Indonesian Journal of Biotechnology, June (2000) 423 - 432

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

Endang Herminajeng[1], Suprayogi Udayana[2] and Wihaskoro Sosroseno[3]

1) Department of Oral Medicine, Faculty of Dentistry, Gadjah Mada University, Yogyakarta, Indonesia
2) Department of Periodontology, Faculty of Dentistry, Gadjah Mada University, Yogyakarta, Indonesia
3) Department of Oral Biology, Faculty of Dentistry, Gadjah Mada University, Yogyakarta, Indonesia

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 tooth were then carried out. Sub-gingival plaque samples were obtained from the selected tooth 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 (r^2 = 0.7981), severity of gingival inflammation (r^2 = 0.6703), and gingival pocket depth (r^2 = 0.7787), but weakly with increased sub-gingival calculus (r^2 = 0.2659) and tooth mobility (r^2 = 0.4201) scores. The levels of this*

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 periodontopathic bacteria. It has been well documented that the course of CIPD is significantly associated with the specific types of periodontopathic 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; Kojima *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 (Sosroseno and Herminajeng, 1995; Page and Kornman, 1997; Sosroseno *et al.*, submitted). These abilities of *P. gingivalis* to induce the host immune response and tissue destruction suggest a crucial role of this periodontopathogen 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 periodontopathic and enteric bacteria have been produced and used to detect the presence of sub-gingival plaque *P. gingivalis* using flow-cytometric analysis and immunofluorescence screenings (Shelburn *et al.*, 1993; Kamiya *et al.*, 1994; Ni Eidhin 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 (Clerehugh *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 periodontal diseased subjects.

Material 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 without 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 subjects.

Dental plaque and calculus scores. The presence of supra- and infragingival plaque and calculus was assessed visually. One tooth with possible higher scores was selected. An assessment of dental plaque score at the selected tooth were then carried out by using 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 (no 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 a Loe and Silness's gingival index (Spolsky, 1990). At the selected tooth, gingival status was scored from 0 to 3 by following criteria; 0 = normal gingiva; 1 = mild inflammation with no gingival bleeding; 2 = moderate inflammation characterized by redness edema and bleeding on probing; 3 = severe inflammation characterized by redness, edema, 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 (SU) 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 = 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 scalier, 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 in sterile PBS and coated on the ELISA plates in triplicate (Nuncl, 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-streptavidine (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 H_2SO_4 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 dan 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, mean 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

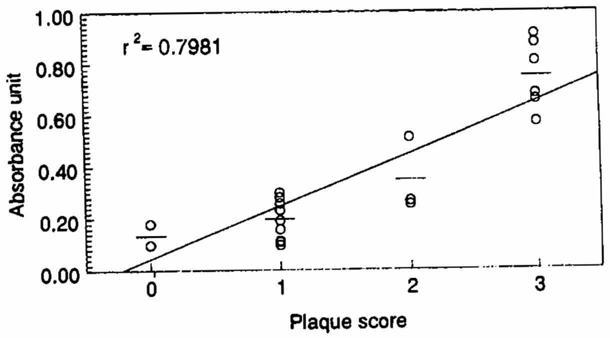

Figure 1. The association between dental plaque and sub-gingival plaque *P. gingivalis* levels. The degree of association (the value of r2) between both parameters is displayed on the left top. Dots and bars represent individual samples and mean of absorbance units, respectively. The levels of *P.gingivalis* are depicted as absorbance unit.

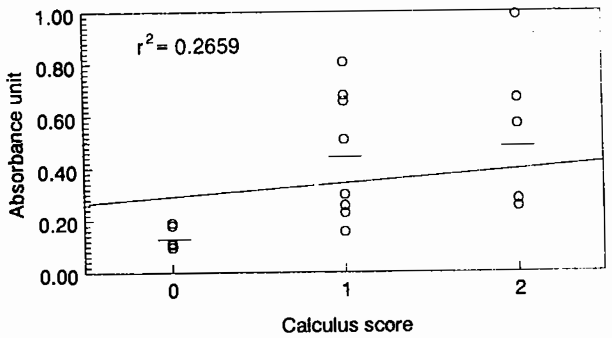

Figure 2. The association between sub-gingival calculus and the levels of subgingival plaque *P. gingivalis*. Notes are similar to Fig. 1.

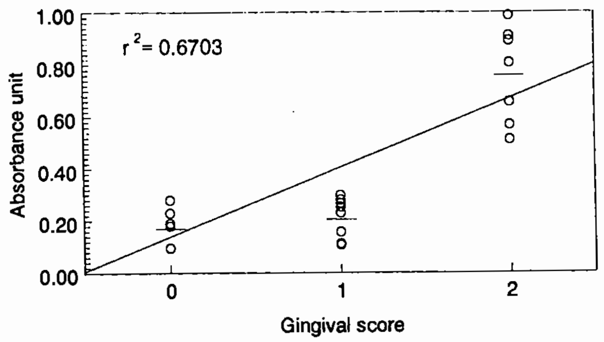

Figure 3. The association between gingival status and the levels of sub-gingival plaque *P.gingivalis*. Notes are similar to Fig. 1.

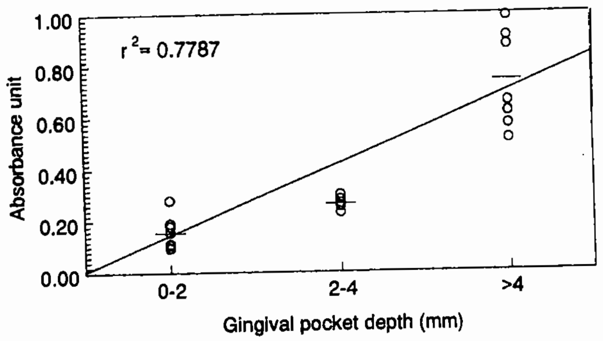

Figure 4. The association between gingival pocket depth and the levels of subgingival plaque *P. gingivalis*. Notes are similar to Fig. 1.

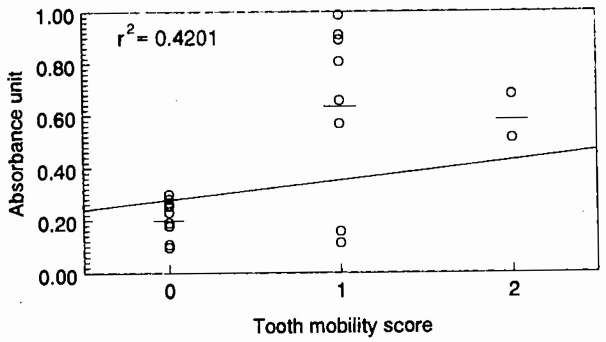

Figure 5. The association between tooth mobility and the levels of sub-gingival plaque *P.gingivalis*. Notes are similar to Fig.1.

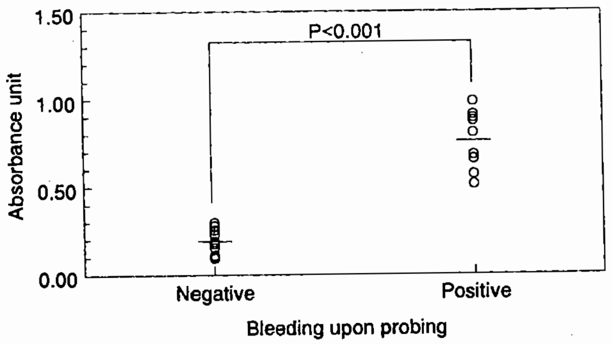

Figure 6. The levels of sub-gingival plaque *P.gingivalis* in subjects with negative or positive bleeding upon probing. Notes are similar to Fig. 1, except that the value of signihcant differences (p<0.001) is displayed.

that significant differences of means were only seen in the subjects graded 2 and 3 ($p<0.05$). Indeed, a multiple regression analysis between plaque scores and the values of AU showed a positive association (r^2 = 0.7981), suggesting that the higher grades of plaque may be present, the higher

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 (r^2 = 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 was 0.1797, 0.1995, 0.7629, respectively. No statistically differences could 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 sub-gingival plaque *P. gingivalis* (r^2 = 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 24 mm and >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 0.2614 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* (r^2 = 0.7787).

In the present study, tooth mobility was recorded due to the fact that it may exemplify tooth attachment loss and the course of CIPD (Staller and Laudenbach, 1980; Giargia 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 differences between that of the former 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 plaques is due to the fact that the time course of dental plaque maturation parallels with distinct colonized bacterial types and virulence. For examples, 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; Clerehugh *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 that of 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 salivary 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 Hammarstrom, 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 (Patters *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 subgingival 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 Kornmann, 1997). Furthermore, *P. gingivalis* possesses potent virulent factors such as LPS capable to inducing periodontal tissue destruction (Page and Kornmann, 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 (Giargia 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. If 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 bacterium, 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 alveolar 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 (Noiri *et al.*, 1997). In 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; Gemmed *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 lesional healing process, suggesting a protective role of antigen-specific antibodies in the *P. gingivalis*- induced dorsal 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 sub-gingival plaque is harbored by mixed types of virulent periodontopathic bacteria, such as *A. actinomycetemcomitans*, indicating that the development of CIPD is not merely induced by a single oral bacteria. Indeed, *A. actinomycetemcomitans*, predominantly detected in the advanced periodontal diseased sites, possesses pathogenic factors capable to stimulating host defenses and both periodontal hard and soft tissue destruction (Christersson *et al.*, 1992a; Kamma *et al.*, 1995; Fives-Taylor *et al.*, 1996). Hence, detection of all sub-gingival 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 sub-gingival plaque according to plaque maturation and extension (Wolff *et al.*, 1994, Page and Kornmann, 1997), suggesting that each clinical stage of CIPD is associated with specihc types of periodontopathogens. Detection of *P. gingivalis* in the sub-gingival 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 specific to *P. gingivalis*-derived LPS could differentiate the clinical stages of periodontal disease, i.e. early and advanced lesion (Clerehugh *et al.*, 1997; Herminajeng, *et al.*, 1999) highlights this contention.

In conclusion, the present study has demonstrated that increased levels of subgingival plaque *P. gingivalis* were 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 sub-gingival 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.

Acknowledgments

The authors gratefully thank to Prof. Greg Seymour and Dr. Phil Bird (Brisbane) for providing antigens and antibodies. Excellent technical assistance of Mrs. Eka is acknowledged. This work was partly supported by "Hibah Bersaing VI" from the Ministry of Education and Culture and "RUT III" from the Ministry of Research and Technology, the Indonesian government.

References

Baelum. V., Fejerskov, O. and Manji, F. (1988) Periodontal disease in adult Kenyans. *J. Clin. Periodontol.* 15: 445-452.

Bird, P.S., Gemmell, E., Polak, B., Paton, R.G., Sosroseno, W. and Seymour, G.J. (1995) Protective immunity to *Porphyromonas gingivalis* infection in a murine model. *J. Periodontol.* 66: 351-362.

Brown, C.M., Hancock, E.B., O'Leary, T.J., Miller, C.H. and Sheldrake, M.A. (1991) A microbiological comparison of young adults based on relative amounts of subgingival calculus. *J. Periodontol.* 62: 591-597.

Christersson, L.A., Fransson, C.L., Dunford, R.G. and Zambon, J.J. (1992a) Subgingival distribution of periodontal pathogenic microorganisms in adult periodontitis. *J. Periodontol.* 418-425.

Christersson, L.A., Grossi, S.G., Dunford, R.G., Machtel, E.E. and Genco, R.J. (1992b) Dental plaque and calculus: Risk indicators for their formation. *J. Dent. Res.* 71: 1425-1430.

Clerehugh, V., Seymour, G.J., Bird, P.S., Cullinan, M., Drucker, D.B. and Worthington, H.V. (1997) The detection of *Actinobacillus actinomycetemcomitans*, *Porphyromonas gingivalis* and *Prevotella intermedia* using an ELISA in an adolescent population with early periodontitis. *J. Clin. Periodontol.* 24: 5744.

Fives-Taylor, P., Meyer, D. and Mintz, K. (1996) Virulence factors of periodontopathogen *A. actinomycetemcomitans*. *J. Periodontol.* 67 (Suppl.): 291-297.

Frisskopp, J. and Hammarstrom, L. (1980) A comparative, scanning electron microscopic study of supragingival and subgingival calculus. *J. Periodontal* 51: 553-562.

Gemmell, E., Bird, P.S., Bowman, J.J.D., Xu, L., Polak, W., Walsh, L.J. and Sieymour, G.J. (1997) Immunohistological study of lesions induced by *Porphyromonas gingivalis* in a murine model. *Oral Microbiol. Immunol.* 12: 288-297

Giargia, M., and Lindhe, J. (1997) Tooth mobility and periodontal disease. *J. Clin. Periodontol.* 24:785-795.

Gmur, R. and Guggenheim, B. (1994) Interdental supragingival plaque - a natural habitat of *Actinobacillus actinomycetemcomitans*, *Bacteroides forsythus*, *Camplylobacter rectus*, and *Prevotella nigrescens*. *J. Dent. Res.* 73:1421-1428.

Herminajeng, E., Udayana, S. and Sosroseno, W. (1999) An ELISA-based detection of *Porphyromonas gingivalis* in the dental plaques of periodontal diseased patients. *I. J. Biotech.* December: 303-310.

Kamiya, I., Okuda, K. and Hara, K. (1994) Flow-cytometric identification and detection of *Porphyromonas gingivalis* by a LPS specific monoclonal antibody. *J. Periodontol.* 65: 309-315.

Kamma, J.J., Nakou, M. and Manti, F.A. (1995) Predominant microflora of severe moderate and minimal periodontal lesions in young adults with rapidly progressive periodontitis. *J. Periodont. Res.* 30: 66-72.

Kojima, T., Yasui, S. and Ishikawa, I. (1993) Distribution of *Porphyromonas gingivalis* in adult periodontitis patients. *J. Periodontol.* 64: 1231-1237.

Mombelli, A., McNabb, H., and Lang, N.P. (1991) Black-pigmenting gram negative bacteria in periodontal disease. I. Topographic distribution in the human dentition. *J. Periodont. Res.* 26: 301-307.

Ni Eidhin, D. and Mouton, C. (1994) The lipopolysaccharide of *Porphyromonas gingivalis* is not antigenically cross-reactive with that of other species. *J. Dent. Res.* 73: 661-670.

Noiri, Y., Ozaki, K., Nakae, H., Matsuo, T. and Ebisu S. (1997) An immunohistochemical study on the localization of *Porphyromonas gingivalis*, *Campylobacter rectus* and *Actinomyces viscosus* in human periodontal pocket. *J. Periodont. Res.*32: 598-607.

Page, R.C. and Kornmann, K., (eds). (1997) The pathogenesis of periodontitis. *Periodontology 2000.* 14:1 -248.

Patters, M.R., Landesberg, R. L., Johansson, L-A., Trummel, C.L. and Robertson, P. B. (1982) *Bactercides gingivalis* antigens and bone resorbing activity in root surface fractions of periodontally involved teeth. *J. Periodont. Res.* 17: 122-130.

Riviere, G. R., Smith, K.S., Tzagaroulaki, E., Kay, S. L., Zhu, X., DeRouen, T.A. and Adams, D.F. (1996) Periodontal status and detection frequency of bacteria at sites of periodontal health and gingivitis. *J. Penodontol.* 67: 109-115

Shelburn, C.E., Sandberg, G.P., Binsfeld, C.A., Wolff, L.F. and Curry, R.A. (1993)

Monoclonal antibodies to lipopolysaccharide of four oral bacteria associated with periodontal disease. *J. Periodont. Res.* 28: 1-9.

Sosroseno, W. and Herminajeng, W. (1995) The immunopathology of chronic inflammatory periodontal disease. *FEMS Immunol. Med. Microbiol.* 10: 171-180.

Spolsky, V. (1990) The epidemiology of gingival and periodontal disease. In *Glickman's clinical periodontology* (F.A. Carranza, ed.) pp.302-329. W.B. Saunders, Philadelphia

Stoller, N.H. and Laudenbach, K.W. (1980) Clinical standardization of horizontal tooth mobility. *J. Clin. Periodontol.* 7: 242-250.

White, D.J. (1997) Dental calculus: recent insights into occurrence, formation, prevention, removal, prevention, removal and oral health effects of supragingival and subgingival deposits. *Eur. J. Oral. Sci.* 105: 508-522.

Wolff, L.F., Liljemark, W.F., Pihistrom, B.L., Schaffer, E.M., Aeppli, D.M. and Bandt, C.L. (1988) Dark-pigmented *Bacteroides* species in subgingival plaque of adult patients on rigorous recall program. *J. Periodont. Res.* 23: 170-174.

Wolff, L., Dahlen, G. and Aeppli, D. (1994) Bacteria as risk markers for periodontitis. *J. Periodontol.* 64: 498-510.

Zambon, J.J., Reynolds, H.S., Chen, P. and Genco, R.J. (1985) Rapid identification of periodontal pathogens in subgingival dental plaque. *J. Periodontol.* 56 (Suppl): 32-40.