Rapid chair-side test for detection of *P. gingivalis*

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Abstract

Porphyromonas gingivalis is a keystone pathogen of chronic periodontitis and its intra-oral levels have been shown to predict disease progression (activity). An accurate and sensitive chair-side (point of care) test to determine disease activity is critical for early intervention and clinical management of disease. This study aimed to develop a rapid, chair-side, saliva-based, detection of *P. gingivalis*. Monoclonal antibodies (mAbs) to the A1-adhesin domain of the *P. gingivalis* RgpA-Kgp proteinase-adhesin complex were screened by ELISA and microbial flow-cytometry, with two mAbs shown to recognise all laboratory and clinical strains tested, without significantly cross-reacting with other oral bacteria tested. Using these mAbs an immunochromatographic device was produced and shown in pre-clinical studies to detect, in inoculated saliva, all *P. gingivalis* laboratory strains and clinical isolates tested. The device was able to detect $\geq 1 \times 10^5$ *P. gingivalis* cells/mL. In a patient age-sex matched control clinical cohort *P. gingivalis* levels in saliva, as measured by real-time PCR, positively correlated with *P. gingivalis* levels in subgingival plaque ($r = 0.819$; $p < 0.01$) and clinical parameters of disease ($r = 0.633$; $p < 0.01$). A positive device result strongly correlated with *P. gingivalis* levels $> 1 \times 10^5$ cells/mL in saliva ($r = 0.778$; $p < 0.001$) and subgingival plaque ($r = 0.715$; $p < 0.001$) with a sensitivity, specificity, positive/negative predictive values and accuracy of 95.0%, 93.3%, 90.5%, 96.6% and 94.0%, respectively. The device result also positively correlated ($r = 0.695$; $p < 0.01$) with disease severity as measured by probing depth. Detection of *P. gingivalis* in saliva was found to be rapid taking 3 min from sample collection.

Introduction

Chronic periodontitis is a major public health problem in all societies and is estimated to affect 47% of the adult (≥ 30 years) US population, with 38% having moderate/severe disease (Eke et al. 2012). Diagnosis of disease activity is critical in disease management. However, conventional clinical indices such as probing depth (PD), clinical attachment loss (CAL) and bleeding-on-probing (BOP) used to define periodontal cases are poor predictors of disease progression (activity) (Byrne et al. 2009; Savage et al. 2009). Changes in the microbial composition of subgingival plaque have been demonstrated to be associated with disease prevalence/severity (Byrne et al. 2009; Socransky et al. 1998). Three subgingival plaque bacteria *Treponema denticola*, *Tannerella forsythia* and in particular *Porphyromonas gingivalis* have been found to be closely associated with severe forms of disease (Byrne et al. 2009; Hong et al. 2015; Kirst et al. 2015; Socransky et al. 1998). Increases in *P. gingivalis* and *T. denticola* in subgingival plaque have been shown to predict disease progression 3 months before clinical detection by PD and/or CAL (Byrne et al. 2009). The RgpA-Kgp proteinase-adhesin complex is a major *P. gingivalis* virulence factor and is up-regulated in active sites (O'Brien-Simpson et al. 2003; Yost et al. 2015). RgpA-Kgp complex proteinase activity is a defining *P. gingivalis* phenotype and we have recently shown that the RgpA/Kgp A1-adhesin domains are highly conserved across all laboratory and clinical *P. gingivalis* strains investigated (O'Brien-Simpson et al. 2003). Further, as part of the RgpA-Kgp complex the A1-adhesin resides on the cell surface, outer membrane vesicles and is antibody accessible (O'Brien-Simpson et al. 2000; O'Brien-Simpson et al. 2009; Veith et al. 2014).

Previous chronic periodontitis activity tests determining proteinase activity, latex bead agglutination, presence of *P. gingivalis* by ELISA, DNA probes and real-time PCR have used subgingival plaque for analysis (Boyer et al. 1996; Eick and Pfister 2002; Loesche et al. 1990; Loesche et al. 1992; Nisengard et al. 1992). These subgingival plaque-based assays have had limited success due to low specificity and/or sensitivity, long development times or the requirement to send samples to a laboratory service for analysis which does not provide point-of-care utility. Saliva, regarded as a reservoir for oral bacteria, is an easy and non-invasive biological fluid to collect, and is being investigated to aid in the detection of a range of diseases (Hu et al. 2008; Zhang et al. 2016). Using real-time PCR, it has been shown that the levels of *P. gingivalis* in saliva and subgingival plaque are closely associated and that these levels also correlate with disease prevalence/severity/activity (Boutaga et al. 2007; Byrne et al. 2009; He et al. 2012; Nomura et al. 2012; Saygun et al. 2011). Lateral-flow immunochromatographic based-assays are considered as a gold standard in point-of-care diagnostics being portable, fast, simple to use and typically having high sensitivity, specificity and accuracy (Mohd Hanafiah et al. 2013; Ngom et al. 2010).

The aim of this study was to develop and evaluate an immunochromatographic device using A1-adhesin monoclonal antibodies (mAbs) to detect *P. gingivalis* in saliva and compare that with real-time PCR detection of the bacterium in saliva and subgingival plaque as well as with clinical indices of disease in a case-control study.

Materials and Methods

Subjects, ethics approval, clinical examination and subgingival plaque sampling

This clinical study conformed to the STROBE guidelines for case-control studies (<http://www.strobe-statement.org>). Ethics approval was obtained from the University of Melbourne Health Sciences Human Ethics Subcommittee (Approval number 1136780). Fifty untreated subjects diagnosed with moderate-severe chronic periodontitis (CP) provided informed consent and were enrolled into the study from the Periodontal clinic of the Royal Dental Hospital of Melbourne in 2012. At examination, the periodontal probing depths at six sites/tooth were recorded using a Williams probe. Pocket depth (PD), recession (REC), clinical attachment level (CAL), bleeding on probing (BOP) and suppuration were recorded for each site. All CP subjects exhibited at least one pocket ≥ 5 mm in each quadrant and were classified as moderate to severe disease based on CAL and PD measurements (Darby et al. 2000; Eke et al. 2012). The CP patients exhibited $> 5\%$ of sites that bled on gentle probing, clear radiographic evidence of bone loss and had an average modified gingival index (MGI) ≥ 1 , representative of at least mild inflammation at the papillary and marginal gingivae (Lobene et al. 1986). Exclusion criteria were; periodontal treatment in the last 12 months, antibiotics or anti-inflammatory drug therapy in the last 3 months, smoking in the last 12 months, any related systemic disease/condition (e.g. diabetes) and individuals with < 20 teeth. Patients were age and sex matched with control subjects from the staff and patients of the Royal Dental Hospital of Melbourne. The control subjects were clinically periodontally healthy as defined as having no PD >3 mm. The control subjects exhibited $< 5\%$ of sites that bled on gentle probing, no radiographic evidence of bone loss and had an MGI < 1 , representative

of an absence of inflammation at the papillary and marginal gingivae. The number of subjects (50 cases with 50 controls) was based on our previous work (Byrne et al. 2009). The sample size was calculated based on comparisons of prevalence of *P.gingivalis*-positive sites in progressing (diseased) and non-progressing (healthy) subjects using the G*Power version 3.1 (<http://www.pscho.uni-duesseldorf.de/abteilungen/aap/gpower3/>) sample size package (Faul et al. 2007), based on a z-test for comparing two independent proportions; an alpha-value of 0.05 and power of 0.80. Subgingival plaque samples were taken using a sterile curette from the deepest six sites in each subject from the test and control groups. Each plaque sample was immediately placed into a coded tube containing 200µL of TE buffer (10mM tris(hydroxymethyl)-methylamine chloride, 1mM EDTA) on ice and stored at -80°C.

Bacterial strains, antigen and monoclonal antibody preparation

The following methodology is described in the Appendix: Bacterial culture and enumeration, purification of the RgpA-Kgp complexes; preparation of outer membrane vesicles (OMVs), formalin-killed *P. gingivalis* cells, recombinant A1-adhesin domain and peptide epitope antigens; and monoclonal antibody (mAb) production.

Enzyme-Linked Immunosorbent Assay (ELISA), plaque and saliva bacterial DNA extraction and real-time PCR.

ELISAs of *P. gingivalis*, bacterial DNA extraction of plaque and saliva samples and real-time PCR were performed as described in the Appendix.

Saliva collection and detection device

A saliva sample was taken at the examination before any treatment was commenced. The detection device supplied by GC Corporation (Tokyo, Japan) consisted of; 1, the test device containing A1-adhesin mAb-1, 2, treating solution [A1-adhesin mAb-2 conjugated gold particle], 3, accessories [paraffin gum, cup, pipette and a graduated mixing container]. Subjects chewed the paraffin gum for 1 min and drooled into a coded plastic cup. Following this 250µL of saliva was placed in the mixing container and mixed with 100µL of the treating solution for 10s. The saliva/treating solution was then applied to the sample window of the test device by an analyst blind to the code to minimise potential bias and the outcome read at 90 secs from application. The remainder of the saliva sample was stored at -80°C for further analysis by real-time PCR. Photographs of the devices were taken using the FujiFilm Las 3000 imager (Fuji Photo Film Co. Tokyo, Japan) using a standard 1/30 sec exposure.

Statistical analysis

All participants, samples and data were coded and the data were entered and locked before the code was released. SPSS statistical software (SPSS version 22, SPSS Inc, Chicago, IL, USA) was used for all statistical tests. All data were analysed for homogeneity of variance using Levene's test. The level of statistical significance was set at $\alpha = 0.05$. Comparisons of categorical or continuous data were made by chi-squared and independent t-tests, respectively. Categorical variables were: test device outcome – negative and positive outcomes ascribed values of 0 and 1 respectively; periodontal health status – health and disease ascribed values of 0 and 1 respectively. Continuous variables were PD and PCR data for saliva and plaque

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samples. Correlations of non-parametric data were analysed by Spearman's rank correlation. Correlations between categorical or continuous data were made by point-biserial correlation.

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Results

*Selection and specificity of monoclonal antibodies for the *P. gingivalis* detection device*

Mice were immunized with recombinant A1 domain protein antigen (PA) and synthesized peptide epitope antigens (EA) representing sequences from the RgpA-Kgp complex to generate B-cell hybridomas. After initial B-cell hybridoma screening, 39 mAbs were selected for further screening and purified by Protein A/G FPLC, concentrated, and purity assessed by SDS-PAGE (Appendix Figure A1). Figure 1A and Appendix Figures A2 and A3, show that 6 mAbs (PA1/1, PA2/2, EA1/4, EA1/6, EA3/4 and EA5/7) had the highest affinities for formalin-killed *P. gingivalis* cells.

Using a titration of viable *P. gingivalis* cells as the adsorbed antigen, three mAbs (EA1/4, EA1/6 and PA2/2) had the strongest binding with detection limits of 6.5×10^3 , 6.5×10^3 and 2.0×10^2 *P. gingivalis* cells, respectively (Figure 1B). PA2/2 mAb was found to not cross-react with the EA1 peptide antigen (Figure 1C). Microbial-flow cytometry showed that EA1/6 and PA2/2 mAbs bound viable *P. gingivalis* cells to a greater extent than EA1/4 mAb (Figure 2A). EA1/6 and PA2/2 mAbs were found to bind all of the *P. gingivalis* type strains tested and recognize only *P. gingivalis* cells in a panel of periodontal/oral bacteria (Figure 2B, 2C). Hence, EA1/6 and PA2/2 mAbs were used in the manufacture of the *P. gingivalis* detection device.

*Characterisation and specificity of the *P. gingivalis* detection device*

To characterise the detection device, saliva, free of *P. gingivalis* (undetectable by RT-PCR) was inoculated with different *P. gingivalis* strains. Initial trials found that the device gave a rapid detection response within a time range of 20-90 seconds

and some false positives from 180 seconds following saliva application. All detection device results were recorded at 90 seconds from saliva application in both the characterisation of the device and with the patient and control cohort study (below). The detection device was able to detect *P. gingivalis* W50, 381 and ATCC33277 at 5×10^4 cells/mL (Figure 3A and Appendix Figure A4), and 5×10^5 cells/mL of *P. gingivalis* A7A1-28 strain (Appendix Figure A4). We further inoculated saliva with the RgpA-Kgp complex and outer membrane vesicles (OMVs) from *P. gingivalis* at proteinase activity levels equivalent to specific *P. gingivalis* whole cell proteinase activity levels. The device was able to detect the RgpA-Kgp complex and OMVs at equivalent cell levels of 5×10^4 cells/mL and 5×10^5 cells/mL, respectively (Appendix Figure A5). Using 5×10^5 cells/mL as a detection limit, Figure 3B, shows that all *P. gingivalis* clinical isolates and laboratory strains, tested, were detected by the device.

Characterisation of the study population

A total of 100 participants, meeting inclusionary criteria, were enrolled in the study with 50 of these untreated but diagnosed with moderate to severe chronic periodontitis (CP) who were matched for age and sex with 50 periodontally healthy participants. All participants provided a saliva sample, subgingival plaque samples and underwent a periodontal examination. As shown in Table 1, there was a significant difference in the average CAL, BOP and pocket depth, percent sites with 4-6 mm or >7 mm pocket depths in the CP group when compared with the healthy cohort. Using real-time PCR as a standard analysis technique, the CP group had a greater prevalence of *P. gingivalis* in subgingival plaque and saliva than the control group (Table 1). Further both the number of *P. gingivalis* cells/mL and as a

proportion of total bacteria were significantly ($p < 0.001$) higher in subgingival plaque and saliva of the CP group compared with the control group (Table 1).

*Correlation between detection of *P. gingivalis* using the detection device and Real-Time PCR in the study population*

In evaluating the *P. gingivalis* detection device we initially compared the levels of *P. gingivalis* in saliva and subgingival plaque using the real-time PCR data and then compared that with clinical parameters. A significant ($p < 0.01$) positive correlation between saliva and subgingival plaque for both the number of *P. gingivalis* cells/mL ($r = 0.819$) and as a proportion of total bacteria ($r = 0.633$) were found for the whole study group. In both saliva and subgingival plaque the *P. gingivalis* cells/mL and the percent of *P. gingivalis* of total bacteria were positively correlated with clinical indices of disease (Appendix Table A2). Of the CP group 40 subjects tested positive for *P. gingivalis* by real-time PCR and 39 subjects were positive with the device. In the control group 2 subjects tested positive for *P. gingivalis* by real-time PCR and both were also positive with the device.

Figure 3C shows the outcomes of the saliva detection device for the CP subjects T1-T24 which shows positive and negative outcomes representative of the whole study cohort. Using point biserial analysis, the positive and negative outcomes from the *P. gingivalis* detection device were compared with saliva and subgingival plaque real-time PCR data, and with clinical diagnosis (CP or control) (Table 2). A positive correlation was found between the detection device result and level of *P. gingivalis* cells/mL in saliva ($r = 0.778$, $p < 0.001$) and as a proportion of total bacteria in saliva ($r = 0.715$, $p < 0.001$). Furthermore, a positive correlation was also found between the detection device result and the level of *P. gingivalis* in

subgingival plaque ($r = 0.771$, $p < 0.01$) and the proportion of *P. gingivalis* of the total bacterial load in subgingival plaque ($r = 0.746$, $p < 0.01$). In saliva and subgingival plaque a positive detection device outcome corresponded to $>1 \times 10^5$ *P. gingivalis* cells/mL, whereas a negative outcome corresponded to $<1 \times 10^5$ *P. gingivalis* cells/mL (Table 2). Using the threshold of 1×10^5 *P. gingivalis* cells/mL the sensitivity, specificity, positive and negative predictive values for the device were 95.0%, 93.3%, 90.5% and 96.6%, respectively with an accuracy of 94.0% for saliva which was similar to the values using subgingival plaque (Table 2).

Correlation between detection of P. gingivalis using the detection device and clinical indices and diagnosis

The detection device outcome was found to positively correlate with mean probing depth ($r = 0.695$, $p < 0.01$). In the CP group the mean probing depth ($r = 0.290$, $p < 0.05$), the percent sites with 4-6 mm ($r = 0.306$, $p < 0.05$) and ≥ 7 mm pockets ($r = 0.373$, $p < 0.01$) significantly and positively correlated with the device result. In both the CP and control groups a positive device result corresponded to $>1 \times 10^5$ *P. gingivalis* cells/mL and $>10\%$ *P. gingivalis* of total bacteria in subgingival plaque, whereas a negative outcome corresponded to $<1 \times 10^5$ *P. gingivalis* cells/mL and $<10\%$ *P. gingivalis* of total bacteria (Table 2). Using clinical diagnosis of disease, the *P. gingivalis* detection device had a sensitivity, specificity, positive and negative predictive values of 81.3%, 94.0%, 92.9% and 83.9%, respectively with an accuracy of 87.8%.

DISCUSSION

Early detection and minimal intervention are key objectives in modern dentistry in the management of oral diseases. Recent microbiome analyses of subgingival plaque samples taken from patients with CP have confirmed the presence of *P. gingivalis* in microbiological communities associated with disease (Hong et al. 2015; Kirst et al. 2015). Furthermore, intra-oral levels of *P. gingivalis* have been shown to be predictive of periodontitis progression (disease activity) (Byrne et al. 2009), hence the chairside assessment of *P. gingivalis* intra-oral levels may assist in the early detection of disease risk. Using mAbs to the A1 adhesin of *P. gingivalis* a chair-side (point-of care) immunochromatographic device has been developed for detection of *P. gingivalis* in saliva. The device exhibited a high level of sensitivity and specificity with 94.0% accuracy in detecting *P. gingivalis* at a level $>1 \times 10^5$ cells/mL. Further the device was found to have a 87.8% accuracy in confirming the clinical classification of CP status, which is superior to the reported 34.5% accuracy of diagnosing CP by dental graduates (Sajadi 2015). The participants in the current study with CP had not received treatment in the previous 12 months and so would be expected to exhibit a higher degree of disease activity (progression of attachment loss) compared with the control participants classified as periodontally healthy using conventional clinical indices. This potential differential in disease activity between the two groups was reflected in the results obtained with the saliva *P. gingivalis* detection device.

The use of saliva as the biological material for screening has obvious advantage as sample collection is non-invasive, which facilitates rapid screening as evidenced here with the whole procedure from sample collection to test outcome taking 3 min. A number of studies have suggested that saliva could be used for the

detection of a variety of biomarkers that may assist in the diagnosis of chronic periodontitis (Ebersole et al. 2015; Izadi Borujeni et al. 2015; Saygun et al. 2011; Slots and Slots 2011). In this study we have shown that the level of *P. gingivalis* in saliva either as cells/mL or as a proportion of total bacterial load correlated with the levels of *P. gingivalis* in subgingival plaque which corroborates earlier studies showing this close association (Boutaga et al. 2007; He et al. 2012; Nomura et al. 2012; Saygun et al. 2011). Furthermore, the level of *P. gingivalis* in subgingival plaque has been positively associated with CP (Darveau et al. 2012; Hong et al. 2015; Kirst et al. 2015; Socransky et al. 1998) and above threshold levels it can predict disease progression (Byrne et al. 2009). A recent systematic meta-analysis of the literature reported that there was moderate evidence that a consortium including *P. gingivalis* was associated with peri-implantitis (Perez-Chaparro et al. 2016). Hence a chairside detection device for *P. gingivalis* may have utility to assist in the determination of risk for both CP and peri-implantitis.

Earlier chair-side microbial-based tests for the screening of CP risk have included determining subgingival plaque levels of proteinase activity using N-benzoyl-DL-arginine-2-naphthylamide (BANA) tests, agglutination of latex beads and whole cell bacterial ELISAs (Boyer et al. 1996; Loesche et al. 1990; Nisengard et al. 1992). In the majority of these previous tests a high sensitivity has come at the expense of specificity or vice versa (Boyer et al. 1996; Eick and Pfister 2002; Hemmings et al. 1997; Kaman et al. 2012). This was found not to be the case for the current detection device which had a sensitivity and specificity of 95.0% and 93.3% respectively. Targeting the major and conserved antigen, A1-adhesin domain of the *P. gingivalis* RgpA-Kgp complex resulted in the high sensitivity and specificity. The detection device not only recognised whole cells but also products that are

released such as OMVs and the RgpA-Kgp complex. Further the saliva detection device was able to recognise all of the *P. gingivalis* type strains and clinical isolates with no appreciable/significant cross-reaction with other oral bacteria tested. A criticism of periodontitis screening assays/devices has been that they do not distinguish between quiescent and active disease (Ji and Choi 2015). However, it has been shown that the composition of the microbiome of subgingival plaque, in particular an increase in the level of *P. gingivalis* above threshold levels is strongly associated with risk of active disease (progression of attachment loss) (Byrne et al. 2009). By adjusting the monoclonal antibody concentrations the detection limit for the device was set to capture this threshold using clinical plaque and saliva samples. The detection threshold for the device was 1×10^5 *P. gingivalis* cells/mL in saliva.

Conclusion

A chair-side (point-of-care) *P. gingivalis* detection device was equivalent to that of real-time PCR in detection of *P. gingivalis* at levels reported to be associated with CP progression (disease activity) (Byrne et al. 2009). With very high sensitivity, specificity and accuracy the *P. gingivalis* detection device may have utility as an adjunctive chair-side method for the assessment of risk for CP and peri-implantitis.

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Author contributions

Neil O'Brien-Simpson, Ivan Darby and Eric Reynolds supervised the project. Neil O'Brien Simpson, Kate Burgess and Eric Reynolds wrote the manuscript with editing and intellectual input from Ivan Darby, Gail Brammar and Jason Lenzo. Neil O'Brien-Simpson and Gail Brammar performed the mAb purification and ELISA assays, Jason Lenzo performed the flow cytometry assays, Gail Brammar performed the real-time PCR studies and Kate Burgess performed the clinical examinations. Eric Reynolds obtained the funding and was responsible for project administration and leadership.

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Figure Legends

Figure 1. Screening of monoclonal antibodies. (A) Binding of PA mAbs to *P. gingivalis* cells in an ELISA. (B) Binding of the 6 candidate mAbs to different numbers of *P. gingivalis* in an ELISA. Red bar indicates 2 x above background O.D. (C) Binding of EA1/4, EA1/6 and PA2/2 to EA1 peptide epitope. All data are expressed as the mean and standard deviation and are representative of 3 separate experiments.

Figure 2. Characterisation of lead mAbs. (A) mAb binding to viable *P. gingivalis* whole cells determined by microbial flow-cytometry. # = $P < 0.01$, comparison of EA1/6 and PA2/2 to EA1/4 opsonisation of *P. gingivalis*. Data are expressed as the mean and standard deviation and are representative of 3 separate experiments. (B) Binding of the two lead mAbs to *P. gingivalis* fimbrial strains by ELISA. Data are expressed as the mean and standard deviation and are representative of 3 independent assays. (C) Binding of the two lead mAbs to periodontal and oral bacteria by ELISA. Data are expressed as the mean and standard deviation and are representative of 3 independent assays.

Figure 3. Representative photographs of the immunochromatographic device in the validation and clinical phases of testing. (A) Determination of the detection limit of the device for saliva inoculated with different cells/mL of *P. gingivalis*. (B) Detection of 5×10^5 cells/mL of laboratory and clinical isolates of *P. gingivalis* inoculated in

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3 saliva. (C) Representative photographs of the device post application of patient
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5 saliva, T1 to T24. Photographs were taken 90 seconds post-application of saliva.
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Table 1. Demographics of the study population including clinical and microbiological parameters.

	CP	Control	P value ^a
Demographics			
Age, (years, mean ± SD (range))	52.4 ± 12.1 (26-71)	51.2 ± 12.0 (28-70)	0.90
Ratio males/females	22/28	22/28	-
Clinical indices			
Pocket Depth (Mean ± SD (mm))	3.6 ± 1.0	1.9 ± 0.3	<0.001
CAL (Mean ± SD) (mm)	7.0 ± 2.1	2.1 ± 0.4	<0.001
BOP (Mean ± SD) (%)	60.8 ± 25.3	2.5 ± 2.4	<0.001
Percent sites 4-6 mm pocket depth (%)	38	0	<0.001
Percent sites >7mm pocket depth (%)	12	0	<0.001
Saliva			
<i>P. gingivalis</i> cells/mL (mean ± SD) ^b	1.6 × 10 ⁵ ± 2.3 × 10 ⁵	2.9 × 10 ⁴ ± 4.8 × 10 ³	0.016
Percent <i>P. gingivalis</i> of total bacteria (mean ± SD) ^c	3.4 ± 4.9	<0.1 ± 0.2	<0.001
Prevalence of <i>P. gingivalis</i> (%) ^d	78	8	<0.001
Subgingival plaque			
<i>P. gingivalis</i> cells/mL (mean ± SD) ^b	4.9 × 10 ⁵ ± 1.2 × 10 ⁶	4.1 × 10 ² ± 2.8 × 10 ³	<0.001
Percent <i>P. gingivalis</i> of total bacteria (mean ± SD) ^c	7.7 ± 11.2	0.3 ± 2.1	<0.001
Prevalence of <i>P. gingivalis</i> (%) ^d	82	2	<0.001

^aData analysed by chi-squared and independent t-tests between CP and Control groups (n = 100).

^bAs determined by real-time PCR.

^cThe percentage of *P. gingivalis* cells per total bacterial cell number present in the saliva or subgingival plaque sample.

^dThe percentage of samples positive for *P. gingivalis* as determined by real time PCR (>1 × 10³ cells/mL).

Table 2. Clinical diagnosis and level of *P. gingivalis* in saliva and plaque stratified on device outcome^a.

	<i>P. gingivalis</i> cells/mL stratified on device outcome (mean $\pm$ SD) ^b		Percent <i>P. gingivalis</i> total bacteria stratified on device outcome (mean $\pm$ SD) ^b		P value ^c	Sensitivity (%) ^d	Specificity (%) ^d	PPV (%) ^f	NPV (%) ^g	Accuracy (%) ^h
	positive	negative	positive	negative						
Saliva	$1.9 \times 10^5 \pm 2.5 \times 10^5$	$1.5 \times 10^4 \pm 2.5 \times 10^4$	3.2 ± 2.3	0.8 ± 1.1	0.015	95.0	93.3	90.5	96.6	94.0
Subgingival plaque	$6.2 \times 10^5 \pm 1.3 \times 10^6$	$3.6 \times 10^4 \pm 5.8 \times 10^4$	13.2 ± 2.1	2.3 ± 3.3	0.009	89.7	88.5	83.3	93.1	88.5
Clinical Diagnosis										
CP	$2.1 \times 10^5 \pm 2.5 \times 10^5$	$1.6 \times 10^4 \pm 2.5 \times 10^4$	12.6 ± 11.8^i	4.7 ± 2.8^i	0.011	81.3	94.0	92.9	83.9	87.8
Control	$1.6 \times 10^5 \pm 1.2 \times 10^5$	$2.6 \times 10^2 \pm 1.3 \times 10^3$	14.7 ± 0.8	$<0.1 \pm <0.1^i$	<0.001					

^adata analysed by Spearman's rank correlation, independent t tests or point-biserial correlation

^bAs determined by real-time PCR.

^cP value of the analysis between positive and negative device outcome and the *P. gingivalis* cells/mL data.

^dSensitivity defined in Appendix

^eSpecificity defined in Appendix.

^fPositive Predictive Value (PPV) defined in Appendix

^gNegative Predictive Value (NPV) defined in Appendix

^hAccuracy defined in Appendix.

ⁱSubgingival plaque real-time PCR data.

Figure 1

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190x275mm (300 x 300 DPI)

Figure 2

Figure 2. Characterisation of lead mAbs. (A) mAb binding to viable *P. gingivalis* whole cells determined by microbial flow-cytometry. # = $P < 0.01$, comparison of EA1/6 and PA2/2 to EA1/4 opsonisation of *P. gingivalis*. Data are expressed as the mean and standard deviation and are representative of 3 separate experiments. (B) Binding of the two lead mAbs to *P. gingivalis* fimbrial strains by ELISA. Data are expressed as the mean and standard deviation and are representative of 3 independent assays. (C) Binding of the two lead mAbs to periodontal and oral bacteria by ELISA. Data are expressed as the mean and standard deviation and are representative of 3 independent assays.

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Figure 3

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190x275mm (300 x 300 DPI)

Appendix

Rapid chair-side test the detection of *P. gingivalis*

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Methods and Materials

Bacterial strains and culture conditions

P. gingivalis strains; W50; 381, A7A1-28, ATCC33277, ATCC 53978, ATCC 49417, YH522 and clinical isolates 84-3, RA, 3-3, 3A1, 7B-TORR ,13-1 and 15-9 were obtained from the culture collection of the Oral Health Cooperative Research Centre, The Melbourne Dental School, University of Melbourne, Australia. *P. gingivalis* strains were grown on Horse Blood Agar (HBA) (20 g/L HBA; Oxoid Ltd., Hampshire, UK) supplemented with 10% v/v lysed horse blood (37°C) in an anaerobic N₂ atmosphere containing 5% CO₂ in a MK3 Anaerobic Workstation (Don Whitley Scientific Ltd., Adelaide, Australia). Colonies were inoculated into starter culture comprised of 20 mL sterilised brain heart infusion (37 g/L BHI; Oxoid Ltd., Hampshire, UK) medium supplemented with 5 mg/L hemin and 0.5 mg/L cysteine (McKee et al. 1986) and incubated anaerobically (24 h, 37°C).

Treponema denticola ATCC 35405, *Tannerella forsythia* ATCC 43037, *Aggregatibacter actinomycetemcomitans* ATCC 700685, *Prevotella intermedia* ATCC 25611, *Streptococcus sanguis* NCTC 7863, *Streptococcus mutans* Ingbritt and *Streptococcus salivarius* NCTC 8618 was obtained from the culture collection of the Oral Health CRC, University of Melbourne and GC corporation and grown as previously described (Dashper and Reynolds 2000; Malkoski et al. 2001; Oettinger-Barak et al. 2013; Veith et al. 2009a; Veith et al. 2009b)

All bacterial cells were harvested during late exponential growth by centrifugation (7,000 g, 20 min, 4°C). Bacterial purity was routinely confirmed by Gram stain (Slots 1982). Absorbance of batch cultures were monitored at OD_{650nm} using a spectrophotometer (model 295E, Perkin-Elmer, Germany).

Enumeration of bacterial strains

Bacterial cells were enumerated using the Molecular Probes Bacteria Counting Kit using the manufacturer's protocol where dilutions of the bacterial cells were performed in media (5% w/v trypticase soy broth (TSB) in 0.15 M NaCl). In order to create a standard curve, bacterial cultures of various cell densities as determined by measuring absorbance at a wavelength of 650 nm were used. Where multiple dilutions were examined, the highest cell density of bacteria used was $\sim 1 \times 10^6$ cells/mL. Aliquots of 10 μ L of these dilutions were mixed with 990 μ L cytometry media. Modifications to the manufacturer's protocol included the addition of 10 μ L of the microsphere suspension, to give a final concentration of 1×10^6 microspheres/mL, to each sample before the addition of the provided SYTO BC bacterial stain. Also differing from the standard protocol, the 1 μ L of SYTO BC added was first diluted 1 in 2 in the media. Controls used in this experiment were samples containing bacterial cells with SYTO BC stain but no microspheres, bacterial cells with microspheres but no SYTO BC stain, and microspheres with SYTO BC stain but no bacteria. Flow cytometry analysis was carried out on a Beckman Coulter Cytomics FC 500 flow cytometer equipped with an argon ion laser operating at an excitation wavelength of 488 nm.

Purification of the RgpA-Kgp proteinase-adhesin complexes (RgpA-Kgp complexes) and preparation of outer membrane vesicles

The protein extraction and purification of the RgpA-Kgp complexes (from strain W50) was performed as described previously (Pathirana et al. 2006). Outer membrane vesicles (OMVs) were prepared as described previously (Veith et al. 2014).

Preparation of recombinant A1 adhesin domain and peptide epitope antigens

Recombinant A1 adhesin domain (PA) was prepared as previously described (Frazer et al. 2006). Peptide epitope antigens (EA) from the RgpA-Kgp complex were synthesised by

solid phase peptide chemistry using Fmoc protocols and once purified conjugated to diphtheria toxoid as previously described (O'Brien-Simpson et al. 2000; O'Brien-Simpson et al. 2004).

Preparation of formalin-killed P. gingivalis W50 (FK W50)

P. gingivalis W50 culture was harvested (6,500 g, 4°C), washed once with phosphate buffered saline (PBS) (0.01 M Na₂HPO₄, 1.5 mM KH₂PO₄ and 0.15 M NaCl, pH 7.4) then pelleted by centrifugation (7,000g, 20 min 4 °C). Bacterial cells were resuspended (gentle shake, 24 h, 22 °C) in 0.5% v/v formal saline on a rocking platform (model RSM6, RATEK Instrument Pty Ltd, Australia). The suspension was centrifuged (7,000g, 20 min 4°C) and resuspended in sterile PBS and this was repeated once. After the second wash, the supernatant was discarded and the cell pellet was resuspended in sterile PBS to obtain a cell density of 2 x 10¹⁰ cells/mL, and protein concentration determined using Biorad Protein Assay Dye Reagent Concentrate (Life Science, NSW, Australia).

Preparation of monoclonal antibodies

Monoclonal antibodies (mAbs) to recombinant A1 protein and peptide epitopes were produced by CSL Ltd (Melbourne, Australia). Monoclonal antibodies were purified from B-cell hybridoma supernatants by buffer exchanging (PD-10 column) into binding buffer (20mM sodium phosphate, pH7.0) which was applied to a HiTrap protein G column installed in an AKTA Basic 10 FPLC. IgG mAb was eluted using a 0.1M glycine-HCl, pH 2.7 at 2mL/min and the eluant was monitored using 280nm absorbance. The collected IgG mAb fractions were pH adjusted (pH 7.0) and concentrated to 1mL and IgG concentration determined by the BioRad protein assay and purity by SDS-PAGE gel electrophoresis using a method as previously described (Pathirana et al. 2006).

Binding of bacteria by mAbs using Enzyme-Linked Immunosorbent Assay (ELISA)

ELISAs were performed using serial dilution of A1-adhesin or peptide epitope specific mAbs as described in Pathirana *et al.* (Pathirana et al. 2007) in triplicate using a solution (5 µg/mL) of either the peptide epitopes, the RgpA-Kgp complexes or FK W50 cells in 0.1M PBS (pH 7.4) to coat wells (16 h, 4°C) of flat-bottom polyvinyl microtiter plates (Microtiter; Dynatech Laboratories, McLean, VA, US). In these experiments the following antibody dilutions were used; a dilution of 1/2000 dilution of goat anti-mouse; IgG (M8642) antibody (Sigma, New South Wales, Australia) was used and a 1/4000 dilution of horseradish peroxidase-conjugated rabbit anti-goat IgG antibody (M5420; Sigma, New South Wales, Australia) was used to develop the ELISA.

Binding of bacteria by mAbs using flow cytometry

P. gingivalis W50 was grown to late exponential phase and harvested by centrifugation (9000 g, 30 mins, 4°C) then washed (x2) with Phosphate Buffered Saline (PBS; Sigma). Bacteria were resuspended at 5×10^7 bacteria/mL in PBS. 1×10^7 bacteria were incubated with 0.1 µg/ml of mAb in a 200 µl volume for 30 mins at 37°C. Bacteria were washed (x2) with PBS before being incubated with the secondary antibody R-Phycoerythrin (PE)-conjugated anti-mouse IgG (BD Biosciences) for 30 mins at 37°C. The bacteria were then washed (x2) with PBS before being resuspended in PBS containing a 1/1000 dilution of Syto9 (Thermo Fisher Scientific). Bacteria were then analysed on a FC500 flow cytometer (Beckman Coulter) equipped with an argon ion laser operating at an excitation wavelength of 488 nm and a red solid state diode laser operating at 635 nm. Bacteria were identified as Syto9 positive events as measured through a 525 nm filter (FL1) and antibody binding measured as PE positive events, as measured through a 575 nm filter (FL2). A mouse isotype control antibody was used as a negative control and all data was analyzed using Kaluza Flow Cytometry Analysis Software (Version 1.2; Beckman Coulter).

Determination of thresholds for different P. gingivalis species and P. gingivalis vesicle and complex

Saliva from a periodontally healthy subject (no detectable *P. gingivalis* by RT-PCR) was inoculated with the following concentrations:- $10^7/250\ \mu\text{L}$, $10^6/250\ \mu\text{L}$, $10^5/250\ \mu\text{L}$ and $10^4/250\ \mu\text{L}$ of whole cell *P. gingivalis* W50, 381, ATCC 33277 and A7A1-28. *P. gingivalis* OMVs and the secreted form of the RgpA-Kgp complex with matched arginine protease activity, using the previously described method (O'Brien-Simpson et al. 1998), to the above concentrations of whole cell *P. gingivalis* W50 were also used. A 125 μL of each test sample were mixed with 125 μL of saliva and then analysed using the immunochromatographic device as described above. The dilutions gave the final cell or cell-equivalent of 5×10^6 , 5×10^5 , 5×10^4 and $5 \times 10^3/\text{mL}$.

Bacterial DNA extraction of plaque and saliva

The six plaque samples from each subject in the test group and the four plaque samples from the control group were pooled. Samples were thawed on ice and vortexed for 10secs. Plaque samples were centrifuged at 10,000g for 10 mins and the supernatant removed. The saliva sample (200 μL) was diluted into 400 μL of TE buffer and centrifuged at 6800 g for 5mins at 4°C and the supernatant removed. The supernatant from saliva and plaque samples were stored at -80°C. The Wizard® Genomic DNA purification kit (Promega, Madison, WI, USA) was used to isolate DNA from plaque and saliva pellet samples using the previously described method (Lam et al. 2014).

Real-time PCR for saliva and plaque samples

Triplicate samples were used for standard curve determination with plaque and saliva samples analysed in duplicate. Real time PCR assays were conducted as previously

described (Lam et al. 2014) using the Rotor Gene 6000 (Corbett Life Science, USA) and the cell numbers of *P. gingivalis* and total oral bacteria/sample/mL were quantified using *P. gingivalis*-specific and universal primers and TaqMan probes listed in Table A1. Individual plaque samples were analysed for each subject, i.e. 6 plaque samples were analysed for each CP and control subject.

Statistical analysis

SPSS statistical software (SPSS version 22, SPSS Inc, Chicago, IL, USA) was used to analyse the data. Comparisons of categorical or continuous data were made by chi-squared and independent t-tests, respectively. Correlations of non-parametric data were analysed by Spearman's rank correlates.

Sensitivity = True positives/(True positives + False negatives) x 100. True positive = positive device detection matches with a positive real-time PCR detection ($>1 \times 10^5$ cells/mL) of *P. gingivalis* cells in the saliva or subgingival plaque sample. False negative = negative device detection with a positive real-time PCR detection ($>1 \times 10^5$ cells/mL) of *P. gingivalis* cells in the saliva or subgingival plaque sample.

Specificity = True negatives/(True negatives + False positives) x 100. True negatives = negative device detection matches with a negative real-time PCR detection ($<1 \times 10^5$ cells/mL) of *P. gingivalis* cells in the saliva or subgingival plaque sample. False positive = positive device detection with a negative real-time PCR detection ($<1 \times 10^5$ cells/mL) of *P. gingivalis* cells in the saliva or subgingival plaque sample.

Positive Predictive Value (PPV) = True positives/(True positives + False positives) x 100.

Negative Predictive Value (NPV) = True negatives/(True negatives + False negatives) x 100.

Accuracy defined as = (True positive + True negative)/(True positive + False positive + False negative + True negative) x 100.

Appendix Figures and Tables.

Table A1. Sequence of the 16S ribosomal RNA oligonucleotide primers/TaqMan probes used for real-time polymerase chain reaction

		Primer/TaqMan probe sequence (5' – 3')	Reference
Forward	<i>P. gingivalis</i>	TGCAACTTGCCTTACAGAGGG	(Gaetti-Jardim et al. 2009)
Reverse	<i>P. gingivalis</i>	ACTCGTATCGCCCGTTATTC	(Gaetti-Jardim et al. 2009)
Probe	<i>P. gingivalis</i>	(6-FAM)-ACCAAAGATTCTACGGTGGAGGATGGG-(TAMRA)	(Gaetti-Jardim et al. 2009)
Forward	Universal	TCCTACGGGAGGCAGCAG	(Nadkarni et al. 2002)
Reverse	Universal	GGACTACCAGGGTATCTAATCCTGTT	(Nadkarni et al. 2002)
Probe	Universal	(6-FAM)-CGTATTACCGCGGCTGCTGGCAC-(TAMRA)	(Nadkarni et al. 2002)

Table A2. Correlation between real-time PCR detection of *P. gingivalis* in saliva and subgingival plaque and clinical indices.

	Subgingival Plaque		Saliva	
	<i>P. gingivalis</i> cells/mL	<i>P. gingivalis</i> % total bacteria	<i>P. gingivalis</i> cells/mL	<i>P. gingivalis</i> % total bacteria
Clinical Indices				
Average PD (Whole study group)	0.725 ^a ;	0.725 ^a ;	0.797 ^a ;	0.790 ^a ;
Average PD (periodontal group)	0.219 ^b ;	0.219 ^b ;	0.600 ^a ;	0.517 ^a ;
PD > 7mm	0.345 ^b ;	0.345 ^b ;	0.590 ^a ;	0.495 ^a ;
Saliva (Whole study group)				
<i>P. gingivalis</i> cells/mL	0.819 ^a ;	0.819 ^a ;	-	-
<i>P. gingivalis</i> total bacteria %	0.541 ^b ;	0.541 ^b ;	-	-
Saliva (CP group)				
<i>P. gingivalis</i> cells/mL	0.262 ^b ;	0.262 ^b ;	-	-
<i>P. gingivalis</i> total bacteria %	0.205 ^c ;	0.205 ^c ;	-	-

a = spearman's correlation r value, p<0.01.

b = spearman's correlation r value, p<0.05.

c = spearman's correlation r value, p=0.062.

Figure A1. Purification of MAbs. Monoclonal antibody supernatants were buffer exchanged (PD-10 column) into binding buffer (20mM sodium phosphate, pH7.0 and applied to a HiTrap protein G column installed in a AKTA Basic 10 FPLC. IgG was eluted using a 0.1M glycine-HCl, pH 2.7 and the eluant was monitored using 280nm absorbance. (A) a typical FPLC profile for the monoclonal antibody supernatants. Collected IgG fractions were pH adjusted (pH 7.0) and concentrated to 1mL and IgG concentration determined by the BioRad protein assay and purity by SDS-PAGE gel electrophoresis. Blue line = IgG fraction monitored at 280nm, Green line = 0.1M glycine-HCl gradient. (B) a typical BIS-Tris electrophoresis gel of the purified Mab

Figure A2. ELISA titration of EA1 peptide specific monoclonal antibodies. (A) EA1 peptide specific antibodies used to probe EA1 peptide as the absorbed antigen by ELISA. . (B) EA1 peptide specific antibodies used to probe *P. gingivalis* whole cells as the absorbed antigen by ELISA. Antibody responses are expressed as the Absorbance (450 nm) obtained minus background, with each point representing the mean $\pm$ standard deviation of three values.

Figure A3. ELISA titration of EA3 and EA5 peptide specific monoclonal antibodies and PA1 protein specific monoclonal antibodies. (A) EA3 peptide specific and PA1 protein specific antibodies used to probe *P. gingivalis* whole cells as the absorbed antigen by ELISA. (B) EA5 peptide specific antibodies used to probe *P. gingivalis* whole cells as the absorbed antigen by ELISA. Antibody responses are expressed as the Absorbance (450 nm) obtained minus background, with each point representing the mean $\pm$ standard deviation of three values.

Figure A4. Representative photographs of the immunochromatographic device in the validation phase of testing. Determination of the detection limit of the major laboratory strains of *P. gingivalis* inoculated in saliva at varying cells/mL concentrations. Photographs were taken 90 seconds post-application of saliva.

Figure A5. Representative photographs of the immunochromatographic device in the validation phase of testing. (A) Determination of the detection limit of the device for saliva inoculated with different cells/mL of *P. gingivalis*. (B) Determination of the detection limit of the device for saliva inoculated with different amounts of *P. gingivalis* OMVs at protease activity equivalency to varying cells/mL of *P. gingivalis*. (C) Determination of the detection limit of the device for saliva inoculated with different amounts of *P. gingivalis* RgpA-Kgp complex at protease activity equivalency to varying cells/mL of *P. gingivalis*. Photographs were taken 90 seconds post-application of saliva.

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