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Association of clinical parameters with periodontal bacteria haemolytic activity

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Abstract

Aims: To determine whether haemolytic activity of subgingival bacteria is associated with periodontitis clinical parameters and to identify which bacteria produce the haemolysins.

Materials and methods: Subgingival plaque samples from twenty-two untreated chronic periodontitis patients were investigated by culture and identified with MALDI-TOF MS.

Results: Total aerobic and anaerobic bacterial viable counts, percentage distribution of α- and β-haemolytic bacteria were significantly elevated in diseased sites in relation to healthy sites (p<0.001). Periodontal pathogens were more frequently detected at diseased sites: *P. gingivalis, T. forsythia, Treponema sp*., *Prevotella sp*., *P. micra*, *Fusobacterium sp*., *Campylobacter sp*., *Capnocytophaga sp*. and *Selenomonas sp.*. Haemolytic unidentifiable species and Gram-positive anaerobes such as *Slackia exigua*, *Solobacterium moorei*, and *Bulledia extructa* were also more frequently detected at diseased sites. In diseased sites, the presence of different haemolytic characteristics was more strongly correlated with clinical measures of disease than the mere absence or presence of specific species. The strongest correlation with probing pocket depth was observed for overall β-haemolytic toxicity (r=0.73, p<0.001).

Conclusion: A strong association was observed between subgingival bacterial haemolytic activity and clinical parameters in patients with chronic periodontitis. Further investigations are warranted to delineate the role of haemolysins in the pathogenesis of periodontitis.

Clinical Relevance

Scientific rationale for study: Periodontal pathogens have been shown to possess haemolytic activity, however to date there has been no clinical study to assess how the collective bacterial haemolytic activity correlates with clinical parameters.

Principal findings: An association was observed between clinical parameters and bacterial haemolytic activity. This association was stronger than the actual presence or counts of specific species. Bacterial haemolytic activity also increased with increasing total bacterial counts.

Practical implications: In periodontally susceptible patients, plaque control is important as it reduces the haemolytic toxicity of the plaque.

Introduction

Plaque-induced periodontitis is acknowledged to be polymicrobial in aetiology, with the host response affecting disease progression (Ramseier et al. 2009). Important periodontopathogens such as *Porphyromonas gingivalis, Prevotella intermedia* and *Fusobacterium nucleatum* are haemolytic (Chu et al. 1991, Beem et al. 1999, Falkler, Jr. et al. 1983). However, studies are lacking on how haemolysis correlates with the clinical parameters of periodontitis.

Bacterial haemolysins are toxins, which interrupt the structural integrity of the erythrocyte, resulting in liberation of haemoglobin and other intracellular metabolites. Haemolysins are also pore-forming toxins and can be cytotoxic for other cells types, such as gingival epithelial cells (Lee et al. 2011) which may allow the bacteria to breach the epithelial barrier facilitating tissue invasion. Therefore bacterial haemolysins may contribute to tissue invasion and the destructive host response seen in periodontitis.

F. nucleatum was the first periodontal pathogen identified to have haemolytic activity (Falkler, Jr. et al. 1983). In fact subgingival plaque containing β-haemolytic subgingival bacteria ≥0.4% were 5.3 fold more likely to have new attachment loss (Haffajee et al. 1991). Similarly, haemolytic variants were more common in diseased site plaque compared with healthy sites within the same individual (Hillman et al. 1993), with a third of periodontitis patients harbouring β-haemolytic streptococci (Flynn & Slots 1993). However, these studies only assessed

β-haemolytic species. Thus, the preliminary evidence suggests an association between periodontitis and bacteria with higher β-haemolytic activity.

While haemolysis is only one of multiple virulence mechanisms of periodontopathogens, it remains a critical mechanism for deriving important nutritional substrates, possibly by violating the periodontal pocket epithelial barrier. The aims of this study were to determine and quantify: a) the bacterial species and their distribution in subgingival plaque; b) the haemolytic activity of these species; and c) the association, if any, between bacterial haemolysis and clinical parameters.

Materials and methods

Patient Selection

Twenty-two patients with untreated chronic periodontitis referred to the Department of Periodontics of the Royal Dental Hospital, Melbourne were recruited in 2012. The inclusion criteria were patients with ≥20 teeth, ≥20 years of age, with at least two non-adjacent periodontal pockets >5mm, each with radiographic bone loss (RBL) more than 20% (Darby et al. 2001). Patients were excluded if they had any systemic illness which affects periodontitis (e.g. diabetes, disorders of the immune system), were pregnant, or were using drugs which may adversely affect periodontal health, or had used systemic antibiotics within 3 months prior to the study. Smokers were not excluded. The study protocol was reviewed and approved by the Human Ethics Committee of the University of Melbourne (HREC 1136794.2). All participants gave written informed consent.

Each participant received a comprehensive periodontal and dental examination from the same examiner (BW), recording probing pocket depth (PPD), recession, attachment loss, bleeding on probing (BOP), suppuration, plaque and gingival index (Loe 1967), mobility and RBL. To determine examiner accuracy initial and repeated measures were compared for pocket depth and recession for 3 patients (Reliability: 0.98 for recession and 0.99 for PPD). The participant's age, gender and smoking status were also recorded.

Sample Collection

Sample size calculation (Power 80%, $p<0.05$), based on the data from (Haffajee et al. 1991), revealed a minimum sample of 40 sites per arm (diseased and healthy sites) was required. Subgingival plaque was collected from 4 sites per patient (2 diseased sites with PPD >5mm,

RBL>20% and BOP; 2 healthy sites with PPD ≤3mm and negative BOP). Prior to collection, Gracey curette tips (cone socket curettes, G. Hartzell & Son, USA) were pre-weighed with a precision balance (Sartorious BL 210S, resolution 0.1mg, Australia), packaged and sterilized. The site was isolated with cotton rolls, supragingival plaque removed, and subgingival plaque collected in a single stroke, using a separate sterile curette for each sample. All samples were transferred immediately into 20 mL pre-reduced, thioglycollate medium (Oxoid, Thermo Fischer, Adelaide, Australia) and immediately placed on ice, stored at 2-8°C, and cultured within 2 hours. The weight of the plaque sample was determined by identifying the increase in weight of the pre-weighed thioglycollate medium and collecting tube.

Culture

Samples were oscillated for 90 seconds with a vortex machine (Ratek, VM1, Australia) and serially diluted (10^4- 10^7) with pre-reduced thioglycollate broth. Aliquots of 0.1 mL from each dilution were spread onto selective and enriched non-selective media and incubated under aerobic and anaerobic conditions (Oxoid ® Anaerogen™ Compact, Australia) at 35^0C. For aerobic incubation, non-selective plain horse blood agar (HBA) medium was used and for anaerobic incubation the following agar media were used:

i) HBA enriched with 0.001% vitamin K1 and 0.001% haemin,

ii) *A. actinomycetemcomitans*: Tryptic Soy Bacitracin Vancomycin (TSBV, (Slots 1982) - Tryptic soy agar 40.0 g, yeast extract 1.0 g, bacitracin, 75.0 mg, vancomycin, 5.0 mg, horse serum, 100.0 ml, distilled water, 1000.0 ml. This was done to allow identification of *A. actinomycetemcomitans* so that their haemolyis could be measured on the original HBA plates.

iii) *Treponema* (Leschine & Canale-Parola 1980): 15% inactivated (4h, 56 degrees Celsius) rabbit serum, 5µl/ml cocarboxylase, 5% horse blood, 0.7% Noble agar, 2µg/ml Rifampicin, 800 U/ml Polymyxin B (antibiotics added after filter-sterilization and autoclaving)

iv) *T. forsythia* (Wyss 1989) - Pancreactic digest of casein, 15.0 g, agar, 15.0 g, soy peptone, 5.0 g, sodium chloride, 5.0 g, hemin (0.1% solution), 5.0 ml, N-acetylmuramic acid, 10.0 mg, Vitamin K1 (1% Solution), 1.0 ml, sheep blood, 50.0 ml, distilled water, 1000.0 ml

v) *P. gingivalis* (Hunt et al. 1986) – Columbia Agar, 42.5 g, hemin (0.1% solution), 5.0 ml, Vitamin K1 (1% solution), 1.0 ml, Agar, 6.5 g, nalidixic acid, 15.0 mg, colistin methanesufonate, 15.37 mg, bacitracin, 10.0 mg, sheep blood, 50.0 ml, distilled water, 1000 ml

As several media were used, for any species detected on more than one plate, the median count was recorded. After incubation (aerobic: 48 hours, anaerobic: 7 days), all culture plates were photographed using a standardized digital technique.

Identification of Microorganisms

The protocol used is based on previous work by others assessing MALDI-TOF MS in comparison with conventional phenotypic identification (Seng et al. 2009) and against 16S rRNA identification (Stingu et al. 2008). Briefly, prior to identification, colony morphology was examined under a stereomicroscope, and similar morphotypes sub-cultured onto HBA for purity checks. Index colonies from purity checks were subjected to protein extraction with 1 µL 70 % formic acid (Sigma Aldrich, Australia), air dried, before overlaying with 1 µL of matrix solution (saturated a-cyano-4-hydroxycinnamic acid (HCCA) mixed with 47.5 % 18 M**Ω** water, 2.5 % trifluoroacetic acid, and 50 % acetonitrile). Once dried, samples were analysed by matrix assisted laser desorption/ionisation time of flight mass spectrometry (MALDI-TOF-MS) equipped with a 60 Hz nitrogen laser. Spectra were recorded in positive linear mode for the mass range of 2000–20000 Da at maximum laser frequency. Raw spectra were analysed using MALDI Biotyper 3.0 software (Bruker Daltonik GmbH, Bremen, Germany) with default settings, without user intervention. Three organisms, *Escherichia coli* (ATCC 25922), *Enterococcus faecalis* (ATCC 19433) and *Candida albicans* were included as internal quality control for each analysis. Identification was considered valid at species level when the score was ≥2; at genus level when the score was between ≥1.7 and <2; and not valid when the score was ≤1.7. All unidentified organisms were stored in tannic acid treated glass beads at -20^{0}C for future reference.

Bacteria were also analysed by their coloured complexes according to Socransky (Socransky et al. 1998).

New Mass Spectra Database

Two new mass spectra were analysed and recorded in the MALDI Biotyper 3.0 software package from the organisms *T. forsythia* (ATCC® 43037) and *T. denticola* (ATCC® 35405). Ethanol (900 µL) was added to each of the two new spectra organisms suspended in 300 µL of 18 M**Ω** RNase-free water, then centrifuged (14,000 *g*) for 2 min. The air dried deposits were treated with 25 µL each with 70% formic acid, and acetonitrile prior to centrifugation (14,000*g*) for a further 2min. The supernatant (1 µL) was transferred onto the MALDI target plate. A minimum of 20 individual spectra from each of the two organisms were checked for spectra uniformity before a new MS profile was created and loaded into the MALDI BioTyper™ V3.0 (Bruker Daltonik GmbH, Bremen, Germany).

Haemolytic Organisms

Basic methods to quantify haemolysis include measuring the diameter of the zone of haemolysis produced, which has a near linear relationship with the concentration of toxin produced (Bramucci & Holmes 1978). To improve the accuracy of such measurements, this study utilized measurements on standardized digital images, captured using a Nikon D40 single-lens reflex camera, 105mm *f*2.8 lens, aperture priority (*f*=2.8), mounted on a stable custom-made stand at the height of 35 cm from the culture plates, which were placed on a fixed base. Measurements of haemolysis were subsequently recorded using image analysis software (Image J version 1.46r, National Institute of Health, USA). The zone of haemolysis (pixel^2) was calculated as colony area subtracted from the haemolysis area ($\pi R^2 - \pi r^2$, R = zone of haemolysis radius = diameter of the zone, D ÷ 2, and r = colony radius = colony diameter, d ÷ 2). Images were split into red, green and blue (RGB) filter channels to enable objective standardized identification of α- and β-haemolysis. White/light zones around colonies in the green channel only were recorded as α-haemolysis, while β-haemolysis was determined as white/light zones in both the green and blue filter channels (Figure 6, supplemental). The measure of haemolytic toxicity was defined as the haemolytic zone multiplied by the colony count for each isolate.

Statistical Analysis

Data distributions were evaluated for violations of normality. All percentage data were arcsine transformed before analysis and non-normally distributed data log transformed for usage in parametric and logistic regression analysis. Nonparametric data were assessed using Mann Whitney U-test. Odds ratios were calculated using the Logit method. Corrections for multiplicity were used where necessary. All measurements were performed by one examiner (BW), and intra-examiner reliability for measuring the zone of haemolysis when assessed using intra-class correlation coefficient was 0.93. These data was analysed using SPSS® Version 18.0. Chicago:SPSS Inc.

Results

The sample population demographics and clinical characteristics of sites sampled are presented in Table 1. A total of 2372 isolates (45 genera, 142 species) were identified. Unidentifiable species consisting of 17.0% of cultivable bacteria were included in the analysis (healthy (H) sites: 13%; diseased (D) sites: 15% $p>0.05$).

Percentage distribution and viable counts

Periodontopathogens were increased in percentage distribution at diseased sites (see Figure 1 and Table 3). Several bacteria not classified as periodontopathogens were elevated in percentage distribution at diseased sites: *Selenomonas sp.*, *Neisseria sp.*, α-haemolytic *Haemophilus sp.* and *Granulicatella adiacens* (Table 3). Mean percentage distribution for *P. micra* and *Fusobacterium sp.* were not different (*P. micra* H: 5.3 ± 17.7%, D: 3.9 ± 7.1%, p=0.48; *Fusobacterium sp.* H: 3.8 ± 7.1%, D: 5.7 ± 6.2%, p=0.19), but were different for their haemolytic variants (α-haemolytic *P. micra* H: 0.4 ± 1.3%, D: 2.8 ± 6.2%, p<0.05; α-haemolytic *Fusobacterium sp.* H: 1.1 ± 3.3%, D: 2.8 ± 3.8%, p=0.03; β-haemolytic *Fusobacterium sp.* H: 0.1 ± 0.7%, D: 1.5 ± 2.5%, p=0.001, Figure 2).

The mean percentage viable counts of α- and β-haemolytic unidentifiable bacteria were elevated in diseased sites (Figure 2). *Streptococcus sp.* comprised a large proportion of the identified bacteria although there was no difference between healthy or diseased sites. However there was an increase in α- and β-haemolytic *Streptococcus sp.* at diseased sites (p<0.05 and p<0.01 respectively). Similarly, although no difference was observed in mean percentage of unidentified species between healthy and diseased sites, more α- and β-haemolytic unidentified species were observed in diseased sites (p<0.001 and p<0.05 respectively, Figure 2).

Associations between *in vitro* bacterial haemolysis and clinical parameters.

Table 2 summarizes the correlation of bacterial haemolysis with clinical parameters. Notably, measures of haemolytic toxicity, in particular total α and β-haemolytic toxicity were more strongly correlated with clinical parameters than the mere counts of bacteria. The correlation coefficient, r, for the red complex count with probing depth was 0.39 (p<0.001) as compared with 0.68 (p<0.001) for red complex β-haemolytic toxicity. A similar finding was observed for orange complex α–haemolytic bacteria. The strongest correlation with probing depth was for total β-haemolytic toxicity [r=0.73, p<0.001, actual data presented in Figure 4 (supplemental)]. Of note is that both α- and β-haemolytic activity were strongly correlated with the total bacteria count, with a near linear increase in haemolytic activity with increasing total bacteria counts (R^2 = 0.91 for α-haemolysis and R^2 = 0.78 for β-haemolysis, Figure 3).

Table 2 also shows the count and percentage distribution of *P.gingivalis* in the healthy and diseased sites. The non-haemolytic count in the healthy sites was 91% and this changed to 10% in the diseased sites. The biggest increase was in the β-haemolytic count, increasing from 7% to 65% of the total *P.gingivalis*. This is graphically presented in Figure 5 (supplemental).

The frequency, percentage distribution and odds ratios are presented in Table 3. The data indicates that the haemolytic variants have greater impact on clinical parameters than the mere presence of the establish pathogens. One example is the increased odds ratio for α- and β-haemolytic orange complex as compared with just the presence of orange complex species (OR = 5.1 for orange complex, 15.3 for α-haemolytic orange complex and 8.3 for β-haemolytic orange complex, Table 3).

Discussion

This study is, to our knowledge, the first to assess bacterial haemolytic activity within healthy and diseased sites in untreated periodontitis patients in relation to clinical parameters. Collectively, the data show that bacterial haemolysis, albeit observed *in vitro*, is strongly associated with the clinical parameters of periodontitis. This data are based on the multiple regression modelling and the relative strength of the correlation coefficients of the haemolytic data with the clinical parameters, compared with that for bacteria counts or presence. Within the same host, the mean percentages of α- and β-haemolytic bacteria are elevated in diseased sites, thus haemolysins may contribute to the site specific nature observed in periodontitis. While some healthy sites did harbour haemolytic species, there were fewer haemolytic isolates in such sites, and the size of the haemolytic zone tended to be smaller, as evidenced by the strong correlation between total haemolytic activity and the clinical parameters. The findings also indicate that haemolytic activity of orange complex bacteria and other undefined bacteria may also be contributors to the development of the clinical features.

The strong correlation between increasing α- and β-haemolytic activity with increasing total bacteria count supports the universally established importance of plaque control in periodontally susceptible patients. The data indicates that the correlation is particularly strong for α-haemolytic bacteria ($R^2 = 0.91$). This may be because many of the primary and secondary colonizers such as *Streptococcus sp.* and *Fusobacterium sp.* are α-haemolytic. In relation to probing pocket depth, although total β-haemolytic toxicity was observed to be most strongly correlated (r = 0.73), α-haemolytic toxicity had a similar strength of correlation (r = 0.72). Likewise, the strength of correlation with pocket depth was similar for β-haemolytic *P. gingivalis* and for α-haemolytic *Fusobacterium sp.* (0.64 and 0.6 respectively*).* While focus has been on tertiary colonizers such as *P. gingivalis,* our findings indicate that further investigation is warranted on the role of haemolysins produced by earlier colonizers and orange complex species.

Current literature indicates the majority of key periodontopathogens possess haemolytic activity (Chu et al. 1991, Beem et al. 1999, Falkler, Jr. et al. 1983). One study observed sites with β-haemolytic species >0.4% had an odds ratio of 5.3 for losing ≥3mm of attachment (Haffajee et al. 1991). Other studies report between a third to three quarters of patients with periodontitis harbour β-haemolytic bacteria (Hillman et al. 1993, Flynn & Slots 1993). Thus the limited clinical studies available on haemolytic bacteria support our observations.

The distribution of cultivable bacteria in this study is similar to studies utilizing culture technique, with dominance of more readily cultivable species such as *Streptococcus, Actinomyces* and *Veillonella* species, with the established periodontal pathogens elevated in diseased sites (Loesche et al. 1985). However, the use of MALDI-TOF MS for bacterial identification did give rise to detection of less common species as well as a proportion of unidentified species, which was not dissimilar to other studies utilizing this technology for identification of clinical samples (El-Bouri et al. 2012).

The orange complex bacteria, many of which are haemolytic (Beem et al. 1999, Falkler, Jr. et al. 1983), are believed to contribute to microbial succession associated with periodontitis (Socransky et al. 1998) as they are elevated in periodontitis patients (Teles et al. 2010). We found that β-hemolytic *Fusobacterium sp.* had a higher odds ratio for disease sites (OR=39) compared with β-hemolytic *P.gingivalis* (OR=29). Moreover, differential gene expression in oral epithelial cells is higher with exposure to *F. nucleatum* compared with exposure to *P. gingivalis* (Milward et al. 2007). Thus our observation that orange complex haemolytic activity is associated with clinical parameters is in agreement with current literature.

The use of specific bacteria to determine disease status or disease progression has been conflicting, with some reports of strong associations (Dzink et al. 1988, Haffajee et al. 1991), and others demonstrating high prevalence of such bacteria, inconsistent with the actual prevalence of periodontal disease (Mombelli et al. 1998, Timmerman et al. 1998). This discrepancy maybe due to differences in bacterial virulence, geographic and ethnic variation or perhaps virulence characteristics such as haemolysis may be more significant than the absence, presence, counts or proportions of various bacterial species, as observed in our study.

More recent studies utilizing molecular approaches indicate certain non-cultivable species are elevated in disease (Colombo et al. 2009). In this study, α- and β-haemolytic unidentified organisms, Gram-positive anaerobes (*Slackia exigua*, *Solobacterium moorei*, *Bulleidia extructa*), and organisms such as *Selenomonas sp.* and *Granulicatella adiacens* were more frequently detected in diseased sites, as observed by others (Booth et al. 2004, Downes et al. 2000,

Goncalves et al. 2012). With the advent of molecular techniques, the list of establish periodontal pathogens has grown considerably, making it increasingly difficult to identify the causative pathogens (Curtis 2014, Perez-Chaparro et al. 2014). Although this study is small, our findings suggest that rather than solving the complex and relentless quest for specific causative species, perhaps the impact of common virulence factors such as haemolysins need to be understood better.

Recent attention has been on disruption of host-microbe homeostasis as a trigger for periodontitis (Hajishengallis & Lamont 2012, Curtis 2014). Bacterial haemolysins, many of which are pore forming toxins, likely disrupt host-microbe homeostasis as they breach the epithelial barrier, increase nutrition availability, trigger host response events and facilitate invasion of the host (Meyer et al. 1997, Okamoto et al. 2000). The proposed notion is that bacterial haemolysins contribute to oral dysbiosis by directly impacting the local host immune response, epithelial barrier integrity and also indirectly amplify tissue inflammation.

This study has several limitations, largely due to cost and time factors. The study population is a convenience sample of consecutive patients, although efforts were made to randomize the sites sampled. The sample size is small but consistent with an 80% power ($p<0.05$) using previously observed differences (Haffajee et al. 1991). A small number of smokers were included in the study sample but no difference was observed in the results with or without that data. The cross-sectional nature also does not allow interpretation of a causative relationship. Furthermore, bacterial haemolysins were not purified or identified, again, due to time and costs constraints. Thus, inferences are only made on the associative nature of bacterial haemolytic characteristics as observed *in vitro* with clinical parameters of periodontitis.

A culture technique was selected as this verifies haemolysin producing microorganisms. While DNA-sequencing methods may reveal haemolysin encoding genes, they do not determine whether the gene is switched on or off, or how much haemolysin is produced. Another advantage of culture is that bacteria are not pre-selected. However, disadvantages include difficulties in identification, and non-cultivable or difficult to culture microorganisms. The sensitivity of culture technique is limited, in this study, to 10^4 colony-forming units, therefore bacteria which were not detected may have been present below this limit. Bacteria such as *A. actinomycetemcomitans* may be associated with periodontitis even at low levels (Casarin et al. 2010). In our study, other *Aggregatibacter* species such as *Aggregatibacter aphrophilus* and *Aggregatibacter segnis* were also detected, on TSBV media which did not allow assessment of their haemolytic activity.

Conclusion

In this study, subgingival bacteria haemolytic activity as measured *in vitro* was strongly associated with the clinical presentation of periodontitis. The data indicates that presence of haemolytic organisms may be more important than the counts, absence, or presence of the organisms. The study further identifies that the haemolytic activity of orange complex and undefined bacteria were also associated with the clinical measures. Bacterial haemolysis is an important virulence factor common to multiple plaque species, and likely contributes to periodontal disease pathogenesis. More research is required to determine the impact of bacterial haemolysins on epithelial integrity and the local host immune system in periodontitis.

**Supplementary data may be accessed by contacting the corresponding author.*

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Table and Figure Legends:

Table 1: Patient Demographics and clinical measures for the healthy and diseased sites.

Table 2: Summary of correlation analyses of bacterial haemolysis with clinical parameters.

Table 3: Frequency, percentage distribution and odds ratios of some bacterial species in healthy versus diseased sites.

Figure 1: Percentage distribution of organisms with viable counts $\geq 10^4$ cfu/ml at diseased versus healthy sites.

Figure 2: Mean percentage of total cultivable bacteria (%) for various bacterial species at diseased versus healthy sites.

Figure 3: Scatterplot of total α- or β-haemolytic bacteria count against total bacteria count in each sample.

Figure 4: Scatterplot of actual data values of total beta-haemolytic activity (counts of bacteria multiplied by haemolytic zone in pixels^2) in relation to probing pocket depth, R=0.73, p<0.001.

Table 1: Patient Demographics and clinical measures for the healthy and diseased sites.

Characteristics	**Measure (Mean ± S.D.)**		
Number of patients	22		
Smokers	6		
Age (years)	52.6 ± 10.7		
Age range (years)	31-70		
Male: Female Ratio	6: 16		
Percentage of sites >5mm	20.7 ± 18.1		
Full mouth bleeding score	34.4 ± 15.5		
Number of teeth/implants	25.5 ± 3.3		
Number of missing teeth	6.5 ± 3.3		
Characteristics	**Healthy (Mean ± S.D.)**	**Diseased (Mean ± S.D.)**	**p-value**
Number of sampling sites	44	44	1.0
Probing pocket depth (mm)	1.7 ± 0.8	5.8 ± 1.1	<0.001
Recession (mm)	0.2 ± 0.6	1.1 ± 1.5	<0.001
Probing attachment levels (mm)	1.9 ± 1.1	7.0 ± 2.1	<0.001
Bleeding on probing (%)	0	100	<0.001
Radiographic bone loss (%)	3.0 ± 7.0	44.3 ± 23.6	<0.001
Gingival index	0.4 ± 0.5	2.0 ± 0.7	<0.001

Statistical methods: T-test.

Table 2: Summary of correlation analyses of bacterial haemolysis with clinical parameters

Bacteria counts/ toxicity measures	**Correlation coefficient r, p-value**		
	Attachment Loss (mm)	**% Radiographic Bone Loss**	**Probing Depth (mm)**
Total β-haemolytic bacteria count	0.22, 0.04	0.23, 0.03	0.33, 0.002

Bacteria counts/ toxicity measures	Correlation coefficient r, p-value		
	Attachment Loss (mm)	**% Radiographic Bone Loss**	**Probing Depth (mm)**
Total β-haemolytic zone area	0.48, <0.001	0.39, <0.001	**0.58, <0.001**
Total β-haemolytic toxicity	**0.63, <0.001**	**0.58, <0.001**	**0.73, <0.001**
Total α-haemolytic bacteria count	0.22, 0.007	0.26, 0.015	0.28, 0.007
Total α-haemolytic zone area	0.50, <0.001	0.46, <0.001	0.50, <0.001
Total α-haemolytic toxicity	**0.68, <0.001**	**0.63, <0.001**	**0.72, <0.001**
Red complex count	0.30, 0.005	0.33, 0.002	0.39, <0.001
Red complex α-haemolytic toxicity	0.47, <0.001	0.44, <0.001	**0.51, <0.001**
Red complex β-haemolytic toxicity	**0.59, <0.001**	**0.54, <0.001**	**0.68, <0.001**
Orange complex count	0.24, 0.028	0.32, 0.003	0.32, 0.002
Orange complex α-haemolytic toxicity	**0.51, <0.001**	**0.53, <0.001**	**0.56, <0.001**
Orange complex β-haemolytic toxicity	0.21, 0.05	0.18, 0.1	0.36, 0.001
P. gingivalis count	0.32, 0.003	0.34, 0.001	0.42, <0.001
P. gingivalis α-haemolytic toxicity	0.45, <0.001	0.42, <0.001	0.48, <0.001
P. gingivalis β-haemolytic toxicity	**0.57, <0.001**	**0.52, <0.001**	**0.64, <0.001**
Fusobacterium sp. count	0.35, <0.001	0.28, 0.009	0.46, <0.001
Fusobacterium sp. α -haemolytic toxicity	**0.55, <0.001**	**0.54, <0.001**	**0.60, <0.001**
Fusobacterium sp. β-haemolytic toxicity	0.34, <0.001	0.27, <0.02	0.46, <0.001
P. micra count	0.33, 0.002	0.29, 0.006	0.37, <0.001
P. micra α-haemolytic toxicity	0.42, <0.001	0.44, <0.001	0.39, <0.001
Prevotella sp. counts	0.21, <0.001	0.19, 0.002	0.30, 0.004
Prevotella sp. α-haemolytic toxicity	0.41, <0.001	0.37, <0.001	0.48, <0.001
Prevotella sp. β-haemolytic toxicity	0.46, <0.001	0.14, <0.001	**0.54, <0.001**
Unidentified sp. counts	0.38, <0.001	0.32, <0.001	0.39, <0.001
Unidentified sp. α-haemolytic toxicity	0.48, <0.001	0.40, <0.001	0.49, <0.001
Unidentified sp. β-haemolytic toxicity	0.49, <0.001	0.43, <0.001	0.47, <0.001
Bulleidia extructa β -haemolytic toxicity	0.36, <0.001	0.37, <0.001	0.38, <0.001

Distribution of Haemolytic counts	**Health**	**Diseased**	**p**
P. gingivalis count	$1.3x10^5 \pm 5.4x10^5$	$9.1x10^6 \pm 1.7x10^7$	<0.001
P. gingivalis α-haemolytic count %	$2.3x10^3 \pm 1.5x10^4$ (2%)	$2.3x10^6 \pm 6.2x10^6$ (25%)	<0.02
P. gingivalis β-haemolytic count %	$9.1x10^3 \pm 4.6x10^3$ (7%)	$6.0x10^6 \pm 1.5x10^7$ (65%)	<0.01
P. gingivalis non-haemolytic count %	$1.2x10^5 \pm 5.4x10^5$ (91%)	$8.7x10^5 \pm 3.4x10^6$ (10%)	0.16

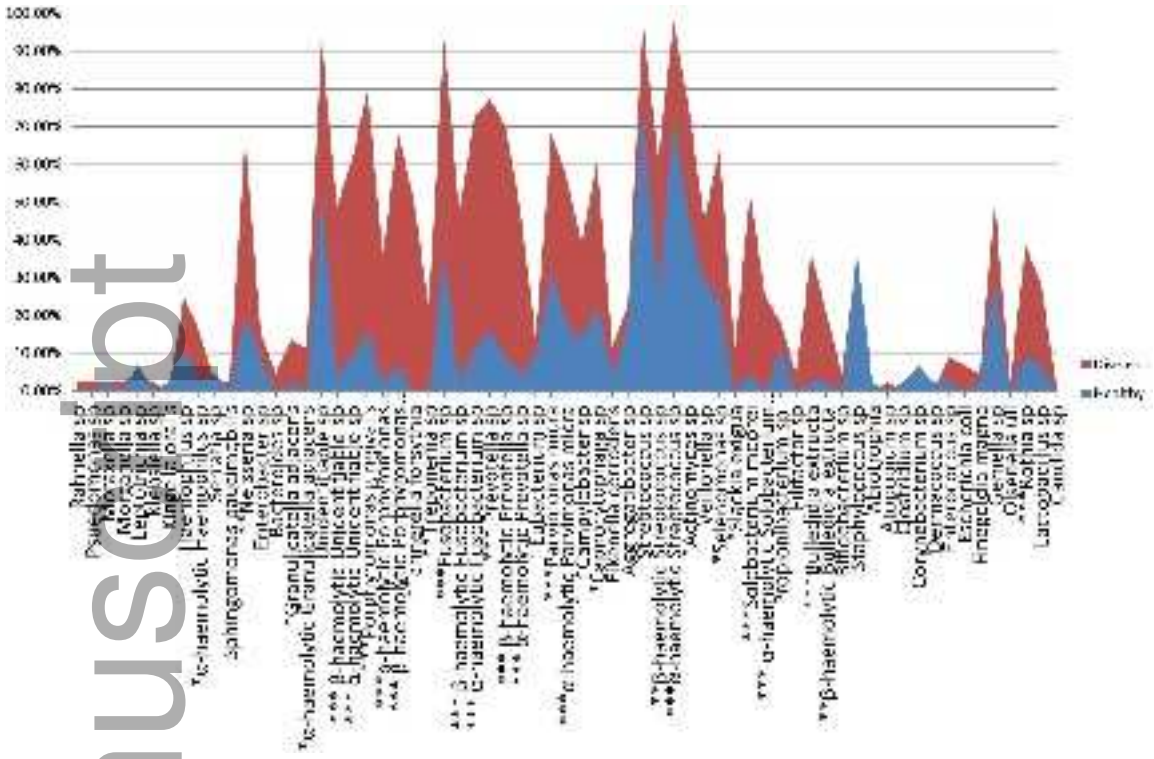
Toxicity = Total bacterial count x size of haemolytic zone (pixels^2), Statistical methods: Pearson product moment correlation. Correction for multiplicity – accept only those p-values <0.01 as significant. Highlighted boxes denote r values>0.5, and p-values<0.001

Table 3: Frequency, percentage distribution and odds ratios of some bacterial species in healthy versus diseased sites

Type of organism	Healthy		Diseased		Odds ratio (95% CI) p-value
	N	%	N	%	
α-haemolytic bacteria	32	72.7	44	100	Chi^2 = 13.8, <0.001
β-haemolytic bacteria	1	2.3	43	97.7	39.3 (4.8-320) <0.001
Red complex	7	15.9	39	88.6	41.2 (11.8-144) <0.001
α-haemolytic red complex	1	2.3	17	38.6	27.1 (3.3-331) 0.002
β-haemolytic red complex	3	6.8	33	75	41.0 (10.4-162) <0.001
P. gingivalis	7	15.9	35	79.6	20.6 (6.8-62.1) <0.001
α-haemolytic P. gingivalis	1	2.3	16	36.4	24.6 (2.9-201.7) <0.001
β-haemolytic P. gingivalis	3	6.8	30	68.2	29.3 (7.5-113.2) <0.001
Orange complex	16	36.3	38	86.4	5.1 (2.0-13.0) <0.001
α-haemolytic orange complex	4	9.1	32	72	15.3 (4.5-51) <0.001
β-haemolytic orange complex	4	9.1	15	45.5	8.3 (2.5-27) <0.001
Fusobacterium sp.	16	36.4	41	93.2	23.9 (6.2-91.6) <0.001
α-haemolytic Fusobacterium	5	11.4	32	72.7	20.8 (6.5-66.3) <0.001
β-haemolytic Fusobacterium	1	2.3	21	47.7	39.3 (4.8-320.2) <0.001
P. micra	14	31.8	30	68.2	4.6 (1.8-11.4) <0.001
α-haemolytic P. micra	8	18.2	25	56.8	5.9 (2.2-15.8) <0.001
β-haemolytic P. micra	1	2.3	8	18.2	9.6 (1.1-82.5) <0.001
Prevotella sp.	7	15.9	34	77.3	17.9 (6.1-53.3) <0.001
α-haemolytic Prevotella sp.	2	4.5	21	47.7	19.2 (4.0-91.2) <0.001
β-haemolytic Prevotella sp.	4	9.1	31	70.5	23.8 (7.0-81.8) <0.001
Bulleidia extructa	1	2.3	16	36.4	24.7 (3.0-201.0) <0.001
Selenomonas sp.	10	22.7	28	63.6	13.7 (4.4-42.3) <0.001
Solobacterium moorei	2	4.5	23	52.3	23.0 (4.8 – 109.3) <0.001
Campylbacter sp	6	13.6	17	38.6	3.9 (1.4 - 11.6) <0.006
Capnocytophaga sp	10	22.7	27	61.3	5.4 (2.1 - 13.9) <0.001
Neisseria sp	9	20.5	30	68.2	8.3 (3.1 - 22.2) <0.001
Haemophilus α-haemolytic sp	1	2.3	7	15.9	8.1 (0.92 - 71.3) <0.02
Granulicatella adiacens	1	2.3	6	13.6	6.8 (0.75 - 60.8) <0.03
T.forsythia	0	0	10	22.7	Chi^2 = 11.3, <0.001
Treponema sp	0	0	17	38.6	Chi^2 = 22.1, <0.001
Slackia exigua	0	0	5	11.4	Chi^2 = 5.3, <0.02

Unidentified sp.	23	52.3	41	93.2	12.5 (3.3-47.3) <0.001
α-haemolytic unidentified	4	9.1	27	61.4	15.8 (4.7-53.3) <0.001
β-haemolytic unidentified	2	4.5	21	48.8	21.0 (4.4-99.8) <0.001

Statistical methods: Odds ratios using the C-logit method, Chi^2 where one group had 0% or 100%.



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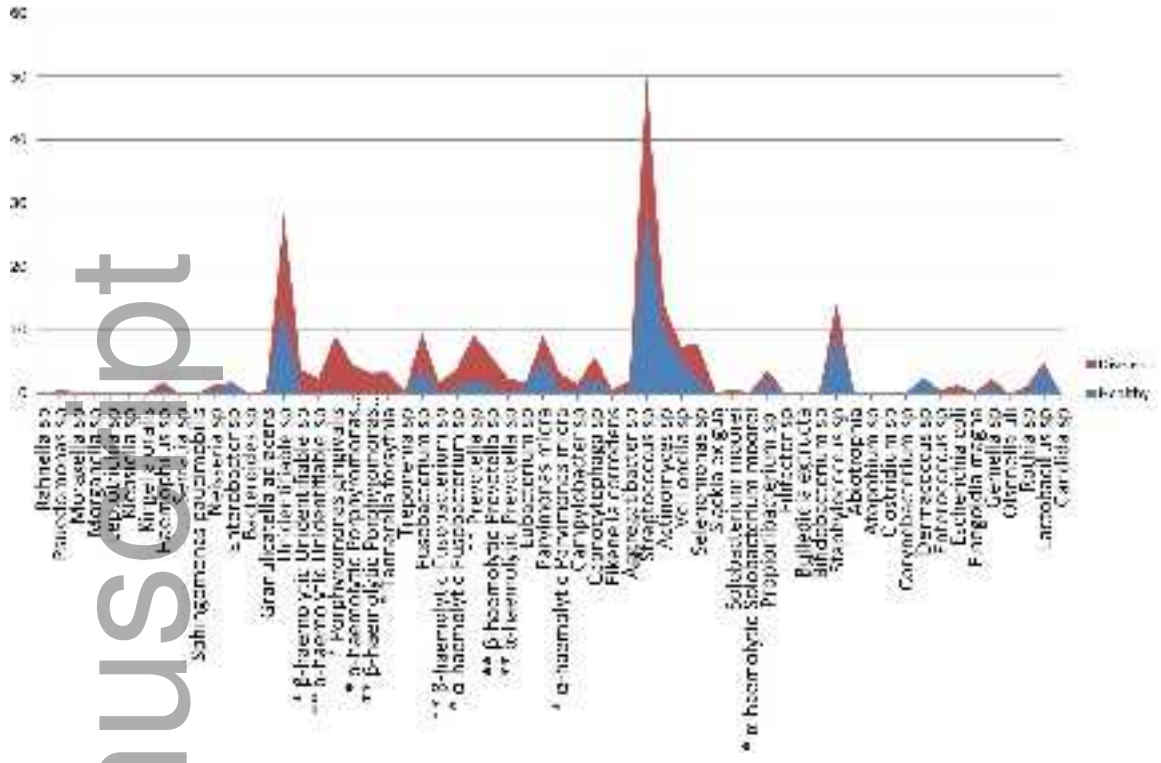

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Figure 3: Scatterplot of total α- or β-haemolytic bacteria count against total bacteria count in each sample

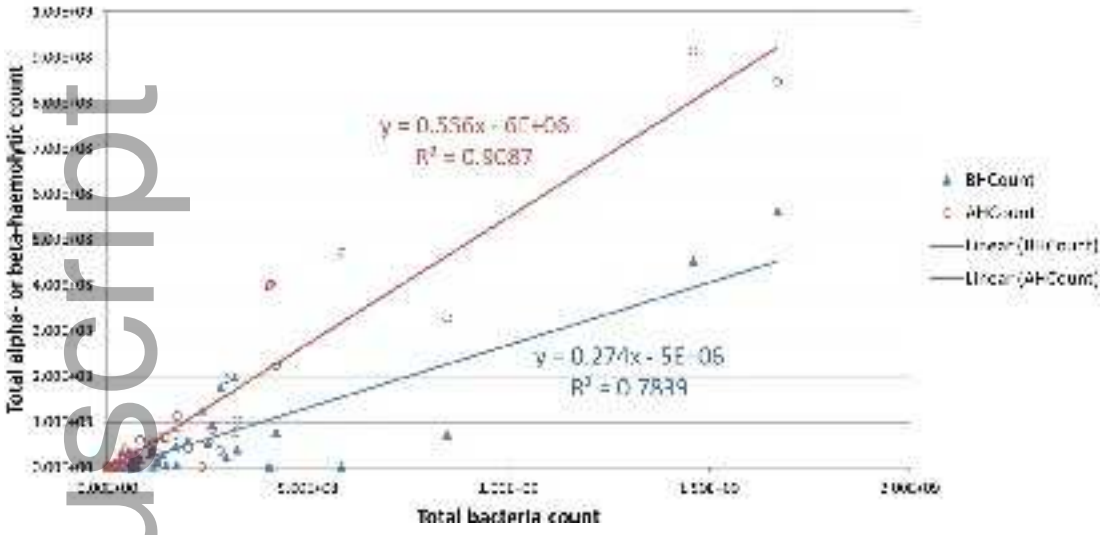

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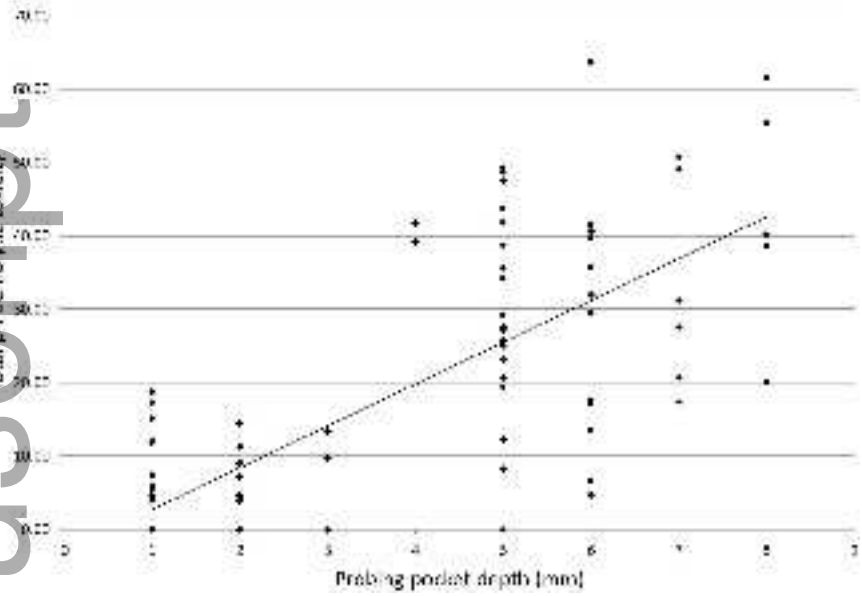
Figure 4 Scatter plot of actual data values of total beta-haemolytic activity [counts of bacteria multiplied by haemolytic zone in pixels^2] in relation to probing pocket depth, R=0.732, p<0.001.



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