Black-Pigmented *Bacteroides* spp. in Human Apical Periodontitis

MARKUS HAAPASALO,¹,²*, HELENA RANTA,¹,³ KARI RANTA,¹ AND HAROUN SHAH⁴

Departments of Cariology,¹ and Bacteriology and Immunology,² University of Helsinki, and National Public Health Institute,³ SF-00280 Helsinki 28, Finland; and Department of Oral Microbiology, London Hospital Medical College, London E1 2AD, United Kingdom⁴

Received 10 February 1986/Accepted 11 April 1986

The incidence of black-pigmented (BP) *Bacteroides* spp. in 62 human dental root canal infections (35 acute and 27 clinically asymptomatic cases of apical periodontitis) in 57 adults was studied. Altogether 37 strains of BP *Bacteroides* were found in 31 infections, always in mixed anaerobic infections. Two different BP *Bacteroides* species were present in six infections. *B. intermedius* was most frequently isolated (15 of 62 canals; 24%) followed by *B. denticola* which was present in 12 cases. Asaccharolytic BP *Bacteroides* species, *B. gingivalis* and *B. endodontalis*, were found in eight cases. BP *Bacteroides* species were found both from symptomatic and asymptomatic infections, but there were also several symptomatic cases from which BP *Bacteroides* species were not isolated. *B. gingivalis* and *B. endodontalis* were present only in acute infections, *B. intermedius* was found both in symptomatic and asymptomatic infections, and *B. denticola* occurred mostly in asymptomatic infections. BP *Bacteroides* species were isolated initially from 9 of the 11 teeth with symptoms at 1 week, but only from 22 of the 51 teeth that were symptomless at 1 week. Two strains of *B. denticola* were resistant to penicillin G at a concentration of 2.4 µg/ml, but the MIC of penicillin G for all other strains was 0.6 µg/ml or lower. Forty-two randomly selected patients received penicillin V (oral administration, 650 mg, three times daily) during the first week of endodontic therapy. Penicillin had no effect on the occurrence of symptoms after 1 week compared with the control group (20 patients).

Black-pigmented (BP) *Bacteroides* species are normal inhabitants of the gastrointestinal tract and oral cavity of humans and are also commonly present in mixed anaerobic infections (5). The essential role of BP *Bacteroides* species in human opportunistic infections has long been established (15, 24, 28). Their pathogenic potential may be related to the production of hydrolytic enzymes such as collagenase and endotoxin and inhibition of the phagocytic function of polymorphonuclear leukocytes (8, 10, 25–27). Results of studies in animals indicate interspecies differences in the virulence potential of BP *Bacteroides* species (20, 29). The asaccharolytic species *B. gingivalis* and *B. asaccharolyticus* and the weakly saccharolytic species *B. intermedius* have been shown to induce more severe infections than strains previously classified as *B. melaninogenicus* subsp. *melaninogenicus*.

Advances in the taxonomy of anaerobic bacteria and especially of BP *Bacteroides* species, however, have brought some uncertainty to the evaluation of the virulence of these bacteria. Originally one species, *B. melaninogenicus* is now known to comprise eight species (human strains) on the basis of differences in biochemical characteristics, lipid analyses, DNA base composition, and DNA homology (3, 9, 11, 30). The occurrence of the newly described species of BP *Bacteroides* in endodontic infections, however, is not known.

The virulence potential of bacteria in vivo is difficult to assess. In many respects dental root canal infections offer good conditions for studying the relative significance of a single bacterial species or combinations of species. Because of its anatomy, the root canal lacks normal flora, and specimens can be successfully obtained without mucosal or salivary contamination. Also, the number of different species in root canal infections is relatively low (12, 17, 23, 35).

Information has accumulated about the diversity of the flora, but it is only recently that the root canal has been used as a model for opportunistic infection. Induced root canal infections have been studied in animals (4). In studies on human infections the composition of the flora have been compared with the severity of infection. It has been suggested that the occurrence of BP *Bacteroides* species and symptoms may be closely related (G. Sundqvist, Ph.D. thesis, University of Umeå, Umeå, Sweden, 1976). Griffee et al. (6) also found a good correlation between the occurrence of symptoms and BP *Bacteroides* species in periapical osseitis. Whether interspecies differences do occur is not clear because of the diagnostic methods used (6) and the small number of infections studied.

Penicillin has been the drug of choice in the treatment of endodontic infections. Recent studies, however, have demonstrated high frequencies of resistant strains also in bile-sensitive *Bacteroides* species, including BP *Bacteroides* species (16, 18, 21). However, susceptibilities of the different species of BP *Bacteroides* are usually not given.

The aim of this study was to examine the occurrence of BP *Bacteroides* species and symptoms in human root canal infections with special reference to recent taxonomic advances. Also, the sensitivity patterns of BP *Bacteroides* spp. to penicillin as well as the effectiveness of orally administered penicillin V in the treatment of apical periodontitis were studied.

**MATERIAL AND METHODS**

**Subjects.** Fifty-seven adult patients referred to the Department of Cariology and Endodontics, University of Helsinki, participated in the study. Teeth with one root canal and with diagnosed apical periodontitis were included (62 teeth). Informed consent was obtained. Reasons for exclusion were (i) antibiotic therapy within the previous 3 months, (ii) a periodontal pocket over 4 mm deep, (iii) previous endodontic therapy of the affected tooth, (iv) previous

* Corresponding author.
TABLE 1. Frequency of isolation and key characteristics used in the identification of BP Bacteroides species in 62 cases of apical periodontitis

<table>
<thead>
<tr>
<th>Species</th>
<th>No. of strains</th>
<th>Indole production</th>
<th>Starch hydrolysis</th>
<th>Agglutination of erythrocytes</th>
<th>Cellobiose fermentation</th>
<th>G + C content (mol%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Asaccharolytic</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>B. gingivalis</td>
<td>6</td>
<td>+</td>
<td>+</td>
<td>NT</td>
<td>NT</td>
<td>45–49</td>
</tr>
<tr>
<td>B. endodentalis</td>
<td>2</td>
<td>+</td>
<td>–</td>
<td>NT</td>
<td>NT</td>
<td>51–52</td>
</tr>
<tr>
<td>Saccharolytic</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>B. intermedius</td>
<td>15</td>
<td>+</td>
<td>+</td>
<td>NT</td>
<td>NT</td>
<td>NT</td>
</tr>
<tr>
<td>B. denticola</td>
<td>12</td>
<td>–</td>
<td>+</td>
<td>NT</td>
<td>–</td>
<td>51–54</td>
</tr>
<tr>
<td>B. loescheii</td>
<td>2</td>
<td>–</td>
<td>+</td>
<td>NT</td>
<td>+</td>
<td>48</td>
</tr>
</tbody>
</table>

* A total of 37 strains were isolated from 31 canals (50%). B. intermedius was isolated in three cases together with B. gingivalis, in two cases with B. denticola, and in one case with B. loescheii.

** Human type A, B, and O sheep erythrocytes.

† NT, Not tested.

contamination of the root canal by saliva (perforated canals), and (v) hypersensitivity to penicillin. Patients with any generalized disorders were also excluded. The mean ages of men and women were 36 and 42 years, respectively.

Sample collection and bacterial diagnosis. The tooth was cleaned with pumice and isolated from the oral cavity with a rubber dam. For disinfection of the teeth and the rubber dam, 10% H₂O₂ and 0.5% chloroform gluconate in 70% ethanol were used. Paper points (Mailfer, Bellauges, Switzerland) were washed with chloroform before sterilization to avoid the possible inhibitory effect of unsaturated fatty acids to the bacterial growth. After perforation of the cavity, the paper point was inserted into the canal to the approximate apex region. The infectious material was immediately spread over the agar plates with the paper point. The following media were used for initial cultivation: (i) kanamycin (75 μg/ml), vancomycin (7.5 μg/ml), laked blood (sheep blood) agar (KVLB) (19); (ii) a nonselective medium containing horse blood (5%), bacteriological agar no. 1 (Oxoid Ltd., Hampshire, England), yeast extract (10 g/liter; Oxoid), menadione (0.5 mg/liter), cystein (500 mg/liter), glucose (2 g/liter) and peptone (10 g/liter); penicillin (0.072 g/liter); and natrium chloride (5 liter) (MCGP medium); and (iii) chocolate agar containing hemolysed horse blood (8%), Trypticase soy agar (BBL Microbiology Systems, Cockeysville, Md.), Mueller-Hinton agar (BBL), and IsoVitalex (10 mg/liter; BBL). KVLB and MCGP plates were prereduced and used within 3 days after preparation. The plates were immediately placed in an anaerobic jar (BBL) and incubated in an anaerobic atmosphere (gas generating kit; Oxoid) at 37°C.

Anaerobic jars were opened after 7 days, and subcultures were made. Primary plates were incubated for up to 3 weeks. The identification of species of BP Bacteroides was based on the following tests: aerotolerance, Gram stain, sensitivity to kanamycin (100 μg; Rosco, Taastrup, Denmark) and vancomycin (5 μg; Rosco), colony morphology, pigmentation patterns, hydrolysis of starch and esculin (19), fermentation of carbohydrates (API 20A; API, Montalieu-Vercieu, France), production of indole (19), agglutination tests, DNA base composition. For inoculum for biochemical tests, strains were grown on MCGP plates (similar to MCGP but without Lab-Lemco powder, peptone, or natrium chloride in the medium).

Agglutination tests. Agglutination tests for the asaccharolytic strains were performed with washed human types A, B, and O sheep erythrocytes. A total of 25 μl of a 2% (vol/vol) suspension of erythrocytes in phosphate-buffered saline was pipetted on microscopic slides (kept on ice), and bacteria harvested from MCGP plates after 3 days of anaerobic incubation were added. Slides were occasionally rotated, and hemagglutination was registered after 30 min.

DNA isolation and determination of base composition. Cells of all asaccharolytic and saccharolytic indole-negative strains grown either in BM medium (liquid medium containing Trypticase [10 g/liter; BBL], proteose peptone [10 g/liter; Oxoid], yeast extract [5 g/liter; Oxoid], natrium chloride [5 g/liter], hemin [5 mg/liter; Sigma], menadione [0.5 mg/liter; Sigma]), and bovine serum [20 ml/liter; Oxoid]) or on blood agar plates were collected by centrifugation, washed twice with 0.05 M Tris–0.005 M EDTA–0.05 M NaCl (pH 8.0; TES buffer), and suspended in the same buffer containing 50 μg of proteinase K (E. Merck AG Darmstadt, Federal Republic of Germany) per ml. Lysis was achieved with sodium dodecyl sulfate (final concentration, 1 mg/ml) treatment for 20 min at 60°C, and the DNA was purified by the method of Marmur (14).

The mole percent of G + C of purified DNA samples was estimated in triplicate by thermal denaturation in standard saline citrate (pH 7.0) with a Gilford model 240 spectrophotometer and a Gilford 2527 temperature programmer, Escherichia coli B (Sigma Chemical Co., St. Louis, Mo.) was used as a reference DNA. The G + C content was calculated from the following equation: mole% G + C = 50.9 + 2.44 × (Tm [melting temperature of test strain] – Tm of E. coli B).

Penicillin sensitivity tests. Sensitivity of BP Bacteroides species to penicillin was determined by the agar dilution method. Cultures were inoculated onto Mueller-Hinton agar (BBL) containing 8% hemolyzed horse blood, hemin (33 mg/liter), and IsoVitalex (10 ml/liter; BBL). A single colony of a pure culture was used as an inoculum, which was spread with a platinum loop on the penicillin plate. Penicillin (penicillin G) concentrations were 0 (control plate), 0.036, 0.072, 0.15, 0.3, 0.6, 1.2, and 2.4 μg/ml. Sensitivity testing was performed in duplicate in an anaerobic chamber (model 1024 anaerobic system; Forma Scientific) containing H₂ (10%), CO₂ (5%), and N₂ (85%). The results were recorded under a stereo microscope at low magnification after 2 and 3 days of incubation. Staphylococcus aureus ATCC 29213 and Enterococcus faecalis ATCC 29212 were used as controls.

Dental therapy. To locate the apical constriction, an electrical apex meter (Dahlin Dentometer; Apollonia, Copenhagen).
hagen, Denmark) was used, and the measurement was confirmed radiographically. Canals were filled with calcium hydroxide and sealed with a temporary filling between appointments. Forty-two randomly selected patients received penicillin V (650 mg, three times daily) for 7 days. Symptoms were registered at the beginning (0 weeks) and at 1 week. Pain, swelling, or both; open sinus tract; and tenderness to percussion were regarded as signs of an acute infection.

RESULTS

Frequencies of isolation, key biochemical characteristics, and the occurrence of symptoms when BP Bacteroides species were present are given in Tables 1 and 2. All BP Bacteroides strains were isolated from mixed anaerobic infections. The number of anaerobic species usually varied from three to seven, and the number of facultative species varied from zero to one. Symptoms were registered in 35 teeth (56%). One or more species of BP Bacteroides were found in 19 (54%) of the symptomatic cases and in 12 (44%) of the asymptomatic cases. Symptoms were present in 11 teeth 1 week after the first examination. BP Bacteroides species were isolated from nine of these teeth at the beginning of the treatment (Table 2). In the tenth tooth a radicular cyst was diagnosed by the radiograph. After the second examination all patients became symptomless.

The reduction in the number of symptomatic cases was comparable in the penicillin (42 teeth) and control (20 teeth) groups between the first and second examinations. The proportions of acute infections declined from 55 to 19% in the penicillin group and from 30 to 15% in the control group. Susceptibilities of BP Bacteroides species to penicillin are given in Table 3. Only two strains of B. denticola were found to be resistant.

DISCUSSION

In previous reports of root canal infections the incidence of BP Bacteroides species ranges from 6 to 38% (6, 12, 22, 23, 35). In this study one or two species of BP Bacteroides were isolated in 31 of 62 teeth (50%) with apical periodontitis. Sampling and cultivation onto the agar plates were performed at chairside. This, together with prolonged incubation of the primary plates to detect delayed pigmentation, may partly explain the high frequency of BP Bacteroides species in our material, in which nearly half of the cases were asymptomatic. When only acute dentigenous abscesses have been studied, higher frequencies also have been found. Aderhold et al. (1) studied acute dentigenous abscess aspirates and isolated BP Bacteroides species in 34 of 50 cases (68%). Of their strains, 15 were identified as B. asaccharolyticus, one strain was identified as B. melaninogenicus subsp. intermedius, and 18 strains were not identified to the species level. In a study of acute periapical abscesses of children (5 to 16 years old) Brook et al. (2) found 9 BP Bacteroides strains in 12 cases. Also in this study only a few strains were diagnosed to the species level. Oguntebi et al. (17) reported two B. intermedius strains from 10 periapical abscesses, and Williams et al. (34) found four strains of BP Bacteroides species in 3 of 10 dental abscesses studied. One of these four strains was identified as B. asaccharolyticus. Von Konow et al. (33) studied needle aspirates of 57 dentoalveolar abscesses (39 periapical, 9 periodontal, and 9 surgical infections) and found BP Bacteroides species in only three cases. The great differences in the frequencies of BP Bacteroides species in these studies may partly reflect differences in sampling, sample processing, and the diagnostic procedures that were employed. However, although BP Bacteroides species are often isolated from acute dentigenous infections, there are also other species or combinations of species capable of inducing an acute phase of infection. Whether the presence of BP Bacteroides species always results in an acute infection cannot be evaluated from the results of these studies because only acute infections were included.

It should be emphasized that not all species of BP Bacteroides readily pigment on horse blood agar. B. denticola, B. loscheii, and B. melaninogenicus often require rabbit or sheep blood to show the characteristic pigmentation (9). In our experience, the pigmentation of asaccharolytic species may also be delayed, and the color of single colonies varies from light grey to dark brown. Colonies of slowly pigmenting BP Bacteroides species have no other characteristics different from many other Bacteroides species. In this study sheep blood was used only in the KVLB medium for initial cultivation, while horse blood was used for other media at this stage. Yet, more BP Bacteroides species were isolated from the MCGP and chocolate agar media than from the KVLB plates. One explanation for this is that vancomycin is inhibitory to some BP Bacteroides species (32). We also noticed that many BP Bacteroides strains pigmented readily on the primary plate when they were adjacent to colonies of other species. When pure cultures of the indole-negative saccharolytic BP Bacteroides species were obtained, the characteristic pigmentation was no longer observed on horse blood agar. After repeated subcultures on MCG medium with horse blood, the pigmentation of most Bacteroides strains reappeared. On laked rabbit blood agar, all strains except one showed the characteristic pigmentation.

The frequencies of B. melaninogenicus, B. denticola, and B. loscheii in root canal infections usually have not been reported. In this study B. denticola was by far the most common representative of this group. B. loscheii was isolated from only two canals, while B. melaninogenicus was totally absent. B. melaninogenicus is very similar to B. denticola if only conventional biochemical tests are used, but these two species can be readily identified by the difference in the DNA base composition (9). It is possible that some strains presented earlier under the name of B. melaninogenicus have in fact been B. denticola.

Results of several studies during the last few years have shown resistance to penicillin among BP Bacteroides species (16, 18, 21). Our strains were all sensitive at a concentration of 0.6 μg/ml, with the exception of two strains of B. denticola. The interspecies differences, as well as the sources of isolates, are usually not reported. However, it is probable that the term clinical material used in many reports refers to nonoral strains. Laatsch et al. (13) studied the susceptibilities of only oral BP Bacteroides species; they

| TABLE 3. MICs for penicillin G of oral BP Bacteroides strains |
|-----------------|-----------|-----------|-----------|
| **Species**     | **No. of strains** | **No. of strains inhibited at the following penicillin G concn (μg/ml):** |
| **B. gingivalis** | 6         | 3         | 3         |
| **B. endodontalis** | 2         | 1         | 1         |
| **B. intermedius** | 12        | 7         | 5         |
| **B. denticola** | 12        | 5         | 3         | 1         | 1         | 2         |
| **B. loscheii** | 2         | 2         |           |           |           |           |
found that half of the strains had a MIC of less than 0.125 μg/ml, and 90% of strains were inhibited at a concentration of 0.5 μg/ml. The results of this study also suggest that penicillin therapy has no substantial effect on the occurrence of symptoms 1 week after the beginning of the treatment, compared with the control group, in cases of uncomplicated periapical infections. Severe dentigenous orofacial infections were not present in our material.

We were not able to confirm the findings of Sundqvist (G. Sundqvist, Ph.D. thesis) and Griffee et al. (6), who suggested that there is a relationship between symptoms and BP Bacteroides species in human periapical osteitis. It was not only that symptoms were present without BP Bacteroides species but we also found an equal amount of symptomless teeth with BP Bacteroides species, and the overall isolation frequency of BP Bacteroides species in our material was high. However, the asaccharolytic oral species B. gingivalis and B. endodontalis were found only in acute infections. We previously reported our B. endodontalis strain as B. dentalis (7), but while our paper was in press the description of B. endodontalis was published (30). Our strains seem to be phenotypically indistinguishable from B. endodontalis, so it was reasonable to use the name B. endodontalis in this report to avoid confusion, although DNA hybridization between the strains has not been done. In a recent study on odontogenic abscesses, Van Winkelhoff et al. (31) found that B. endodontalis was the most frequently isolated BP Bacteroides species after B. intermedius, B. endodontalis was isolated in 9 of 17 abscesses of endodontic origin, B. gingivalis was isolated in only 2 of 17 cases, and B. denticola was not found in any of the abscesses that were studied. In our study B. gingivalis was isolated more frequently than B. endodontalis, but comparisons between these two studies are difficult to make because different materials were used. In our study only 56% of cases were symptomatic, and of them a minority were abscesses.

Our results are in good agreement with the virulence tests of BP Bacteroides species in animals (20, 29), as well as results of in vitro studies of the BP Bacteroides species-polymerophenonuclear leukocyte interaction (26, 27), which suggest that B. gingivalis is the most virulent BP Bacteroides species. The presence of symptoms 1 week after the beginning of treatment appeared to be related to the occurrence of BP Bacteroides species in the teeth when therapy was started. Extrusion of infected root canal contents is known to occur frequently, even with careful cleaning of the root canal space. It is possible that the resistance to phagocytosis by BP Bacteroides species (10, 26) may partly explain the slower disappearance of the symptoms.

The results of this study suggest that the presence of B. gingivalis and B. endodontalis in necrotic root canal is closely related to an acute infection and that fermentative BP Bacteroides species, including B. intermedius, are frequently present both in symptomatic and asymptomatic infections. It is also possible that the risk for persisting symptoms may be greater when BP Bacteroides species are part of the infective flora.

LITERATURE CITED


