

Lysine Gingipain (*kgp*) Biovars of *Porphyromonas gingivalis* Exhibit Differential Distribution on Oral Mucosal Sites[∇]

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A predominant *kgp* biovar colonized subgingival sites and buccal and tongue mucosa in 45 of 56 adults in an isolated community. The presence of biovars 381, W83, and W83v, but not HG66, correlated with the *Porphyromonas gingivalis* load at diseased sites. Biovars W83 and W83v poorly colonized tongue and buccal mucosa.

Porphyromonas gingivalis is a well-documented periodontal pathogen with two surface cysteine proteinases, a lysine (Kgp) and an arginine (RgpA) gingipain, both containing C-terminal hemagglutinin domains that facilitate colonization of the oral cavity by the bacterium (9, 16). Rearrangements within the genome of *P. gingivalis* have created strain-specific, virulence-related genes (5, 13). For instance, the *fimA* gene coding for the fimbrial subunit has been extensively used to categorize clinical isolates. Association of the *P. gingivalis fimA* genotype II with periodontitis has been observed in different cohorts from Japan (1), Germany (2), Brazil (10), and Switzerland (20). While *fimA* genotype II is the most prevalent gene, the *fimA* genotype I from ATCC 33277/381 strains has been reported to be the second most prevalent genotype among German periodontitis patients, while the *fimA* genotype IV gene (from strain W83) is the second most prevalent gene among Japanese periodontitis patients (1). This suggests that differences in frequency of *fimA* alleles depend on geographic location and/or ethnic origin. However, a recent report highlights the role of gingipains and not fimbriae in preferential binding to matrix proteins, a process that could facilitate colonization of the host (9). Previously we reported that *P. gingivalis* could be categorized by differential PCR into four *kgp* biovars: HG66, 381, W83, and W83v, a domain variant of the W83 *kgp* gene (12). Using paired subgingival samples from the most- and least-diseased sites, we showed that the majority of periodontitis patients in our study were colonized by one predominant biovar with only trace amounts of a second detectable in a few subjects. The frequency of occurrence of biovars HG66, 381, W83, and W83v was 8:11:6:5, respectively (12). In support of this observation is a recent study by Inaba and colleagues (4), who reported that *fimA* genotype II clinical isolates from nine Japanese periodontitis patients all possessed the HG66 biovar, while isolates from two patients corresponded to biovars 381 and W83 or biovars 381 and W83v.

In this report, we describe the distribution of *P. gingivalis* biovars on buccal mucosa and dorsum of the tongue, in addition to healthy and diseased subgingival sites, in relation to *P. gingivalis* load. Site-specific distribution of biovars in three healthy and three diseased gingival sites was also studied in eight subjects colonized with a trace amount of a second biovar.

The study comprised 56 adult Aborigines diagnosed with localized periodontitis with a gender ratio of 33 females (age range, 21 to 67 years; median age, 40 years) to 23 males (age range, 22 to 68 years; median age, 43 years). The Aboriginal Medical Research Council of New South Wales and the Executive of the Durri Aboriginal Medical Service in the North Coast region of New South Wales endorsed the proposal to analyze the molecular epidemiology of oral infection in an isolated, high-risk, high-disease-burden community with preserved family units and no clinical intervention. Informed consent was obtained from each subject. The project was also approved by the Human Ethics Review Committee of the Sydney West Area Health Service.

After removal of supragingival plaque, subgingival plaque was collected from each subject with a sterile curette using a standardized approach whereby the curette was placed in the base of the sulcus or pocket and raised vertically against the tooth surface to remove a sample of ~10 μ l. Curette sampling by two experienced calibrated clinicians gave a more accurate representation of pocket microbiota than paper point sampling (18).

Samples were taken from sites adjacent to areas probed for diagnosis and included sites around three teeth that represented the healthiest gingiva (\leq 3-mm probing depth with no bleeding or suppuration) and three sites that represented the most-diseased sites (\geq 4-mm probing depth with bleeding and/or suppuration). The categorization of healthy and diseased site sampling was based on indices of gingival inflammation (gingival index score), clinical attachment levels, gingival recession, bleeding on probing, and probing depth for each tooth (14). Attachment loss in diseased sites ranged from 2 to 6 mm. Radiographic facilities were not available for the study.

Buccal mucosal swabs were collected with a cotton bud rotated from the mid-cheek third molar to the corner of the lip, while tongue swabs were similarly taken from lateral border to

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TABLE 1. Site-specific distribution of *P. gingivalis* *kgp* biovars in subjects colonized with trace amount of a second biovar

Subject	Biovar found in ^a :					
	Healthy subgingival sites			Diseased subgingival sites		
	Site 1	Site 2	Site 3	Site 1	Site 2	Site 3
1	381	381 , W83	381	381 , W83		HG66
2				W83v		W83v , 381
3	HG66 , 381			HG66 , 381	HG66 , 381	HG66 , 381
4	381			381	W83v	W83v , 381
5		HG66	HG66	HG66	381, HG66	HG66
6	HG66		381	381, HG66	HG66 , 381	HG66, 381
7	HG66	381	381	HG66	HG66, 381	HG66, 381
8	HG66	HG66	HG66 , W83	HG66	HG66	HG66

^a A biovar in boldface represents the predominant biovar identified in pooled DNA samples isolated from six subgingival sites representing the three healthiest and three most-diseased sites from a given subject.

lateral border across the anterior dorsum of the tongue. Samples were collected in tubes containing 200 µl phosphate-buffered saline and snap-frozen with dry ice in preparation for extraction of DNA using a QIAamp DNA minikit (Qiagen, Clifton Hill, Victoria, Australia). *P. gingivalis* load and total bacterial load were quantified, and identification of *kgp* biovars was determined as previously described (8, 11, 12). Briefly, total bacterial and *P. gingivalis* loads were determined using the universal primer (300 nM) and probe (175 nM) set or the *P. gingivalis*-specific primers and probe (100 nM), respectively, using the TaqMan PCR core reagents kit (Applied Biosystems) and an ABI-PRISM 7700 sequence detection system (8, 12). The amount of DNA in each sample was measured against *P. gingivalis* DNA standards in the range 3.6 fg to 3.6 ng and converted to cell numbers based on one cell containing 2.37 fg of DNA. *P. gingivalis* load was expressed as per ml for subgingival plaque and for mucosal or tongue samples as previously used (3). For identification of *kgp* biovars, differential PCR was carried out with HotStarTaq master mix (Qiagen, Australia) using the HG66 biovar-specific primer set HG66F and HG66R (amplicon size, 1.9 kb), the 381 biovar-specific primer set *kgp*F and 381R (amplicon size, 3.4 kb), and the W83/W83v biovar-specific primer set *kgp*F and W83R (amplicon size, 3.6 kb for W83 and 2.2 kb for W83v) (12). Total bacterial load (described above) was used as a reference marker for bacterial biomass recovered to verify the sampling technique (15). This standard was preferred over total recovered protein as inflamed sites contain high levels of serum proteins (6, 19) that could have distorted the analysis.

Biovar-specific PCR was carried out using DNA samples isolated from the buccal mucosa, the tongue, and two individually pooled DNA samples isolated from three subgingival sites that represented the three healthiest or the three most-diseased sites, respectively. Due to low loads of *P. gingivalis* in some of the subjects, it was only possible to detect the *kgp* biovar(s) present in the subgingival plaque of 45 subjects using our visual gel-based system (12). Thus, *kgp* biovar 381 was detected in 26 subjects, HG66 in 17 subjects, and biovars W83 and W83v in 7 and 3 subjects, respectively. For 8 of the 45 subjects in which biovars were detected (strong visual band after 40 cycles of PCR), trace amounts of a second biovar (weak visual band after 40 cycles of PCR) were also detected. In the eight subjects possessing two *kgp* biovars, further analysis showed that the predominant biovars in the pooled sam-

ples also predominated at individual sites (Table 1). Similarly, for those subjects harboring *P. gingivalis* in subgingival plaque and where *P. gingivalis* biovars could be determined, the tongue and buccal mucosa were colonized by the same predominant biovar present in subgingival plaque. Biovar 381 was detected on the buccal and tongue mucosa of 8 individuals, HG66 on 12 individuals, and biovar W83v on 1 individual. These observations were in keeping with previous reports suggesting that soft tissue surfaces harboring periodontal pathogens could serve as reservoirs for infection (7, 17). However, in none of the seven subjects colonized subgingivally by biovar W83 could this biovar be detected on either the buccal mucosa or the dorsum of the tongue. The use of total bacterial load as a reference marker for bacterial biomass across the four sites healthy, diseased, mucosal, and tongue indicated that the absence of biovars W83 and W83v on mucosal and tongue sites

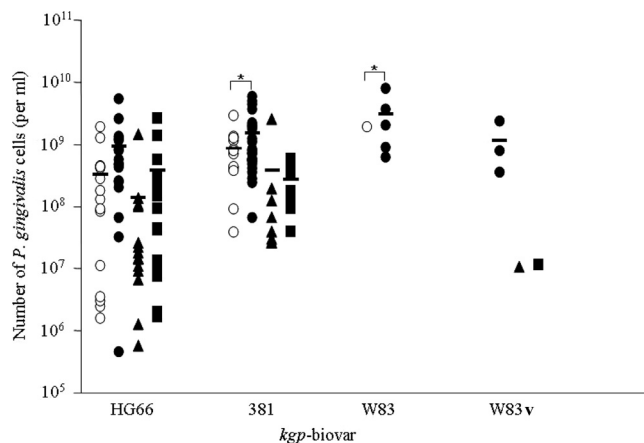


FIG. 1. Relationship between *P. gingivalis* load and *kgp* biovar. Subgingival plaque from the least-diseased (open circles) and the most-diseased sites (closed circles), as well as swabs of buccal mucosa (closed triangles) and the dorsum of the tongue (closed squares) from each subject, was assayed for the number of *P. gingivalis* (expressed as per ml for subgingival plaque and for mucosal or tongue samples) and *kgp* biovar. The mean value for each group is represented by a solid bar, and statistical comparison based on GLMEM (21) between the groups was analyzed. The asterisk denotes significance ($P < 0.0001$). It should be noted that due to the limited number of samples in the biovar W83 data set, the GLMEM model recognizes that the level of significance may be unstable.

was not due to sampling error, as total bacterial loads were not significantly different from those sites colonized by HG66 or 381 biovars (data not shown).

Based on analysis using generalized linear mixed-effects models (GLMEM) (21) for the 45 subjects, there was significantly less detection of biovars 381, W83, and W83v in healthy subgingival sites, buccal mucosa, and tongue than for diseased gingival sites ($P < 0.0001$). Significant correlation was also observed between *P. gingivalis* load and the presence of the 381, W83, and W83v biovars ($P < 0.0001$). For the HG66 biovar, the presence in diseased gingival sites showed no significant correlation to either the presence or absence of this biovar in clinically healthy subgingival sites ($P = 0.3640$) or the tongue surface ($P = 0.0737$) (Fig. 1). In summary, the isolated adult Aboriginal community suffering from chronic periodontitis demonstrated a distribution frequency for the *kgp* biovars HG66 and 381 close to that observed for the multiethnic Sydney metropolitan population (12). The fact that the *kgp* biovars W83 and W83v were poor colonizers of the dorsum of the tongue and buccal mucosa, while the *kgp* biovars HG66 and 381 were capable of colonizing a variety of mucosal environments, might explain the high frequency of these biovars in populations suffering from periodontal diseases. It remains to be determined if this behavior is related directly to structural variation in the hemagglutinin domain of Kgp.

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