An association between the levels of sub-gingival plaque *Porphyromonas gingivalis* and clinical parameters in periodontal diseased patients

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Abstract

The aim of this study was to determine whether the levels of sub-gingival plaque *Porphyromonas gingivalis* were associated with clinical parameters in periodontal diseased patients. Twenty-three adult subjects were participated and intra-oral examinations for recording clinical parameters of the selected teeth were then carried out. Sub-gingival plaque samples were obtained from the selected teeth and the levels of *P. gingivalis* of these samples were detected by ELISA.

The results showed that increased levels of sub-gingival plaque *P. gingivalis* were significantly associated with increased dental plaque scores (r² = 0.783), severity of gingival inflammation (r² = 0.6703), and gingival pocket depth (r² = 0.7787), but weekly with increased sub-gingival *entactins* (r² = 0.2658) and tooth mobility (r² = 0.4201). The levels of this periodontopathogen in the subjects with gingival bleeding upon probing were significantly higher than those of without gingival bleeding (p < 0.001). Since increased grades of these clinical parameters have been strongly associated with severe periodontal disease, these results suggested a crucial role of *P. gingivalis* in this periodontal disease.

Keywords: *Porphyromonas gingivalis* — Periodontal disease — clinical parameters

Introduction

Chronic inflammatory periodontal disease (CIPD) is an inflammatory response of periodontal tissues due to invasive dental plaque periodontopathogenic bacteria. It has been well documented that the course of CIPD is significantly associated with the specific types of periodontopathogenic bacteria. *Porphyromonas gingivalis*, a black pigmented anaerobic bacterium, has been implicated in the destructive stage of CIPD, since an increased number of this bacterium is associated with increased dental plaques, gingival pocket depth, gingival attachment loss and gingival bleeding (Zambon et al., 1985; Mombelli et al., 1991; Xojima et al., 1993; Christersson et al., 1992a; Kamma et al., 1995). Indeed, several major antigens, such as lipopolysaccharide (LPS), derived from this organism have been shown to induce alveolar bone resorption, cell activation, gingival cell-produced proinflammatory cytokines, production of antigen-specific

*This work is dedicated to late Dr. S. Udayana who is everlasting missed by all colleagues and friends.*
antibodies, a L-arginine-dependent nitric oxide production of macrophages and apoptosis (Sosronen and Herminajeng, 1995; Page and Kornman, 1997; Sosronen et al., submitted). These abilities of P. gingivalis to induce the host immune response and tissue destruction suggest a crucial role of this periodontal pathogen in the course of CIPD. Therefore, detection of this bacterium in the dental plaque of periodontal diseased patients for a diagnostic marker of lesional stages and patient risk as well as treatment plans significantly adds extremely important information to complement other laboratory and clinical diagnostic tools.

Murine monoclonal antibodies specific to P. gingivalis-derived LPS with no cross-reactivity to LPS from other periodontally pathic and enteric bacteria have been produced and used to detect the presence of sub-gingival plaque P. gingivalis using flow-cytometric analysis and immunofluorescence screening (Shelburn et al., 1993; Kawanaka et al., 1994; Ni Eadhin and Mouton, 1994). We and others have also previously demonstrated that this periodontopathogen could be semi-quantitatively detected from mixed dental plaque bacteria by ELISA using these monoclonal antibodies (Clerugh et al., 1997; Herminajeng et al., 1999). The aim of the present study was, therefore, to determine the association between dental plaque P. gingivalis levels assessed by ELISA and clinical parameters in periodontally diseased subjects.

Materiai and Methods

Subjects
Subjects were recruited through the Department of Periodontology at the Faculty of Dentistry, Gadjah Mada University, Yogyakarta. Only subjects with at least 20 teeth intact and with no systemic conditions related to sub-acute bacterial endocarditis prophylaxis, antibiotic and steroid therapy in the last 6 months were participated. Prior to entering the study, all subjects were well informed about the aims and scope of the study.

Clinical examinations
The following clinical parameters by using several indices were evaluated in each subject:

Dental plaque and calculus scores. The presence of supra- and infraginal plaque and calculus was assessed visually. One tooth with possible higher scores was selected. An assessment of dental plaque score at the selected tooth was then carried out using the Silness and Loe’s dental plaque index (Spolsky, 1990). Dental plaque was graded into 0, 1, 2, or 3 as judged by absence or presence in one third, two third or whole surface of tooth, respectively. Scoring sub-gingival calculus was graded into 0 (no detectable calculus), 1 (clinically detectable calculus, but the root surface was rough or grainy), or 2 (clinically detectable calculus of the root surface) (Brown et al., 1991).

Gingival status. Degrees of gingival status were evaluated by using a Loe and Silness’s gingival index (Spolsky, 1990). At the selected tooth, gingival status was scored from 0 to 3 by the following criteria: 0 = normal gingiva; 1 = mild inflammation characterized by redness edema and bleeding on probing; 2 = moderate inflammation characterized by redness edema and ulceration and bleeding.

Gingival pocket depth and gingival bleeding. Gingival pocket depth was evaluated on the mesial surface of the selected tooth by measuring the distance from the gingival margin to the bottom of the pocket, using a periodontal probe. Bleeding upon probing at this site was then recorded (Brown et al., 1991).

Tooth mobility. Since no consensus on the precision for measuring clinical tooth mobility has been made, this study used a Miller’s clinical index for such propose at the selected tooth (Stoller and Laudenbach, 1980). In order to obtain consistent measurements, the scoring was made only by one of us (SL) throughout the study. The tooth mobility was scored from 0 to 3 by using the following criteria: 0 = no sign of tooth movement; 1 = first sign of movement; 2 = a tooth crown movement within 1 mm of its normal position; 3 = tooth movement more than 1 mm at any direction or to be rotated.

ELISA
The sub-gingival plaque samples of the selected tooth on the mesial surface were obtained by using a sterile periodontal scaler, immediately immersed in a sterile tube containing 0.5 ml of sterile buffer saline, dispersed by vortexing for 1 minute and subsequently kept in -20°C until used.

ELISA to semi-quantitatively detect the levels of P. gingivalis was essentially described elsewhere (Herminajeng et al., 1999). Briefly, plaque samples were diluted in 1:10 sterile PBS and coated on the ELISA plates in triplicate (Nunc, Denmark). Diluted murine monoclonal antibodies specific to P. gingivalis-LPS were added into the plates which were then incubated for 1 hour at room temperature (RT). Following 3 times washing, diluted biotin-labeled sheep anti-mouse Ig (Sigma, St. Louis) was added and the plates were incubated for 1 hour at RT. Diluted peroxidase-streptavidin (Sigma) was added and following incubation and washing, color was developed by adding TMB solution (Sigma) as described by the manufacturer. After 10 minutes, color was stopped by adding H2SO4, and read with an ELISA reader (Flow Lab., Finland) at a wavelength of 450 nm. As intra-plate negative controls, PBS was added, instead of sheep anti-mouse Ig antibodies. The positive controls were carried out by coating certain wells in the plates with P. gingivalis-LPS. The data from the reading was expressed in absorbance unit (AU).

Statistical analysis
Statistical analysis was carried out as described previously (Mombelli et al., 1991). The mean differences in each clinical parameter were analyzed by an one-way ANOVA using LSD test. An analysis with multiple regression was used to evaluate the degrees of association between the absorbance unit and each clinical parameter. A simple T-test was used to analyze the differences between the absorbance unit of subjects with or without gingival bleeding. All data were computed by using the SPSS software (SPSS Inc., USA).

Results and Discussion

Periodontal diseased subjects
Following the loss of one of us (late dr. S. Udayana), this study was then terminated. Twenty-three adult subjects, consisting of 12 males and 11 females and ranging in age from 15 to 42 years, could finally be recorded. This study was not intended to differentiate the subjects into separate periodontal lesional stages as seen in the previous one (Herminajeng et al., 1999); rather it collected the data from participants regardless the lesional stages.

An association between clinical parameters and the levels of sub-gingival plaque P. gingivalis.

As seen in Fig. 1, scoring the sub-gingival plaques revealed that only 2 subjects had no dental plaques and 9 had minimal plaque score (score 1). Three and six ones had the plaque score of 2 and 3, respectively. Measuring the levels of plaque P. gingivalis as
judged by the values of AU of the healthy subjects was 0.1395. The mean of AU of the subjects with plaque graded 1, 2 and 3 were 0.1926, 0.3443 and 0.7507, respectively. A statistical analysis revealed levels of sub-gingival plaque P. gingivalis may be found.

Out of 23 subjects, dental calculus scores could only be recorded in 19 ones. Six subjects had no calculus (score =0), whilst 8 and 5 subjects had calculus graded 1 and 2, respectively (Fig. 2). The mean of AU of the subjects with calculus graded 0, 1 and 2 was 0.1312, 0.4505 and 0.4720, respectively. An ANOVA test revealed that no statistical differences between the mean of AU from subjects with calculus graded 1 and 2 could be observed (p<0.05), although those from both groups of the subjects were significantly different to those from the subjects with calculus graded 0 (p<0.05). Further analysis also showed a weak association between calculus scores and AU (r² = 0.2659), indicating that increased calculus scores may not necessarily be accompanied by increased levels of subgingival plaque P. gingivalis.

Gingival inflammation measured by the gingival index was observed in 19 subjects. The results showed that 6 subjects had no gingival inflammation and similar number of the subjects had mild inflammation (score =1) (Fig. 3). The remaining subjects suffered a moderate gingival inflammation (score = 2). None of the subjects had severe inflammation (score = 3). The mean of AU from the subjects with no, mild and moderate gingival inflammation were 0.1797, 0.1995, 0.7629, respectively. No statistically differences could be seen when comparing the values of AU from the subjects with no and mild inflammation (p>0.05). However, the mean of AU from these two groups were significantly different to that of the subjects with moderate inflammation (p<0.05). An analysis with the multiple regression indicated that an elevated severity of gingival inflammation may be positively associated with elevated levels of the subgingival plaque P. gingivalis (r² = 0.6703).

Measurements of the gingival pocket depth in each subjects were ranked into 3 groups, i.e. 0-2 mm, 2-4 mm and >4 mm (Fig. 4). Out of 20 subjects recorded for the pocket depth, 8 had a normal depth (0-2 mm). Five and seven subjects had the pocket depth >4 mm and 2-4 mm, respectively. The mean of AU from the first group of subjects was 0.1531, whereas that from the second and third one was 0.2514 and 0.7424, respectively. Further statistical analysis of these different means revealed that the third one was significantly higher than both the first and second one (p<0.01). No statistically differences between the mean of the first and the second group could be observed (p>0.05). These results indicated, therefore, that the sub-gingival plaque samples of the gingival pocket depth above 4 mm were colonized by P. gingivalis much higher than those of the pocket depth less than 4 mm. Indeed, the multiple regression analysis showed that an increased gingival pocket depth may be positively correlated with increased levels of sub-gingival plaque P. gingivalis. In the present study, tooth mobility was recorded due to the fact that it may exemplify tooth attachment loss and the course of CP/DP (Staller and Laudenbach, 1980; Giagia and Lindhe, 1997). Twenty-two subjects were participated and most of them (12 subjects) had no altered tooth mobility at the selected tooth and the mean of AU was 0.2033 (Fig. 5). The mobility graded 1 and 2 were seen in 8 (mean of AU = 0.637) and 2 (mean of AU = 0.5970) subjects, respectively. None of the subjects had tooth mobility graded 3. Test with one-way ANOVA revealed that the mean of AU from the subjects with tooth mobility graded 1 and 2 was significantly much higher than that of the subjects graded 0 (p<0.001), but no difference between the mean mobility grades were observed (p>0.05). These results suggested, therefore, that the number of sub-
gingival plaque. *P. gingivalis* may be increased in altered tooth mobility, but not necessarily be a positive association (r^2 = 0.42011).

Immediately after measurements of the gingival probe depth, gingival bleeding was recorded at the selected tooth. The gingival bleeding was not seen in 14 out of 23 subjects and the positive bleeding could be observed in the remaining ones (Fig. 6). The levels of sub-gingival plaque *P. gingivalis* from the gingival bleeding-positive subjects were significantly much higher than those of the gingival bleeding-negative ones (p<0.001), indicating that the number of this periodontopathogen may be increased in association with spontaneous gingival bleeding, known also as a clinical sign of severe periodontal inflammation.

Dental plaque as the main etiology of CIPD is well known, as seen in a study showing that increased visible dental plaque is accompanied by increased severity of CIPD (Christersson et al., 1992b). Indeed, a strong association between the course of CIPD and dental plaque is due to the fact that the time course of dental plaque maturation parallels with distinct colonized bacterial types and virulence. For example, increased dental plaque maturation is associated with changes from the gram positive aerobic to the gram negative anaerobic bacteria (Wolff et al., 1994). This is true when one considers that the oral gram negative anaerobic bacteria such as *P. gingivalis* and *Actinobacillus actinomycetemcomitans* are naturally colonized in the subgingival plaques (Zambon et al., 1985; Gmur and Guggenheim, 1994). That increased sub-gingival plaque *P. gingivalis* results in increased grades of visible dental plaques which in turn augment the severity of CIPD (Wolff et al., 1988; Christersson et al., 1992a and 1992b; Clerhugh et al., 1997) is, therefore, not surprising and it certainly supports the results of the present study (Fig. 1).

Of interest, although the levels of *P. gingivalis* were elevated in the sub-gingival plaque of the selected site with visible sub-gingival calculus as compared to the sub-gingival calculus-free site, increased sub-gingival calculus scores did not parallel with increased levels of this periodontopathic bacterium (see Fig. 2). The development of dental calculus is preceded by dental plaque accumulation which is subsequently mineralized following the deposition of saliva calcium and phosphate (White, 1997). The newly formed calculus thus contains mineralized bacteria and dental plaque remains however to develop following continuous bacterial colonization on the surfaces of this calculus (Friskopp and Hammarström, 1980). Not surprisingly, a significant association between sub-gingival calculus and periodontal disease has been strongly suggested, since substantial number of plaque *P. gingivalis* on the surfaces of this calculus could still be found and the calculus itself harbored potent mineralized bacteria-derived endotoxins capable to inducing bone resorption (Patterson et al., 1982; Brown et al., 1991). Hence, the simplest explanation of the present study is that sub-gingival *P. gingivalis* levels depend largely upon the degrees of dental plaque formation on the calculus surfaces, but not on its calculus amounts. If oral hygiene is kept in well controlled to prevent newly developed plaque, the number of this periodontopathic bacterium may be stable. This contention remains, however, to be further investigated.

The results of the present study showing that elevated gingival inflammation and gingival pocket depth were associated with increased sub-gingival plaque *P. gingivalis* levels (Figs. 3 and 4) are obvious. The previous findings have demonstrated that the number of sub-gingival plaque *P. gingivalis* of gingival diseased sites as judged by increased gingival index and pocket depth is much higher than that of healthy sites (Wolff et al., 1988; Zambon et al., 1985; Christersson et al., 1992a; Mombelli et al., 1991; Kojima et al., 1993; Riviere et al., 1996). Increased grades of both sub-gingival plaque and calculus lead to increased gingival pocket depth due to extensive loss of periodontal tissue attachment and gingival destruction which in turn create a potential niche for oral anaerobic bacterial growth (Christersson et al., 1992b; Page and Kornman, 1997). Furthermore, *P. gingivalis* possesses potent virulent factors such as LPS capable to inducing periodontal tissue destruction (Page and Kornman, 1997). High gingival inflammatory response induced by this periodontopathogen as a result of increased colonization on sub-gingival plaque is, therefore, imminent.

It has been well documented that altered tooth mobility may be due to trauma from excessive occlusion, incomplete maturation of periodontal membrane during tooth eruption, the influences of pregnancy-associated hormones, and periodontal disease (Giannios and Lindhe, 1997). Despite the fact that an increased tooth mobility may be considered as one of the co-factors to develop severe periodontal disease, the precise association between this clinical parameter and periodontal disease remains, however, uncertain. For example, the works of Baelum and colleagues (1988) have shown that no obvious gingival inflammation in the teeth with some degrees of mobility could be observed. So, several possibilities can be put forward to explain the lack association between increased tooth mobility and the levels of sub-gingival *P. gingivalis* as seen in the present study (Fig. 5). Firstly, increased tooth mobility in several participated subjects did not represent their degrees of gingival inflammation; thus, the levels of tooth mobility did not exemplify that of sub-gingival plaque *P. gingivalis*. Secondly, trauma from occlusion rather than sub-gingival plaque-induced tooth attachment loss might be a primary etiology of increased tooth mobility in some of the subjects. Alternatively, in spite of elevating tooth mobility, the oral hygiene of these subjects might be adequately controlled, thereby preventing increased levels of sub-gingival plaque *P. gingivalis*. Further studies are required to delineate which of these possibilities may primarily occur.

Sub-gingival plaque *P. gingivalis* levels in subjects with bleeding upon probing at the selected tooth appeared to be significantly much higher than those in bleeding-free ones (Fig. 6). These results are in accordance with the previous findings showing that the amount of identified sub-gingival *P. gingivalis* is increased in the subjects with gingival bleeding upon probing (Zambon et al., 1985; Christersson et al., 1992a; Kojima et al., 1993). Most likely, increased levels of this periodontopathogen in the subjects with gingival bleeding upon probing as compared to those without gingival bleeding may represent an increased development and maturation of sub-gingival plaque and elevated severity of gingival inflammation, since the levels of this oral bacterium were also significantly associated with elevated grades of both clinical parameters (see Fig. 1). The significant relationship between sub-gingival *P. gingivalis* and several clinical parameters in periodontal diseased patients seen in the present study indicates a crucial role of *P. gingivalis* in the course of CIPD. It has been hypothesized that virulence factors of this periodontopathic bacteria, such as LPS and outer membrane proteins (OMP), may induce not only both nonspecific and specific immune response to eliminate the invasive bacteria, but also induce bone and periodontal soft tissue destruction at the local site (Sosroseno and Herminajeng, 1995;
Page and Kornmann, 1997). In humans, this bacterium could markedly be detected in the gingival inflamed sites in situ, particularly in the gingival epithelial layer adjacent to the periodontal pocket (Nouri et al., 1997). To observe this, the animal models, injection of live P. gingivalis on the dorsal sites of mice resulted in development of lesions resembling to human periodontal disease (Bird et al., 1995; Genco et al., 1997). Furthermore, in these studies, pre-immunized mice with OMP of this periodontopathogen led to stimulate high levels of serum antigen-specific antibodies and early lesion healing process, suggesting a protective role of antigen-specific antibodies in the P. gingivalis-induced dental lesion. These studies have demonstrated, nevertheless, that P. gingivalis does possess abilities to induce soft tissue destruction in periodontal disease.

It should, however, be kept in mind that subgingival plaque is harbored by mixed types of virulent periodontopathogenic bacteria, such as A. actinomycetemcomitans, indicating that the development of CIPD is not merely induced by a single oral bacteria. Indeed, A. actinomycetemcomitans, predominately detected in the advanced periodontal diseased sites, possesses pathogenic factors capable to stimulate host defenses and both periodontal hard and soft tissue destruction (Christersson et al., 1992a; Kantma et al., 1995; Fives-Taylor et al., 1996). Hence, detection of all subgingival plaque periodontopathogens in the periodontal disease patients for establishment of clinical diagnosis would ideally be carried out. Bulk of evidences have, as yet, shown that only limited types of periodontopathogens colonize in subgingival plaque according to plaque maturation and expansion (Wolt et al., 1994; Page and Kornmann, 1997), suggesting that each clinical stage of CIPD is associated with specific types of periodontopathogens. Detection of P. gingivalis in the subgingival plaque as a representative of advanced periodontal disease associated oral bacteria would, in this respect, seem to be adequate. The fact that an ELISA-based semi-quantitative detection of this periodontopathogen by using monoclonal antibodies to P. gingivalis-derived LPS could differentiate the clinical stages of periodontal disease, i.e. early and advanced lesion (Cleerehugh et al., 1997; Heminajeng et al., 1999) highlights this contention.

In conclusion, the present study has demonstrated that increased levels of subgingival plaque P. gingivalis is significantly associated with increased grades of subgingival plaque, gingival inflammation and periodontal pocket depth as well as bleeding upon probing, but weakly with those of subgingival calculus and tooth mobility. Whether following periodontal treatments, changes of these clinical parameters may be accompanied by altered levels of this periodontopathogen are certainly worth to determine.

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References


Application of card blot as a semi-quantitative detection of genes expression on RNA level

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Abstract

The purpose of this research is to invent a quick, reproducible method of gene expression which are based on RNA-DNA hybridization, through a sensitive, efficient and cost-effective way. In this research, biotin labeled probe were used to measure bcl-2 and c-myc mRNA genes expressions of peripheral blood lymphocytes from patients of Infectious Mononucleosis (IM), Non-lymphoid Lymphoma (NLL) and Non-diagnostic asymptomatic (NDA). The blots were hybridized with biotin labeled probe. Indirect detection system utilizing streptavidin-HRP - Horse Radish Peroxidase (SA- HRP) to bind the biotin. The blocked and hybridized membrane was wets washed with Biotinyl Tyramide (BT) and Diminouviolinkers (DAB) chromogenic substrate that exists in Tyramide Signal Amplification™-Indirect (ISH).

Result of this research showed that card blot can be used in detecting gene on the RNA level with a sum of 20 ng sample randomly and with an effective cost.

Keywords: card blot, dot blot, TSA

Introduction

In the past few years, the hybridization technology of nucleic acid has been evolving rapidly. Either modification or alteration methods were intended to enhance the detection method of nucleic acid from samples observed. The basic pattern of hybridization process is the bound between targeted nucleic acid and labeled nucleic acid probe which are complementing each other in which one of it is bound to immobile matrix. The blotting process of nucleic acid was spelled out to Southern Blot, Northern Blot, Dot Blot, Slot Blot and in situ hybridization (Keller and Manak, 1999). The existence of nucleic acid was detected through appropriate labeling and probing process. The interaction between the probe and the target should be performed in the form of DNA-RNA or DNA-DNA (Farrel, 1993). By way of a strong and specific conjugation between the probe and the conjugate, then the detection process could be observed visually. Conjugate is also able to bind itself specifically with enzymes and moreover