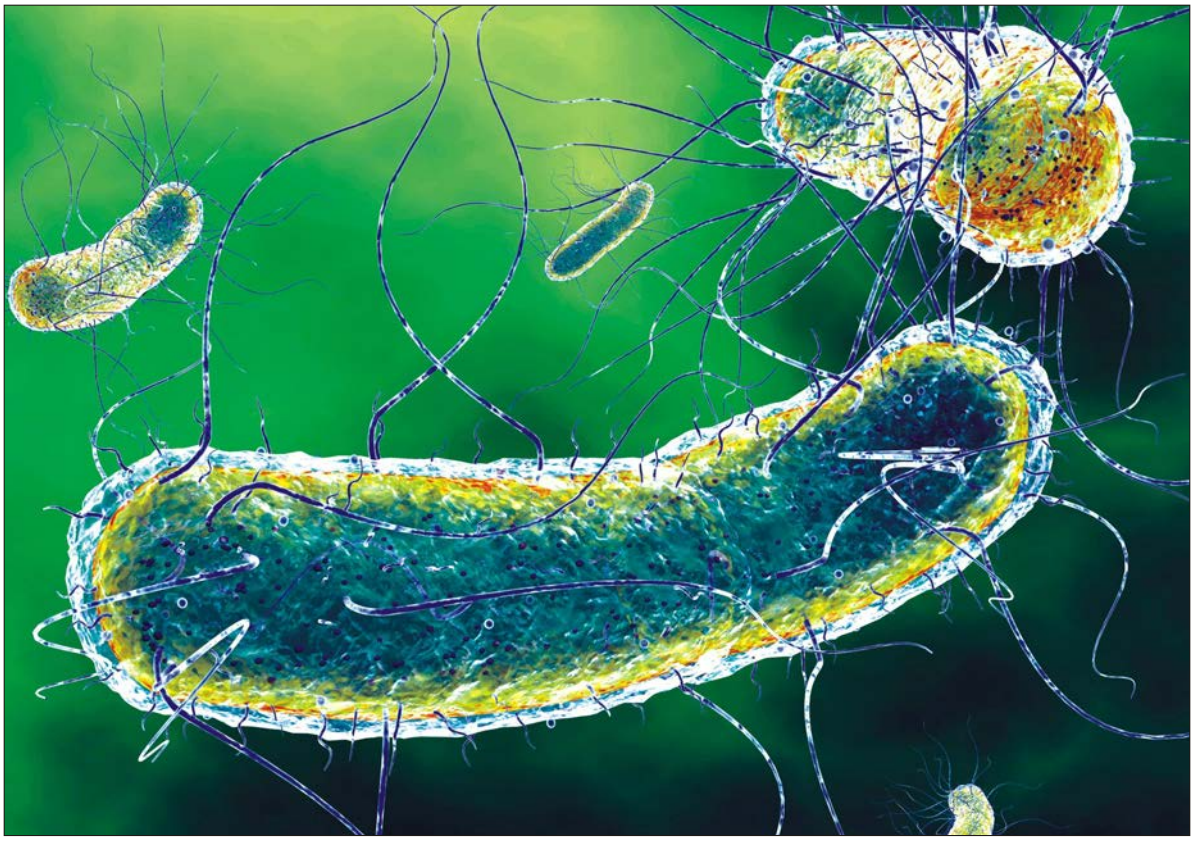

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Pathogenicity of *Porphyromonas asaccharolytica* in Adult Periodontitis of Malaysian Samples

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Abstract: Bacterial toxin of *Porphyromonas asaccharolytica* strains obtained from adult periodontitis were analysed using mouse model. Results from subcutaneous injection with whole bacterial cells of 10^8 and 10^{10} cells mL^{-1} failed to induce any infection. However when bacterial concentration of 10^{12} cells mL^{-1} was used, all *P. asaccharolytica* strains were able to induced the development of localized lesion on balb/c mice. In addition, infected mice appeared cachectic with ruffled hair. Thus, infection with the latter concentration was pathogenic and was dose dependent. The toxin was also found to be heat labile for all strains. Therefore, we conclude that the extracellularly toxin produced by *P. asaccharolytica* of our local isolates which were obtained from adult periodontitis, is virulent and heat labile.

Key words: *Porphyromonas*, *Porphyromonas asaccharolytica*, skin lesion, mice, toxin

INTRODUCTION

Inflammatory reaction due to response to the penetration of bacterial products at the subgingival areas in the periodontal pockets of teeth leads to periodontal disease. Infected individuals may suffer tooth loss and require life-long review. As a result of various improved methods for isolation and classification of oral microorganisms, more groups of bacteria have been shown to be associated with periodontal disease. Microorganisms that are commonly associated with periodontitis are *Porphyromonas* species, *Prevotella* sp., *Bacteroides* sp. Fusobacterium sp. and *Actinobacillus* sp. (Dahlèn and Leonhardt, 2006).

Porphyromonas gingivalis is a black-pigmented gram-negative anaerobe and has been found to have virulence factors that can directly affect the periodontium (Fletcher *et al*., 1994, 1995). Another species in the same genus of Porphyromonas is *Porphyromonas asaccharolytica*. This species can be isolated from a variety of clinical specimens but it is commonly found in urogenital and intestinal tracts (Jousimies-Somer *et al*., 2003). *P. asaccharolytica* showed black pigmented colonies on blood agar and is a Gram negative rod anaerobic bacteria. It is highly resistant to beta lactams antibiotics (Morizano *et al*., 2005). *P. asaccharolytica* has also been reported to have collagenase and trypsin-like activities which is similar to the properties of *P. gingivalis* (http://www.gideononlin.com). However, until to date the contribution and pathogenesis of *P. asaccharolytica* in oral diseases, especially periodontitis is still remain unknown. Thus, the study was undertaken with the aim of identifying *P. asaccharolytica* virulent toxin to analyze its possible contribution to pathogenicity *in vitro*.

MATERIALS AND METHODS

Bacterial Strains and Culture Conditions

All *Porphyromonas asaccharolytica* strains were of previous research collection obtained in year 2005 in University of Malaya, Kuala Lumpur, Malaysia. These samples were collected from deep

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periodontal pocket of 5 mm or more from adult periodontitis patients. All 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. As *Porphyromonas assacharolytica* is anaerobic organism incubation was carried out in anaerobic jars placed in the 37°C incubator for up to 14 days.

Porphyromonas gingivalis ATCC 33277 was used as the positive control whereas PBS (phosphate buffered saline) and saline were used as negative controls in all studies.

Toxin Pathogenicity Analysis

Porphyromonas asaccharolytica isolates were tested for invasiveness in a mouse model as previously described by Neiders *et al*. (1989). 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. Mice were challenged with subcutaneous injections of 0.1 mL of 10^8, 10^{10} and 10^{12} bacterial suspensions at 2 sites 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. A number of 6 mice were used for every strain tested, in order to confirm the accuracy including the reproducibility of the experiment.

Preparation of Unsonicated Bacterial Suspension

All strains were grown overnight at 37°C for 48 h in pre-reduced enriched trypticase yeast extract broth supplemented with 5 μl mL^{-1} hemin and 0.5 μl mL^{-1} menadione. Incubation was carried out under standard anaerobic condition (80%N_2, 10%H_2, 10%CO_2). The cells were sedimented by centrifugation at 12000 rpm for 20 min at 4°C, after which 0.1, 0.2 and 0.3 mL of the unsonicated cell-free filtrate supernatant prepared from each of these isolates were used to inject the mice subcutaneously at 2 dorsal sites of the balb/c mice. Positive and negative controls were always used in the mouse pathogenicity study to ensure results accuracy and to avoid biases throughout the experiments.

Preparation of Sonicated Bacterial Suspension

All strains were grown overnight at 37°C for 48 h in pre-reduced enriched trypticase yeast extract broth supplemented with 5 μl mL^{-1} hemin and 0.5 μl mL^{-1} menadione. Incubation was carried out under standard anaerobic condition (80%N_2, 10%H_2, 10%CO_2).

For preparation of sonicated bacterial suspension, the bacterial cells were washed and resuspended in 1.0 mL 0.85% normal saline and sonicated for 30 sec. The sonication was repeated 3 times after which the suspension was centrifuged and the sonicated pellet was discarded. The supernatant fluid was filtered through a 0.22 μm membrane and retained. 0.1, 0.2 and 0.3 mL of the sonicated cell-free filtrate supernatant prepared from each of these isolates were used to inject the mice subcutaneously at 2 sites about 1 cm lateral on the dorsal surface of the balb/c mice.

Temperature Lability of *Porphyromonas asacharolytica* Toxin

Bacterial cell-free supernatants were heated to 37 and 60°C for 60 and 15 min, respectively. Toxic activity was then tested as described above, with 0.2 mL filtrates injected subcutaneously at two dorsal sites of the balb/c mice after the heat treatment.

RESULTS AND DISCUSSION

Porphyromonas asaccharolytica labelled as Pa1, Pa2, Pa3 and Pa4 were used to study the bacterial toxin pathogenicity, which cause skin lesion in mice. This study was carried out to determine

Table 1: Virulence in *Porphyromonas asaccharolytica* bacterial cells via mice injection at 10^8, 10^{10} and 10^{12} cells mL^{-1} on day 7

Conc. used to inject the mice	Tested samples	Lesion diameter sizes (mm)	Mice health status
10^8	*P. asaccharolytica* Pa1	-	Healthy
	P. asaccharolytica Pa2	-	Healthy
	P. asaccharolytica Pa3	-	Healthy
	P. asaccharolytica Pa4	-	Healthy
	ATCC 33277	-	Healthy
	Neg. controls	-	Healthy
10^{10}	*P. asaccharolytica* Pa1	-	Healthy
	P. asaccharolytica Pa2	-	Healthy
	P. asaccharolytica Pa3	-	Healthy
	P. asaccharolytica Pa4	-	Healthy
	ATCC 33277	-	Healthy
	Neg. controls	-	Healthy
10^{12}	*P. asaccharolytica* Pa1	3.5-4.2 mm	Cachectic
	P. asaccharolytica Pa2	3.0-4.7 mm	Cachectic
	P. asaccharolytica Pa3	3.9-5.0 mm	Cachectic
	P. asaccharolytica Pa4	3.2-4.9 mm	Cachectic
	ATCC 33277	7.0-10.0 mm	Cachectic
	Neg. controls	-	Healthy

Positive control: ATCC 33277, Negative controls: Saline

toxin production among clinical isolates via mice injection of *Porphyromonas asaccharolytica*. Six mice were used for every set of injection for each bacterial strain tested. The toxicity activity from bacterial cell culture was observed as abscesses on the skin of the injected mice. Development and severity of lesion or abscesses were observed and recorded. There was no death occurred after the injection. However, all mice produced localized lesion/abscesses and showed signs of mild to moderate cachexia with ruffled hair on day 3 onwards after getting the injection. It has been shown in this study that the response to develop skin lesion on balb/c mice is similar with previous reports of Van Steenbergen *et al.* (1992) on *Porphyromonas gingivalis* strains and Himratul-Aznita *et al.* (2005) on *Prevotella intermedia* strains obtained from deep periodontal pockets.

The subcutaneous injection with 10^8 and 10^{10} cells mL^{-1} of *Porphyromonas assacharolytica* and *Porphyromonas gingivalis* ATCC 33277 failed to produce any skin lesion. However when 10^{12} cells mL^{-1} cultures used, lesions developed about 3-5 days after infection (Table 1), with varied localised lesion areas, measuring about 3-5 mm in diameter occurred for all mice. Similar results have been shown in another finding of Himratul-Aznita *et al.* (2005) on *Prevotella intermedia* that 10^{12} cells mL^{-1} concentration of bacterial suspension was required to induce infection in mice.

Bacterial cell-free supernatant of sonicated and unsonicated cells were also injected subcutaneously in mice to determine the toxicity effect. It was found that the sonicated and unsonicated cell-free filtrate supernatant prepared from each of these isolates developed lesion when 0.1, 0.2 and 0.3 mL supernatant were used. Thus, suggesting that the isolates produced extracellular toxigenic compounds. For both sonicated and unsonicated cell-free filtrates, all mice produced localized lesions or abscesses at the site of injection starting from day 7 onwards and appeared to be cachectic. The destruction of tissue observed in the study may be correlated to the destruction of the periodontal tissues in individual with periodontitis (Van Steenbergen *et al*., 1982). However, injection with 0.1 mL cell-free filtrate is found to be too little to cause severe skin lesions compared to 0.2 and 0.3 mL filtrate used. In contrast, the use of 0.2 and 0.3 mL cell-free filtrates produced big lesion areas (Table 2) with higher level of severity for all mice. Both sonicated and unsonicated cell-free extracts from all strains used in the study showed a relative increment of volume used to the sizes of abscesses produced. However the distribution of toxigenicity varied among the isolates. Therefore, it is clearly shown in present study that this toxin is produced extracellularly by this bacterial strain. In addition, it

Table 2: Toxigenicity of *Porphyromonas asaccharolytica* isolates from unsonicated and sonicated cell-free filtrates via mice injection

	Size of lesions developed (mm) when injected with					
	Unsonicated cell-free filtrates			Sonicated cell-free filtrates		
Samples	0.1 mL	0.2 mL	0.3 mL	0.1 mL	0.2 mL	0.3 mL
P. asaccharolytica Pa1	0.2-0.4	2.5-4.0	2.5-4.1	0.4-0.5	1.5-3.0	3.0-4.0
P. asaccharolytica Pa2	0.2-0.3	1.0-3.5	3.7-4.9	0.3-0.5	3.1-4.5	3.3-4.5
P. asaccharolytica Pa3	0.3-0.5	2.5-5.0	3.0-5.3	0.4-0.75	1.7-5.2	3.7-5.5
P. asaccharolytica Pa4	0.3-0.4	3.0-4.5	4.0-5.0	0.5-0.6	2.5-4.9	3.5-5.0
ATCC 33277	0.7-1.2	6.0-7.0	6.6-8.1	0.6-1.3	5.8-7.7	7.1-8.9
Neg. controls	0.0	0.0	0.0	0.0	0.0	0.0

Positive control: ATCC 33277, Negative controls: Saline

was found that periodontopathic bacteria is dose dependent in causing skin lesion on balb/c mice and is in agreement with those of other scientist elsewhere (Van Steenbergen *et al*., 1982; Himratul-Aznita *et al*., 2005).

The toxicity activity effect of the toxin towards heat treatment was studied at 2 different temperatures (37°C heated for 60 min and 60°C heated for 15 min). The results showed that the toxin activity of *Porphyromonas asaccharolytica* cell filtrates were stable when heated at 37°C for 60 min. However, the toxicity activity was terminated when the filtrates were heated at 60°C after 15 min, as none of the injected mice developed skin lesion. Similar result has also been obtained in another study on *Prevotella intermedia* toxin which was found to be stable at 37°C (Himratul-Aznita and Ansary, 2006).

CONCLUSION

Therefore, *P. asaccharolytica* isolates from adult periodontitis in our Malaysian sample have toxic activity and the toxin is heat labile, which functioned optimally at 37°C.

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