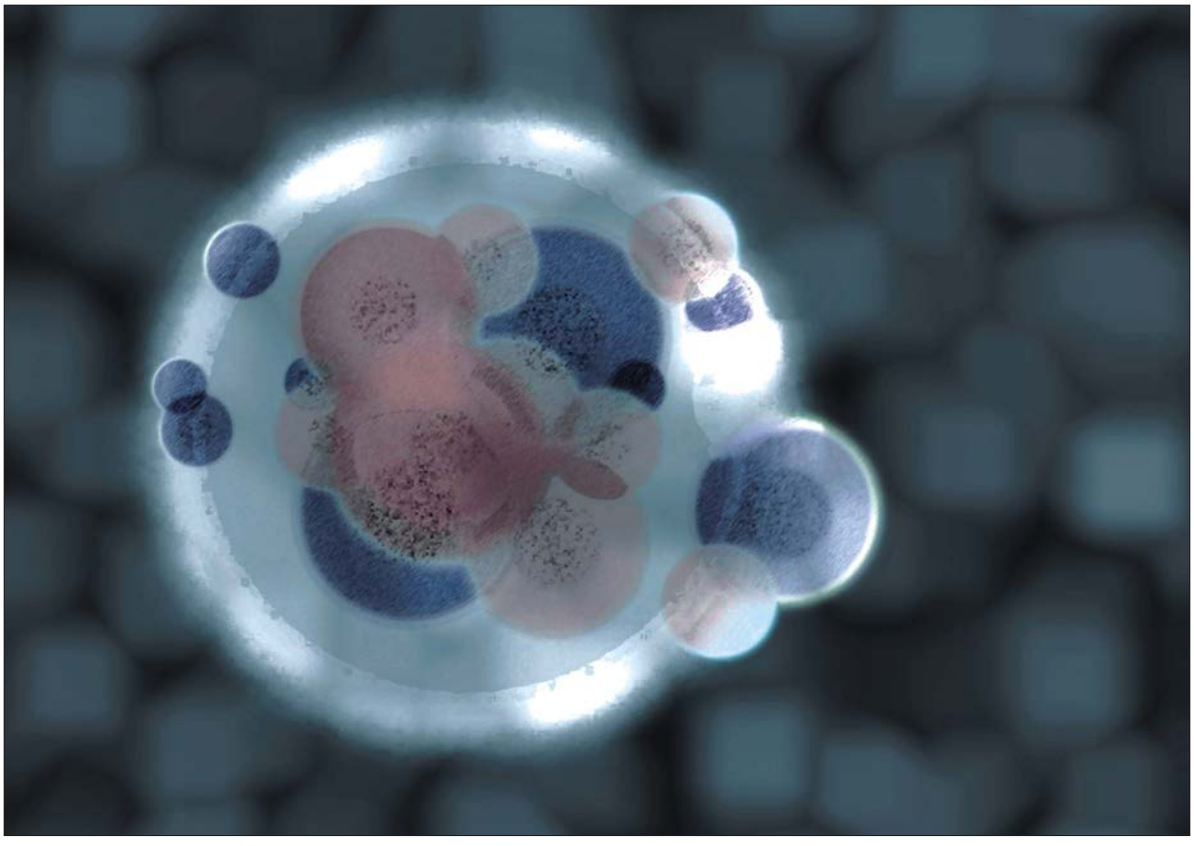

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Effects of Subcutaneous Injection in Mice with Oral *Prevotella intermedia* Clinical Isolates in Malaysia

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Abstract: The study was undertaken with the aims of identifying strains providing virulent toxin and to observe the histological effect of the toxin in mice. Plaque samples were collected from adult periodontitis patients. *P. intermedia* were recovered from subgingival periodontal pocket with depths of 5 mm or greater. Ninety *P. intermedia* isolates were identified based on its bacteriological properties, gram staining and biochemical characteristics. The clinical isolates of *P. intermedia* were assessed for their potential and ability to produce toxin and form skin lesion in balb/c mice. 10^8, 10^{10} and 10^{12} cells mL^{-1} of bacterial suspension were used in the study. No lesion was observed in mice injected with 10^8 cells mL^{-1} and only three *P. intermedia* with concentration 10^{10} cells mL^{-1} were able to induce localized skin lesion in balb/c mice. However, all isolates causes balb/c mice to develop localized skin lesion when 10^{12} cells mL^{-1} was used. Infected mice appeared cachectic and the histological effect of the skin lesion showed that all lesions were localised at the injection site and causes tissue damage with skin necrosis and hair loss.

Key words: *Prevotella intermedia*, mice, skin lesion, hair loss

INTRODUCTION

The most common chronic infectious periodontal disease in human is adult periodontitis. It is found worldwide and is a global public health problem including in Malaysia. Several studies suggested that the major forms of periodontal destruction are associated with subgingival plaque microflora, which include the black pigmented anaerobe bacteria that have been indicated to possess a variety of virulence factors. *Prevotella intermedia* is a pleomorphic rod Gram-negative anaerobic microorganism and is generally accepted as one of the most important putative periodontopathic organisms[1-3]. Recent clinical studies have suggested that there is a strong correlation between the presence of *P. intermedia* and the development of periodontal disease[4-7]. However, its role in the etiology of periodontitis infections is still unclear. Thus, the use of this species for further studies by virtue of their virulent properties can become a useful model to elucidate some of the subtle processes involved in the pathogenicity of periodontal disease. Numerous investigators have been using mouse model to examine the virulence factors of other periodontopathic microorganisms in pathogenesis by subcutaneous infection[8-13]. The animal models currently used were models in which the specific clinical features of periodontal diseases can be demonstrated or those in which the growth of specific etiological agents can be assessed[8]. Perhaps the most widely used animal model to examine pathogenicity of oral black-pigmented organisms is the mouse[9]. Balb/c mice had been reported to be a good mouse model to study the virulence of various periodontopathic microorganisms[10-12]. Thus the study was undertaken with the aims of identifying strains producing virulent toxin and to observe the histological effect of the toxin in mice.

MATERIALS AND METHODS

Bacterial strains and culture conditions: Subgingival plaque samples were obtained from 40 patients diagnosed as suffering from adult periodontitis. Subgingival plaque samples from periodontal sites with signs of inflammation and had probing depths of 5 mm or greater were taken.

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Samples from these sites were obtained by introducing two sterile paper points subgingivally and left for 10 sec after which they were transferred into sterile tubes containing pre-reduced reduced transport fluid with glassbeads. Aggregated microbes were dispersed by vigorous vortexing and plated onto 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. All microbiological manipulations were performed on the open bench with a total time of less than one hour from sample collection to culture incubation.

Within 14 days of incubation, colonies showing brown or black pigmentation were selected for study. All colonies showing various different morphological structures, including several identical colonies were picked from the same isolation plate. Five to ten colonies were picked for identification when numerous black-pigmenting colonies were present on a culture plate. Gram staining, API 20A and BIOLOG Identification System were used to identify *P. intermedia* strains.

In addition to the clinical isolates, *Porphyromonas gingivalis* ATCC 33277 was used as the positive control in all studies.

Virulence analysis: *Prevotella intermedia* isolates were tested for invasiveness in a mouse model as previously described by Neiders *et al.*[11]. Balb/c mice, aged between 8 to 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. Then, the cells were centrifuged, washed twice in sterile phosphate buffered saline (0.147 M NaCl, 0.01 M sodium phosphate) and counted in a Petroff-Hausser chamber. Purity of the suspensions were checked by Gram staining. Mice were then challenged with subcutaneous injections of 0.1 mL of 10^8, 10^{10} and 10^{12} bacterial suspensions at two sites about 1 cm lateral on the dorsal surface. The presence, size, consistency and location of each ulceration lesions were evaluated. Mice were also examined daily to assess their general health status. Six mice were used for every strain tested, in order to confirm the accuracy including the reproducibility of the experiment.

Microbial identification of skin lesion: Sterile cotton swabs were immersed into sterile normal saline and used to swab the lesion areas of all infected mice. Swabs were streaked onto enriched tryptic soy agar supplemented with 5 μL mL^{-1} hemin, 0.5 μL mL^{-1} menadione and 5% defibrinated blood and incubated anaerobically.

Histological procedures: Skin lesions were biopsied and fixed in 10% formalin at room temperature overnight. After fixation, the excised skin lesion were embedded in paraffin before being sectioned into 5 μm thick and stained using haematoxylin and eosin stains.

RESULTS

From 40 adult periodontitis patients sampled, black-pigmented anaerobic bacteria were recovered from 33 patients and another seven patients did not have any growth of black-pigmented bacteria even after ten days of incubation. All black-pigmented colonies obtained produced smooth shiny colonies with diameter between 2 to 4 mm. Pigmentation began about the third day and darkened progressively which finally became black in 10 to 14 days. Colonies were randomly isolated and stored for identification as described above. A total of ninety Gram-negative pleomorphic rods of *P. intermedia* isolates were obtained.

To develop a virulence analysis for *P. intermedia* strains in the study, we used the model of the experimental infection in mice, described for oral *Bacteroides* strains[12]. The toxicity activity from bacterial cell culture was observed as abscesses on the skin of the injected mice.

It was found that injection with 10^8 cells mL^{-1} failed to produce any infection when none of the injected mice developed abscess or lesion or swelling up to day 10. On the other hand, mice injected with 10^{10} cells mL^{-1} of *P. intermedia* started to produce very small lesions which appeared about 10 days after infection for three strains, labeled as PI5, PI16 and PI46 (Table 1). However, with 10^{12} cells mL^{-1} of all *P. intermedia* strains injected subcutaneously, the balb/c mice started to develop lesion as early as day 3 onwards. For all mice injected with different *P. intermedia* strains, lesion varied from small to large area of mild to severe necrosis with scab-encrusted and hair loss that occur on the skin over the abscess. In addition, all infected mice showed signs of mild to moderate cachexia with ruffled hair. Cultivation of the lesions from all infected mice yielded pure cultures of *P. intermedia*. As there was no mortality in any of the mice after the injection, all infected mice were euthanized and killed prior to skin biopsy for histology slide preparation.

A statistical analysis was performed with Fisher's exact test, which analyzes a 2×2 contingency table using two-tailed p-values[14]. The bacterial concentration of 10^{12}

Table 1 : Virulence in *Prevotella intermedia* bacterial cells via mice injection at 10^8, 10^{10} and 10^{12} cells mL^{-1}

Conc. used to inject the mice	Days observed	Tested samples	Mice (lesion) / mice (injected) 6/6	5/6	4/6	3/6	2/6	1/6	0/6	Total number tested samples
10^8	Day 3	*P. intermedia*	-	-	-	-	-	-	90	90
		ATCC 33277	-	-	-	-	-	-	1	1
		Neg. controls	-	-	-	-	-	-	2	2
	Day 5	*P. intermedia*	-	-	-	-	-	-	90	90
		ATCC 33277	-	-	-	-	-	-	1	1
		Neg. controls	-	-	-	-	-	-	2	2
	Day 10	*P. intermedia*	-	-	-	-	-	-	90	90
		ATCC 33277	-	-	-	-	-	-	1	1
		Neg. controls	-	-	-	-	-	-	2	2
10^{10}	Day 3	*P. intermedia*	-	-	-	-	-	-	90	90
		ATCC 33277	-	-	-	-	-	-	1	1
		Neg. controls	-	-	-	-	-	-	2	2
	Day 5	*P. intermedia*	-	-	-	-	-	-	90	90
		ATCC 33277	-	-	-	-	-	-	1	1
		Neg. controls	-	-	-	-	-	-	2	2
	Day 10	*P. intermedia*	-	-	-	-	-	3	87	90
		ATCC 33277	-	-	-	-	-	1	-	1
		Neg. controls	-	-	-	-	-	-	2	2
10^{12}	Day 3	*P. intermedia*	-	2	-	18	30	28	12	90
		ATCC 33277	-	1	-	-	-	-	-	1
		Neg. controls	-	-	-	-	-	-	2	2
	Day 5	*P. intermedia*	1	4	24	29	23	9	-	90
		ATCC 33277	1	-	-	-	-	-	-	1
		Neg. controls	-	-	-	-	-	-	2	2
	Day 10	*P. intermedia*	1	32	35	20	2	-	-	90
		ATCC 33277	1	-	-	-	-	-	-	1
		Neg. controls	-	-	-	-	-	-	2	2

Prevotella intermedia: 90 clinical isolated strains, Positive control: ATCC 33277, Negative controls: PBS, saline

cells mL^{-1} of *P. intermedia* for infection was more significant in causing skin lesion in mice with the two-tailed value which is less than 0.0001 compared to concentration of 10^{10} cells mL^{-1} or 10^8 cells mL^{-1} of *P. intermedia*.

Results from histological analysis revealed that all strains are non-invasive as localized lesions developed at the site of injection. Skin necrosis was also observed which led to sloughing and scab formation, with some strains causing the lesion areas to have complete destruction of the epidermis layer. The localized lesions caused by all *P. intermedia* strains had very heavy infiltration of leukocytes which was walled-off by a connective tissue capsule.

DISCUSSION

Identification of *P. intermedia* species in the oral cavity has been carried out mainly on the basis of colony morphology and biochemical reaction profile using commercialized identification kit. These tests had confirmed all 90 black-pigmented clinical isolates were *P. intermedia*. The environmental conditions and the greater anaerobiosis within the deep periodontal pockets favour the colonization of *P. intermedia*. In this study, all subjects with detectable periodontopathic bacteria were demonstrated to have primarily a single species of black-pigmented organism. However, the incidence of mixed black-pigmented populations was not found in this study, which may be due to the fact that only five black-pigmented colonies from each specimen were speciated, so less prevalent species in our population may have been missed or if a significant proportion of organisms failed to survive during transit to the laboratory and became undetectable. Thus, generally we may have only detected the dominant species at the sampling site.

In this study, the response to develop skin lesion on balb/c mice was dose dependent, in agreement with those of Steenberg *et al.*[12], Kastelein *et al.*[13], Sundqvist *et al.*[15] and Takazoe *et al.*[16].

Subcutaneous injection with *P. intermedia* strains on the dorsal site of balb/c mice induced inflammation when examined histologically, a finding similar to other reported results[11,12,15,17]. In the present study, all *P. intermedia* were non-invasive as the localized lesions developed at the infection site. In 1979, Sundqvist *et al.*[15] reported a similar infection capability, which causes localized skin lesion on mice for a strain of *P. intermedia*[12]. Lesion size varied from small areas to coagulative necrotizing lesion of the epidermis layer in mice receiving high doses. Histological studies on these mice showed that there was accumulation of masses of leukocytes in the dermis layer. The dilation of blood vessels in the area of the inflammation increases blood circulation, allowing increased numbers of phagocytic

inflammatory cells to reach the affected area. Polymorphonuclear Neutrophils (PMN), the most abundant phagocytic cells in blood exhibit chemotaxis and were attracted to the invading microorganisms which will be phagocytized and digested. In this study all infected mice suffered cachexia with ruffled hair and occasionally with hair loss for some of the severely infected mice. This may be caused by inflamed follicles, which were involved in the inflammatory process. Thus, this indicates that all *Prevotella intermedia* isolates in this study are pathogenic and could possibly contribute in causing periodontal disease.

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REFERENCES

1. Cookson, A.L., A. Wray, P.S. Handley and A.E. Jacob, 1996. An investigation into the use of SDS-PAGE of cell surface extracts and proteolytic activity to differentiate *Prevotella nigrescens* and *Prevotella intermedia*. FEMS Microbiol. Lett., 136: 109-115.
2. Gharbia, S.E., M. Haapasalo, H.N. Shah, A. Kotiranta, K. Lounatmaa, M.A. Pearce and D.A. Devine, 1994. Characterization of *Prevotella intermedia* and *Prevotella nigrescens* isolates from periodontic and endodontic infections. J. Periodontol., 65: 56-61.
3. Dahlén, G.M., S. Wikstrom, S. Renvert, R. Gmur and B. Guggenheim, 1990. Biochemical and serological characterization of *Bacteroides intermedius* strains isolated from the deep periodontal pocket. J. Clin. Microbiol., 28: 2269-2274.
4. Van der Weijden, M., F. Timmerman, E. Reijerse, G.N. Wolffe, A.J. van Winkelhoff and U. van de Velden, 1994. The prevalence of *A. actinomycetemcomitans*, *P. gingivalis* and *P. intermedia* in selected subjects with periodontitis. J. Clin. Periodontol., 21: 583-588.
5. Wennstrom, J.L., G. Dahlén, J. Svensson and S. Nyman, 1987. *Actinobacillus actinomy cetemcomitans*, *Bacteroides gingivalis* and *Bacteroides intermedius*: Predictors of attachment loss. Oral Microbiol. Immunol., 2: 158-163.
6. Slots, J., L. Bragd, M. Wikström and G. Dahlén, 1986. The occurrence of *Actinobacillus actinomycetemcomitans*, *Bacteroides gingivalis* and *Bacteroides intermedius* in adults. J. Clin. Periodontol., 13: 570-577.
7. Zambon, J.J., H.S. Reynolds and J. Slots, 1981. Black-pigmented *Bacteroides* spp. in the human oral cavity. Infect. Immun., 32: 198-203.
8. Genco, C.A., C.W. Cutler, D. Kapczynski, K. Maloney and R.R. Arnold, 1991. A novel mouse model to study the virulence of the host response to *Porphyromonas* (*Bacteroides*) *gingivalis*. Infect. Immun., 59: 1255-1263.
9. Holt, S.C., L. Kesavalu, S. Walker and C.A. Genco, 1999. Virulence factors of *Porphyromonas gingivalis*. Periodontology, 20: 168-238.
10. Katz, J., D.C. Ward and S.M. Michalek, 1996. Effects of host responses on the pathogenicity of strains of *Porphyromonas gingivalis*. Oral Microbiol. Immunol., 5: 309-318.
11. Neiders, M.E., P.B. Chen, H. Suido, H.S. Reynolds, J.J. Zambon, M. Shlossman and R.J. Genco, 1989. Heterogeneity of virulence among strains of *Bacteroides gingivalis*. J. Periodont. Res., 24: 192-198.
12. Van Steenbergen, T.J.M., P. Kastelein, J.J.A. Touw and J. de Graff, 1982. Virulence of black-pigmented *Bacteroides* strains from periodontal pockets and other sites in experimentally induced skin lesions in mice. J. Periodont. Res., 17: 41-49.
13. Kastelein, P., T.J.M. van Steenbergen, J.M. Bras and J. de Graff, 1981. An experimentally induced phlegmonous abscess by a strain of *Bacteroides gingivalis* in guinea pigs and mice. Antonie van Leeuwenhoek, 47: 1-9.
14. GraphPad QuickCalcs Program, GraphPad Software, Inc. (http://www.graphpad.com/quickcalcs/)
15. Sundqvist, G., M.I. Eckerbom, A.P. Larsson and U.T. Sjören, 1979. Capacity of anaerobic bacteria from necrotic pulps to induce purulent infections. Infect. Immun., 25: 685-693.
16. Takazoe, I., M. Tanaka and T. Homma, 1971. A pathogenic strain of *Bacteroides melaninogenicus*. Arch. Oral Biol., 16 : 817-822.
17. McKee, A.S., A.S. McDermid, A. Baskerville, A.B. Dowsett, D.C. Ellwood and P.D. Marsh, 1986. Effect of hemin on the physiology and virulence of *Bacteroides gingivalis* W50. Infect. Immun., 52: 349-355.