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Pathogenic Capability of *Prevotella intermedia* Recombinant Clone Expression in *Escherichia coli*

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Abstract: The study was undertaken with the aims of identifying *Prevotella intermedia* virulent strains from adult periodontitis and to detemine its virulent gene via molecular analysis. The clinical isolates were assessed for their potential and ability to produce toxin and form skin lesion in balb/c mice. Infected mice appeared cachectic and the histological effect of the skin lesion showed that all lesions were localized at the injection site and causes tissue damage with skin necrosis and hair loss. *Prevotella intermedia* UMD 5 was the most virulent isolate and thus was selected for shotgun cloning. Partially digested genomic DNA (2-9 kb) from *P. intermedia* UMD 5 was cloned into the *Bam* HI site of *E. coli* pUC 18. The inserted gene for all recombinant clones was expressed in balb/c mice. It was found that only one clone was able to induce localized skin lesion in mice. The cloned gene expressed well by inducing skin lesion in mice when incubated in aerobic environment, but the expression was better in combination of aerobic followed with anaerobic environment, producing bigger skin lesion area in balb/c mice. Thus, the presence or absence of O_2 did not suppress the level of toxin production for anaerobic bacterial gene cloned in the aerobic system. Histological effect of the skin lesion showed that lesions were localized at the injection site and causes tissue damage with skin necrosis and hair loss. In addition, the recombinant plasmid was found to be stable in the host system. The clone was further analyzed via restriction analysis and was found to have a DNA insert of 4 kb.

Key words: *Prevotella intermedia*, pathogenic, periodontitis, recombinant clone DNA

INTRODUCTION

Adult periodontitis is a global public health oral disease caused by subgingival plaque microflora which causes the inflammation of the gingival tissues, that could lead to tooth loss (Kilian, 1981; Zambon, 1996; Van der Weijden *et al*., 1994). One of the most important periodontopathic bacteria is *Prevotella intermedia* and many studies have suggested a strong correlation of the presence of this bacteria towards the development and progression of periodontal disease (Wennstrom, *et al*., 1987; Slots *et al*., 1986; Himratul-Aznita *et al*., 2005; Himratul-Aznita and Ansary, 2006). *Prevotella intermedia* is a gram negative rod anaerobe that is involved in the pathogenesis of periodontal disease and is highly prevalence in periodontal pockets (Slots, 1981). The virulent properties of *P. intermedia* is important in the pathogenicity of periodontal disease (Himratul-Aznita *et al*., 2005; Himratul-Aznita and Ansary, 2006) and thus, this study was undertaken with the aims of identifying *Prevotella intermedia* virulent strains from adult periodontitis and to detemine its virulent gene via molecular analysis.

MATERIALS AND METHODS

Bacterial strains and culture conditions: All *Prevotella intermedia* strains were of previous research collection obtained from deep periodontal pocket of 5 mm or more from adult periodontitis patients. All *P. intermedia* strains were revived from -80°C storage and thawed to room temperature before being grown on enriched tryptic soy agar supplemented with 5 μl mL^{-1} hemin, 0.5 μl mL^{-1} menadione and 5% defibrinated blood and incubated in anaerobic jars placed in the 37°C incubator for up to 14 days.

Virulence analysis: *Prevotella intermedia* isolates were tested for invasiveness in a mouse model as previously described by Neiders *et al*. (1989). Balb/c mice, aged

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between 8-12 weeks old were provided by The Central Animal House, Faculty of Medicine, University of Malaya for use in this study, under the ethical code of (BM/09/04/03/WHA(R)). Strains were grown for 18 h using media and anaerobic environment as described above. The cells were then centrifuged and washed twice in sterile phosphate buffered saline (0.147M NaCl, 0.01M sodium phosphate) before being counted in a Petroff-Hausser chamber. 0.1 mL of 10^{12} cells mL^{-1} bacterial suspensions was injected subcutaneously in balb/c mice at 2 different locations on the dorsal surface. Each ulceration lesions were evaluated based on the presence of lesion, size, consistency and location of the developed skin lesion. Daily assessment on the mice general health status was carried out. Six mice per group were used for every test, in order to confirm the accuracy including the reproducibility of the experiment.

Histological procedures: Biopsied skin lesion was fixed in 10% formalin at room temperature and left overnight. Following that, the excised skin lesion was embedded in paraffin. Sectioned of 5 μm thick was carried out and haematoxylin and eosin stains were used as the staining reagent.

Shotgun cloning: The total genomic DNA of *Prevotella intermedia* was partially digested using *Sau* 3A restriction enzyme to generate fragments of 2-10 kb. Following this, the DNA fragments were ligated with vector pUC 18 and transformed in aerobic host system of *E. coli* JM 101.

Recombinant clones oxygen requirement: Two sets of recombinant clones were grown in Brain Heart Infusion (BHI) broth and the first set (Set A) was incubated for 24 h in oxygen at 37°C and the second set (Set B) was incubated for the first 12 h in aerobic condition with oxygen supply and the next 12 h in anaerobic condition, without oxygen at 37°C, before being tested for virulence in mice. The gene expression was then determined via skin lesion in balb/c mice. Both sets (A and B) of the same recombinant clone were used to inject balb/c mice independently with 0.1 mL of 10^{12}cells mL^{-1} subcutaneously into two dorsal sites of male balb/c mice for lesion development.

Plasmid stability test: The plasmid stability test was carried out following the modified method of Lanka and Barth (1981). The recombinant clone was checked for the presence of the cloned plasmid by using the Genispin Plasmid Miniprep Extraction Kit, BST Techlab. The clone was then subcultured following the same protocol as above and the same steps were carried out.

RESULTS AND DISCUSSION

Toxicity activity of *Prevotella intermedia* were observed from subcutaneous injection on balb/c mice with 10^{12} cells mL^{-1} of *P. intermedia* which resulted with skin lesion. Balb/c mice developed various sizes and severity of skin lesion from mild to severe necrosis with scab-encrusted. In addition, some infected mice suffered hair loss and were cachexic which is in agreement with other reported results (Van Steenbergen *et al*., 1982; Kastelein *et al*., 1981; Sundqvist *et al*., 1979; McKee *et al*., 1986) by many scientist elsewhere. The hair loss might be caused by the inflammation of hair follicles that were involved in the inflammatory process.

All strains produced localized lesion and was confirmed by histology analysis. In addition, the localized lesion that developed at the injection sites was non-invasive. However, some strains had lesion which completely destroy the epidermis layer. Heavy infiltration of leukocytes was also observed due to the skin inflammation.

Following the mouse pathogenicity studies, strain UMD 5 was found to be the most virulent strain and thus was used for further analysis. Partially digested genomic DNA with fragments ranging from 2-9 kb from *P. intermedia* UMD 5 was shotgun cloned into the *Bam* HI site of *E. coli* pUC 18. The resulting recombinant molecules were introduced into *E. coli* JM101 by transformation. Results showed that 51 recombinant clones have plasmid DNA ranging from 0.5-6.1 kb. All recombinant clones were tested for their expression in balb/c mice, but only one recombinant cloned expressed the inserted gene successfully. It was found that the cloned gene expressed well by inducing skin lesion in mice when incubated in aerobic environment but the expression was better when the clone was incubated in combination of aerobic followed with anaerobic environment, producing bigger skin lesion area in balb/c mice. A similar scenario was reported by Borgeau *et al.* (1992) on protease gene expression of anaerobic *P. gingivalis* which has also been cloned in *E. coli* system. Thus, it is concluded that the presence or absence of oxygen did not suppress the level of toxin production of *P. intermedia* clone. In addition, toxic activity was increased when incubated in combination of both environments. Incubation in aerobic environment is able to express the inserted gene of *P. intermedia*, however, with prolonged exposure to oxygen, it has been observed to reduce the toxic activity of the recombinant clone.

The recombinant clone from both incubation conditions was then subjected to plasmid stability testing to ensure the recombinant plasmid, which was cloned in

E. coli is stably maintained and no detectable plasmid loss was found. Thus, this concludes that the cloned plasmid from the anaerobic *P. intermedia* was stably maintained and is well express in *E. coli* host whether being incubated with or without oxygen.

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