UK), under the following conditions; 50^{o}C for 2 minutes, 95^{o}C for 2 minutes, followed by 40 cycles of 95^{o}C for 3 seconds and 59^{o}C for 30 seconds. Data analysis was carried out using StepOne software V2.3, (Life Technologies). Baseline threshold values of the samples were adjusted to correspond with the equivalent standard curve; Ct values were then used to approximate the number of corresponding colony forming equivalents (CFEs) based on standard curves created from serial two-fold dilutions of each bacterial species.

Statistical analysis

Graph production, data distribution, and statistical analysis were performed using GraphPad Prism (v.4; La Jolla, CA) or IBM SPSS statistics (v.21; Chicago, IL). Since continuous data including real-time qPCR determination of bacterial cell numbers on dentures did not conform to a normal distribution, the Mann-Whitney U-test was used for comparisons between the different denture-wearer subsets. The Spearman bivariate correlation analysis was used to determine relationships between the Newton Grade of inflammation and bacterial numbers.

RESULTS

Patient demographics

Of the 130 patients that participated in this study, 84 were female. The mean patient age was 70.4 years, with an average denture age of 4.5 years. Clinical diagnoses indicated that 37% of participants were suffering from DS. The majority of patients

(68%) wore complete maxillary dentures, while 32% had a partial maxillary denture with ≥1 natural teeth remaining.

Detection of respiratory pathogens

The dentures of 84 patients (64.6%) carried potential respiratory pathogens. The following six were identified: *S. aureus, S. pneumoniae, P. aeruginosa, H. influenzae B, S. pyogenes* and *M. catarrhalis* (Table 1). *S. aureus* was the most prevalent, with 67 patients (51.2%) being shown to carry this pathogen. Of these 67 isolates, re-testing using primers specific for the *mecA* gene demonstrated that two (3%) were MRSA. *H. influenzae B, P. aeruginosa, S. pneumoniae, S. pyogenes*, and *M. catarrhalis* were also detected within denture plaque of our patients (Table 2). Fifty-eight patients (44.6%) had dentures colonized by a single pathogen, 24 patients (18.5%) were colonized by two pathogens, and two patients (1.5%) were colonized by three. None of the samples were shown to be positive for *L. pneumophila, C. pneumoniae* or *K. pneumoniae.*

Quantifying respiratory pathogens

Standard curves of known quantities of bacteria were prepared for each pathogen; R^2 values ranged from 0.97 to 0.99. Table 2 shows the mean CFE counts of each organism detected on dentures. In terms of overall proportion of denture plaque, *P. aeruginosa* was found to be the most abundant, with a mean count of 4.3×10^6 CFE when present. This was followed by *S. pneumoniae* and *S. aureus*, which were detected at relatively high levels of 2.5×10^5 CFE/denture and 1.3×10^5 CFE/denture respectively. Conversely, *H. influenzae B, S. pyogenes* and *M. catarrhalis*, where present, were all detected at levels lower than 10^5 CFE/denture. The total number of bacteria residing on dentures shown to harbor respiratory pathogens was calculated using the 16S gene, with mean quantities ranging between 7.97×10^9 to 4.5×10^{10} CFE/denture. As a proportion of the overall plaque, *P. aeruginosa* was most abundant contributing to 1.5×10^{-2} % of the total. The remaining pathogens all contributed to less than 0.001% of the complete plaque sample

The relationship between DS, denture hygiene, and presence of respiratory pathogens was investigated. In relation to DS, we determined the prevalence of respiratory pathogens among healthy and diseased patients, based on clinical presentation using Newton's classification (Table 3). The overall prevalence of these

was similar between the healthy and diseased groups. Variation between individual species was also investigated; however, no statistical significance was found. In terms of denture hygiene, a greater proportion of dentures left in patients' mouths overnight were found to carry *S. pneumoniae*, and an increased median number of *S. pneumoniae* were detected on those dentures ($p = 0.041$ and $p = 0.038$, respectively). In contrast, the proportion of dentures positive for *P. aeruginosa* and numbers of *P. aeruginosa* found on dentures left in overnight was lower than dentures that were removed ($p = 0.038$ and $p = 0.04$, respectively).

DISCUSSION

Improvements in health care in the last 100 years have led to a growing population of elderly residents. This aging population, particularly those aged 65 and over, are more susceptible to developing pneumonia, accounting for approximately 90% of deaths in the elderly.[6] Many cases of pneumonia are related to oral bacteria emanating from the oropharynx.[12] Given that 70% of UK adults older than 75 years old wear dentures, it is clear that these individuals are at greater risk of developing life-threatening infections due to putative respiratory pathogens residing upon their denture.[13] This recent study showed that patients who wear their denture overnight double their risk of pneumonia. In this paper, using qPCR for the first time on denture plaque from a large cohort of denture wearers, we report that almost 65% of these individuals harbored significant quantities of pathogens capable of causing respiratory infections. This was regardless of existing oral disease status, and therefore suggests that even denture wearers with healthy palatal mucosa and good oral hygiene may harbor respiratory pathogens on their dentures.

Given the dense and complex microbial population of the oral cavity, the presence of similar genera and species is problematic for accurate quantitative microbial counts on selective agars.[14] Therefore, we adopted an alternative non-culture based method, which employed a qPCR-based approach to identify a panel of defined respiratory pathogens. This targeted molecular approach enabled us to rapidly assess the presence and quantity of defined respiratory pathogens.[15,16] While there are numerous putative respiratory pathogens, our study focused on nine key bacterial pathogens widely implicated in respiratory infections. We showed that almost 65% of patients' dentures were positive for one or more of these pathogens. This is in line with a recent report indicating that 64% of the healthy patient group contained

respiratory pathogens based on culture-based methodology, though only nine patients were assessed in this study.[17] It is possible that other microorganisms capable of causing pneumonia were not detected due to the limitation of a targeted qPCR approach, as colonization and infection of the respiratory tract is possible by a wide variety of human pathogens. Indeed, microbiome analysis of the entire denture specimen would enable a detailed characterization of potential pathogens, though to date no studies have reported this information. Nonetheless, our qPCR approach gives an initial insight into the risk associated with colonized dentures.

qPCR has proved an important tool, as in this study we have shown a high prevalence of *S. aureus* (51.2%), which an earlier culture-based study of 50 patients' dentures reported as only 10%.[14] This disparity is likely due to improved detection abilities of qPCR, as in our parallel study using culture techniques *S. aureus* was detected in only 20% of samples (data not shown). *H. influenzae* was also detected in 15.3%, whereas Sumi et al[14] were unable to detect this organism at all. *P. aeruginosa* was also detected in significant levels (11.5%), similar to a study of institutionalized and hospitalized patients. *S. pneumoniae* was detected on dentures from 6.9% of patients, which is much lower than the prevalence reported by Abe et al[15] (63%); however, this discrepancy is probably due to the non-specific nature of the primers in Abe's study.[15] We used the *cspA* gene specifically, instead of the generic primers, to minimize detection of closely related streptococcal species. Collectively, these organisms were detected in relatively low abundance in relation to total bacterial flora ($<6 \times 10^{-4}$ %), indicating that these bacteria only occupied limited niches upon the denture surface. Interestingly though, *P. aeruginosa* comprised 0.015% of the total denture microbiome, which may relate to its resistance to standard mechanical and chemical cleaning methods when grown on denture acrylic.[18]

DS is a disease affecting between 30 and 70% of denture wearers. The etiology of DS is complex, and the microbial composition of denture plaque is not yet fully understood.[19] We were interested to identify whether respiratory pathogen colonization of denture plaque was a more common occurrence in those with DS; however, our results revealed a similar prevalence of respiratory pathogens between healthy and diseased individuals (89.2% and 85.8%, respectively), suggesting that DS had no influence on the likelihood of respiratory pathogen colonization. Thus, despite published studies showing the prevalence of AP to be higher in patients who do not follow adequate dental hygiene measures,[20,21] our study found no difference in the

prevalence of respiratory pathogen carriage in those with and without signs of DS, suggesting that the presence of the denture was sufficient risk in itself.

The high prevalence of *S. aureus* residing on dentures is a concern, particularly in light of the emergence of drug-resistant strains. In this study we found two patients positive for the MRSA *mecA* gene (1.5%). Our prevalence rate is low, but similar to a previous study conducted in Scotland in which 5% of denture wearers were found to carry MRSA in their oral cavity.[22] This may be cause for concern, as many over-the-counter oral hygiene antimicrobials are ineffective against MRSA biofilms.[23] *S. pneumoniae* was shown to be significantly more abundant on dentures that were kept in their owner's mouths overnight, a habit practiced by a large proportion (55%) of the patients in this study. This is particularly important given that currently 20 to 30% of *S. pneumoniae* worldwide are multidrug resistant.[24] In addition, 11.5% of patients were carriers of *P. aeruginosa*, a notorious cause of life-threatening pneumonia for intensive care patients with a mortality rate of 44.5%.[25] Oropharyngeal *P. aeruginosa* isolates have also been shown to have a high rate of antibiotic resistance.[26] Given that it is common practice to soak dentures in water overnight, this may be an inadvertent source of contamination, and some form of disinfectant such as hypochlorite for non-metal containing prostheses, should be included.[27]

CONCLUSION

This study has shown that dentures are a reservoir for respiratory pathogens in the oral cavity, thus increasing the risk of developing AP. This study used a robust method of sampling the denture microflora and characterizing it using culture-independent techniques. We have shown a high prevalence of putative respiratory pathogens on the dentures of ambulatory adults, a finding that could explain the source of infection in some cases of AP. Adoption of routine oral hygiene practices including mechanical cleaning, and the use of antiseptic cleansing agents could help to reduce the risk of respiratory infection among the elderly population.[28] Further detailed microbiological investigations using next generation sequencing will further aid our understanding of the risks associated with ono-complicance of denture cleanser regimens.

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Table1: Respiratory pathogen primers used for qPCR

Species	**Type strain**	**Media/Conditions**	**Gene**	**Primer sequence**	**Amplicon Size**
S. aureus	DSMZ 1104	BHI, aerobic	*SAR0134*	**F** - ATTTGGTCCCAGTGGTGTGGGTAT **R** - GCTGTGACAATTGCCGTTTGTCGT	143
MRSA		BHI, aerobic	*MecA*	**F** – AACCACCCAATTTGTCTGCC **R** - TGATGGTATGCAACAAGTCGTAAA	135
H. influenzae B	DSMZ 11969	MHB + 0.4% haemophilus test medium, 5% CO_2	*GryB*	**F**–CTTACGCTTCTATCTCGGTGATTAATAA **R** – TGTTCGCCATAACTTCATCTTAGC	138
P. aeruginosa	PA14	LB, aerobic	*RpoD*	**F** – GGGCGAAGAAGGAAATGGTC **R** – CAGGTGGCGTAGGTGGAGAA	178
S. pneumoniae	DSMZ 14377	BHI, 5% CO_2	*CspA*	**F** – ACGCAACTGACGAGTGTGAC **R** – GATCGCGACACCGAACTAAT	352
M. catarrhalis	DSMZ 11994	BHI, aerobic	*OmpCD*	**F** – ACACGCAACTCTTGACGAAG **R** – CTGAGCCTGTCATTGAGGAA	180
S. pyogenes	DSMZ 20565	BHI, 5% CO_2	*SpeB*	**F** – TGC TAAAGTCGCTACGGTTG **R** – GAATTGATGGCTGATGTTGG	148
C. pneumoniae	ATCC VR-1360D DNA	NA	*MomP*	**F -** TTACTTAAAGAAACGTTTGGTAGTTCATTT **R** – TAAACATTTGGGATCGCTTTGAT	154
K. pneumoniae	DSMZ 12059	TSB, anaerobic	*PhoE*	**F** – AGAATTCAGATTCCCAACGG **R** – ACAAGAACGCGAACAAACTG	167
L. pneumophila	DSMZ 25038	YEB with BCYE supplement, 5% CO_2	*Mip*	**F** – CAATGTCAACAGCAATGGCTGCAAC **R** – CTCATAGCGTCTTGCATGCCTTTAGCC	160
16S			*16S*	**F** – ACTCCTACGGGAGGCAGCAGT **R**- TATTACCGCGGCTGCTGGC	198

Table 2: Prevalence and quantity of respiratory pathogens colonizing dentures

Species	**Positive samples**	**Prevalence (%)**	**Average CFE**	**Average total 16S CFE**	**Proportion of 16S (%)**
*S. aureus**	67	51.2	$1.3x10^{5}$	$4.5x10^{10}$	$2.8x10^{-4}$
H. influenzae B	20	15.3	$2.4x10^{4}$	$1.7x10^{10}$	$1.4x10^{-4}$
P. aeruginosa	15	11.5	$4.3x10^{6}$	$3.34x10^{10}$	$1.5x10^{-2}$
S. pneumoniae	9	6.9	$2.5x10^{5}$	$4.4x10^{10}$	$6.3x10^{-4}$
M. catarrhalis	1	0.8	$2x10^{3}$	$7.97x10^{9}$	$2.5x10^{-5}$
S. pyogenes	1	0.8	$3.7x10^{4}$	$3.2x10^{10}$	$1.2x10^{-4}$
C. pneumoniae	ND**	ND	ND	ND	ND
K. pneumoniae	ND	ND	ND	ND	ND
L. pneumophila	ND	ND	ND	ND	ND

* Two *S. aureus* samples were found to be MRSA positive.
**ND = Not detected

Table 3: Prevalence of respiratory pathogens in plaque of patients with healthy palatal mucosa and those with denture stomatitis

	Healthy		**Denture stomatitis**	
Species	**Patients**	**Prevalence (%)**	**Patients**	**Prevalence (%)**
S. aureus	40	49.4	27	55.1
H. influenzae B	16	19.8	4	8.2
P. aeruginosa	10	12.3	5	10.2
S. pneumoniae	3	3.7	6	12.2
M. catarrhalis	ND*	ND	1	2.0
S. pyogenes	ND	ND	1	2.0
C. pneumoniae	ND	ND	ND	ND
K. pneumoniae	ND	ND	ND	ND
L. pneumophila	ND	ND	ND	ND
MRSA	ND	ND	2	4.1
OVERALL	69	85.2	44	89.8

*ND = Not detected