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# Alkali-resistant bacteria in root canal systems

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The aim of this study was to isolate and identify alkali-resistant bacteria from the dentin of infected root canals. Bacteria from homogenized dentin powder made up from infected root canal walls from human teeth were cultured on buffer-enriched Brain Heart Infusion agar supplemented with 4% sheep blood (BHI-blood agar), adjusted to pH 7.0, 9.0 or 10.0. Incubation took place for 7 days at 37°C in an anaerobic glove box. Bacterial strains selected according to colony and morphology were subcultured in buffer-enriched BHI broth adjusted to pH 9.0, 10.0 or 11.0 to confirm their growth as alkali-resistant bacteria. Polymerase chain reaction amplification using specific primer sets and 16S rDNA sequence analysis was performed for identification of alkali-resistant isolates. In the present study, 37 teeth extracted from 37 patients were used for preparation of the dentin powder samples. Bacteria were detected in 25 samples when standard BHI-blood agars (pH 7.0) were used. Of these, 29 strains from 15 samples were alkali resistant, 25 strains growing at pH 9.0 and 4 at pH 10.0. The alkali-resistant strains included *Enterococcus faecium* (10 strains) and *Enterococcus faecalis* (2 strains), *Enterobacter cancerogenus* (1 strains), *Fusobacterium nucleatum* (1 strains), *Klebsiella ornithinolytica* (2 strains), *Lactobacillus rhamnosus* (2 strains), *Streptococcus anginosus* (2 strains), *Streptococcus constellatus* (3 strains), and *Streptococcus mitis* (2 strains). Three strains were also identified as bacteria of genus *Firmicutes* or *Staphylococcus* at the genus level. The present study showed that many bacterial species in infected root canal dentin were alkali-resistant at pH 9.0 and/or pH 10.0, and belonged mainly to the genus *Enterococcus*.

Key words: 16S rDNA sequence; alkali-resistant bacteria; endodontics; infected root canal; polymerase chain reaction

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Calcium hydroxide, $Ca(OH)_2$, has been used in endodontics since 1920 (11). $Ca(OH)_2$ has a strong bactericidal effect on oral bacteria (2) and it is acknowledged to be one of the most effective antimicrobial dressing agents in endodontic treatment (2, 10).

Although $Ca(OH)_2$ can exert lethal effects on endodontic microorganisms (2, 7, 28), some bacteria can survive in root canal systems (2, 8, 22) and can be isolated from infected root canals after $Ca(OH)_2$ root canal filling (25, 27). The surviving bacteria may be alkali resistant, although most microorganisms are killed following exposure to the high pH environment provided by $Ca(OH)_2$ (10). Those that survive may have been exposed to low levels of $OH^-$ because of a remote location relative to the $Ca(OH)_2$ filling. In fact, it has been suggested that complete elimination of bacteria using $Ca(OH)_2$ medicaments from root canal systems is not always possible (2, 8, 22) and not all bacteria isolated from root canals after application of $Ca(OH)_2$ may be alkali resistant. Thus, it is necessary to determine the resistance of endodontic bacteria to high pH values in order to designate them as true alkali-resistant isolates.

Anaerobic bacteria predominate in infected root canals, and their isolation requires strict culture conditions in an anaerobic glove box or similar effective anaerobic culture systems (1, 14, 29). Circumferential $CO_2$ inside the anaerobic glove box may lower the pH of culture media to a neutral pH and result in failure to maintain the high pH of culture media. To study possible alkali-resistant bacteria, experimental procedures should be established that maintain a high pH throughout the culture of such bacteria.

In the present study, we have established bacteriologic procedures to make it possible to maintain a high pH of culture media for at least 10 days. With these procedures, we have isolated and identified alkali-resistant bacteria from infected root canal dentin.

## Material and methods

### Bacterial samples

Thirty-seven extracted teeth were collected from 37 systemically healthy patients (22 male and 15 female; mean 53.3 years

old, range 16–80 years old) who attended several private dental clinics in Niigata and the dental hospital of Niigata University. The study included extracted teeth that were diagnosed clinically as 'hopeless' because of severe root canal infection. After extraction, teeth were coated with blue inlay wax and transferred as soon as possible to an anaerobic glove box (Model AZ-Hard, Hirasawa, Tokyo, Japan) containing 80% $N_2$, 10% $CO_2$, and 10% $H_2$.

After making shallow notches with a sterile diamond disk along the long axis of the tooth to facilitate splitting, the tooth was split with a pair of sterile forceps. Dentin powder from the root canal was obtained using sterile steel round bars at low speed (< 120 r.p.m.) (4, 13), and was used in the bacteriologic study.

#### Culture media

In the present study, Brain Heart Infusion (Difco Laboratories, MD)–yeast–extracted blood (sheep, 4%) agar (BHI-blood agar) plates and BHI broth were used as culture media for the bacteria.These media were enriched with buffer solution instead of tap water to maintain the high pH during culture of the bacteria. To strengthen the buffering capacities of the culture media, buffer solutions were made by mixing 0.2 M $KH_2PO_4$ and 0.2 M KOH for pH 7.0, 0.2 M $NaHCO_3$ and 0.2 M $K_2CO_3$ for pH 9.0, and 10.0, 0.2 M $Na_2HPO_4$ and 0.2 M KOH for pH 11.0, respectively, and used for preparation of the buffer-enriched media.

BHI broth media, also enriched with buffering capacities as described above, were efficient in maintaining the proper alkaline pH when the media were filter-sterilized, put into vials (3 ml) and placed in the entrance-chamber of the anaerobic glove box for 1 week. Loose capping of the vials facilitated gas exchanges of media in the presence of anaerobic gas (10% $H_2$ and 90% $N_2$) and catalysts. The broth media were then transferred to the main chamber (80% $N_2$, 10% $CO_2$, and 10% $H_2$) of the anaerobic glove box and the vials tightly capped until used.

#### Isolation and culture of alkali-resistant bacteria

The dentin powder of the root canals was homogenized in 40 mM phosphate buffer solution (1 ml), pH 7.0. After serial 10-fold dilution with the same buffer, 0.1 ml aliquots of original and diluted samples were inoculated onto a buffer-enriched BHI-blood agar (pH 9.0 or 10.0) plate for isolation of alkali-resistant isolates.

After incubation in the anaerobic glove box for 1 week at 37°C, bacterial strains were selected according to morphologic differences of the colonies, and purified. These bacterial strains were also inoculated into the buffer-enriched BHI broth adjusted to pH 9.0, 10.0 or 11.0 to confirm that they were alkali resistant.

#### Extraction of bacterial DNA

The cells of alkali-resistant bacteria harvested from the buffer-enriched BHI-blood agar (pH 9.0 or 10.0) plates were suspended with sterilized saline for washing in a tube. After centrifugation, the bacterial cells were treated with 200 µl of InstaGene Matrix (Bio-Rad, Hercules, CA), and genomic DNA was extracted according to the manufacturer's protocol.

#### Identification of isolates

Specific polymerase chain reaction (PCR) amplifications were carried out in 50 µl of reaction mixture containing 25 µl of TaKaRa Premix Taq solution (Takara, Tokyo, Japan), 4 pmol of the specific primer set and the template DNA extracted from the bacterial cells. Amplifications were performed on the Programmable Thermal Controller PTC-100 (MJ Research, Watertown, MA) with 35 cycles at 94°C for 60 s, 54°C for 90 s, and 72°C for 90 s. The specific primer sets selected in the present study were 5′-ATC AAG ACA GTT AGT CTT TAT TAG-3′ and 5′-ACG ATT CAA AGC TAA CTG AAT CAG T-3′ for the 941-bp product of *Enterococcus faecalis* (16), 5′-TTG AGG CAG ACC AGT TGA CG-3′ and 5′-TAT GAC AGC GAC TCC GAT TCC-3′ for the 658-bp product of *Enterococcus faecium* (16), 5′-GCG ATT GAT GGT GAT ACG GTT-3′ and 5′-ATC GCA TCA CAA GCA CCA ATC-3′ for the 796-bp product of *Enterococcus gallinarum* (3), 5′-CGG GGA AGA TGG CAG TAT-3′ and 5′-CGC AGG GAC GGT GAT TTT-3′ for the 484-bp product of *Enterococcus casseliflavus* (3), 5′-GCC TTT ACT TAT TGT TCC-3′ and 5′-GCT TGT TCT TTG ACC TTA-3′ for the 224-bp product of *Enterococcus flavescens* (3) and 5′-GTT ATT AGG GAA GAA CAT ATG TG-3′ and 5′-CCA CCT TCC TCC GGT TTG TCA CC-3′ for the 750-bp product of *Staphylococcus* species (15).

The sensitivities of the PCR amplification with these primer sets were confirmed by reference DNAs extracted from *E. faecalis* ATCC 19433$^T$, *E. faecium* ATCC 19434$^T$ and *Staphylococcus aureus* ssp. *aureus* ATCC 12600$^T$. After PCR amplification, these amplicons were separated by electrophoresis in a 1.8% agarose gel and visualized by staining with ethidium bromide. It was confirmed that these specific primer sets did not produce any amplicons with DNA templates from other bacterial species or genus in the PCR reactions.

For the cycle sequence method of 16S rDNA sequence analysis, a Thermo Sequenase fluorescent labeled primer cycle sequencing kit (Amersham Pharmacia Biotech, Buckinghamshire, UK) was used with the 11 universal primer sets labeled with Cy-5, following the manufacturer's protocol (9). These sequences of the 16S rDNA were analyzed with a DNA sequencer (ALFexpress, Amersham Pharmacia Biotech). The segmented nucleotide sequences of 16S rDNA were connected by using Seqman in the LASERGENE computer program (DNA Star, Madison, WI). The 16S rDNA sequences of alkali-resistant bacteria in this study were compared with the established sequences searched with BLAST algorithms in GenBank. Sequence similarity was analyzed by the clustal method, which is programmed by Megalign in the LASERGENE computer program. A 16S rDNA sequence similarity of 98% was used as the cut-off point for positive identification of taxa (20). If the bacterial strain had less than 98% 16S rDNA sequence similarity with the most closely related bacterial species, that taxa were identified at the genus level.

## Results

Our preliminary experiments showed that the pH of BHI-blood agar plates (12) decreased from pH 10.0 to pH 8.5 and from pH 9.0 to pH 7.5 within 1 week stored in an anaerobic glove box, when tap water was used for the preparation of the media (standard BHI-blood agar plate). Even pH 7.0 had decreased to pH 6.5 within 1 week. In this study, the buffer (0.2 M $NaHCO_3$ and 0.2 M $K_2CO_3$)-enriched pH 9.0 BHI-blood agar plates were found to be efficient in maintaining the alkaline pH for at least 10 days in an anaerobic glove box. Buffer-enriched BHI-blood agar plates at pH 10.0 (0.2 M $NaHCO_3$ and 0.2 M $K_2CO_3$) and pH 11.0 (0.2 M $Na_2HPO_4$ and 0.2 M KOH) plates were lowered to pH 9.0 within 1 day (Fig. 1). Buffer-enriched BHI-blood agar could not be stabilized at pH 12.0 or higher (data not shown).

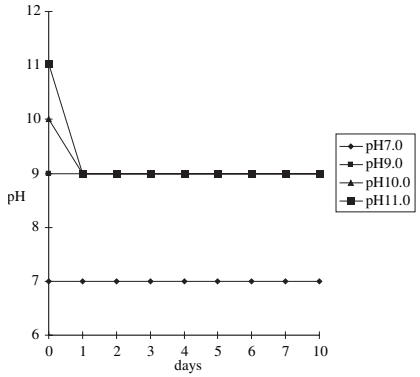


*Fig. 1.* pH changes of the buffer-enriched BHI-blood agar plates, showing that the pH 7.0 and pH 9.0 BHI-blood agar plates were found to be efficient at maintaining the pH for 10 days in an anaerobic glove box. The pH of the buffer-enriched pH 10.0 and 11.0 BHI-blood agar plates were lowered to pH 9.0 within 1 day.

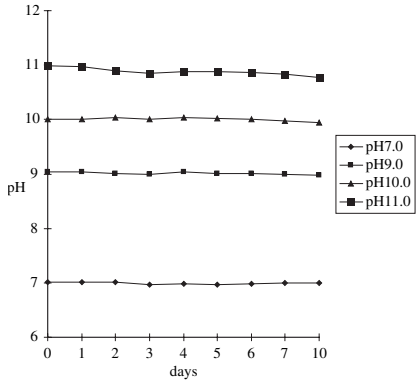


*Fig. 2.* pH changes of the buffer-enriched BHI broth media, showing that these broth media were efficient at maintaining the proper alkaline pH 9.0, 10.0 and 11.0 for 10 days in an anaerobic glove box.

In contrast, on the other hand, with the buffer-enriched BHI broth media, the proper alkaline pH of 9.0, 10.0, and 11.0 was maintained during the experiments (Fig. 2) when the media were prepared and maintained as described under 'Material and methods'. And these buffer-enriched broth media were further used to confirm alkali-resistant bacterial strains from the infected root canals in the anaerobic glove box.

Of 37 teeth, 25 (67%) were shown to be culture-positive on the standard BHI-blood agar plates and the buffer-enriched BHI-blood agar plates adjusted to pH 7.0. The total bacterial recovery on the standard BHI-blood agar plates (mean $9.2 \times 10^4$, range $5.5 \times 10^5 - 1.0 \times 10^1$; $n = 25$) was similar to that of the buffer-enriched BHI-blood agar plate adjusted to pH 7.0 (mean $8.3 \times 10^4$, range $7.0 \times 10^5 - 1.0 \times 10^1$; $n = 25$) (Table 1). This result indicated that a high concentration of buffer components did not alter the growth of bacteria taken from infected root dentin.

*Table 1.* Bacterial recovery from infected root dentin

| BHI-blood agar plates | CFU/sample (n = 25) | | |
|---|---|---|---|
| | Mean | Range | Median |
| Standard pH 7.0 | $9.2 \times 10^4$ | $5.5 \times 10^4 \sim 1.0 \times 10^1$ | $5.0 \times 10^3$ |
| Buffer-enriched | | | |
| pH 7.0 | $8.3 \times 10^4$ | $7.0 \times 10^5 \sim 1.0 \times 10^1$ | $3.7 \times 10^3$ |
| pH 9.0 | $2.2 \times 10^4$ | $3.2 \times 10^5 \sim 1.0 \times 10^1$ | $1.0 \times 10^4$ |
| pH 10.0 | $1.0 \times 10^4$ | $1.3 \times 10^5 \sim 1.0 \times 10^1$ | $3.0 \times 10^2$ |

Total bacterial recovery on the buffer-enriched BHI-blood agar plate at pH 9.0 ranged from $3.2 \times 10^5$ to $2.0 \times 10^1$, and corresponded to 29% (± 30%, $n = 25$) of that at pH 7.0. At pH 10.0, recovery ranged from $1.3 \times 10^5$ to $1.0 \times 10^1$ and corresponded to 18% (± 27%, $n = 25$) of that at pH 7.0 (Table 1).

In all, 109 strains were selected and purified on the basis of morphologic differences of the colonies obtained from the buffer-enriched BHI-blood agar plates (pH 9.0 or 10.0). It is possible, in the present study, that the alkaline pH condition of the agar plates might have been lowered if the bacteria had produced acids as end products around the colonies after growing. After changing from a high alkaline condition to low pH, some non-alkali-resistant bacteria might grow in that lower pH condition. This would occur only under mixed culture conditions. To confirm the alkali resistance, these 109 strains isolated from the buffer-enriched BHI-blood agar plates were inoculated to the buffer-enriched BHI broth media and incubated in the anaerobic glove box for 7 days at 37°C.

Among the 109 strains, 29 isolates (27%) were further confirmed to grow at pH 9.0 in the buffer-enriched BHI broth and 4 isolates (4%) also grew at pH 10.0. These isolates were further identified by molecular methods as alkali-resistant bacteria. These alkali-resistant bacteria were isolated from 15 of the 25 teeth (60%).

Of 29 isolates, 12 isolates were classified as genus *Enterococcus*, composed of 10 strains of *E. faecium* and 2 strains of *E. faecalis*. The other 17 isolates were assigned to *Enterobacter cancerogenus*, *Fusobacterium nucleatum, Klebsiella ornithinolytica, Lactobacillus rhamnosus*, *Streptococcus anginosus*, *Streptococcus constellatus*, *Streptococcus mitis*, *Firmicutes* species, and *Staphylococcus* species by PCR analysis using specific primer sets or 16S rDNA sequence analyses (Table 2).

## Discussions

BHI-blood agar plate is one of the most efficient media supporting the growth of oral bacteria, especially anaerobic species (12, 13). But if the pH of that medium is adjusted to a high pH by using tap water, the pH of the medium was easily lowered during storage inside an anaerobic glove box, as shown in our preliminary experiment. Therefore, instead of tap water for preparation of the medium, we used buffer solutions to maintain a high pH of the medium during culture of bacteria in an anaerobic glove box. These buffer-enriched BHI-blood agar plates were sufficient to maintain the high pH of the media for 10 days in the anaerobic glove box. We also established that the buffer-enriched BHI broth system could maintain a high pH during bacterial culture. These buffer-enriched BHI media did not change the recovery of bacteria from any sample when compared with standard BHI media at pH 7.0. In the present study, alkali-resistant bacteria were detected on the buffer-enriched BHI-blood agar plates, and their capacities for alkali resistance were confirmed in the buffer-enriched BHI broth media.

When root canals are filled with $Ca(OH)_2$ medicaments and a high alkaline environment is provided, almost no bacteria can survive (10). However, a few studies (24, 26) indicated that the alkaline pH of root canal systems may be lowered by components in the exudate of bacterial

*Table 2.* List of alkali-resistant bacteria

| Bacterial species | No. of isolates | |
|---|---|---|
| | pH 9.0 | pH 10.0 |
| *Enterobacter cancerogenus* | 1 | |
| *Enterococcus faecalis* | 2 | 1 |
| *Enterococcus faecium* | 10 | |
| *Fusobacterium nucleatum* | 1 | |
| *Klebsiella ornithinolytica* | 2 | |
| *Lactobacillus rhamnosus* | 2 | |
| *Streptococcus anginosus* | 2 | |
| *Streptococcus constellatus* | 3 | 2 |
| *Streptococcus mitis* | 2 | |
| *Firmicutes* species | 1 | |
| *Staphylococcus* species | 3 | 1 |
| Total | 29 | 4 |

products such as acids. Chemical substances in dentin, such as proteins, phosphate and $CO_2$, may also cause a drop in the alkalinity in the root canal system. If so, bacteria, even though isolated from root canal systems after $Ca(OH)_2$ filling, will not always be alkali resistant. On the other hand, even alkali-resistant bacteria may easily be killed when exposed to the high alkalinity of $Ca(OH)_2$ (2, 7, 28). Thus, bacterial survival may depend on the location in the lesions. Such bacteria which could grow at pH 9.0 or pH 10.0 in the present study, are most likely candidates to survive in the root canal system after $Ca(OH)_2$ filling.

There are several reasons why bacteria survive in a high pH environment. Evans et al. (5) have reported that survival of microorganisms in $Ca(OH)_2$ appears to be unrelated to stress-induced protein synthesis, but a functioning proton pump is critical to survival at high pH. Also, the bacterial tolerance to pH changes may be due to the activation of specific proton pumps, specific enzymatic systems and/or buffering devices, which help to keep the internal pH practically constant (21). In addition, it is probable that some bacteria may be protected from medicaments in closed dentinal tubules, and some bacterial cells may be covered by structural polymers.

In the present study, microorganisms of various genera and species were clearly isolated and identified as alkali-resistant bacteria from infected root dentin. Twenty-nine strains of alkali-resistant bacteria were isolated from root canal dentin of 15 teeth, and *Enterococcus* species was most often isolated, in agreement with previous reports (18, 30).

Recent advances in molecular techniques based on direct amplification of the 16S rRNA gene from DNA extracted from bacterial cells have made it possible to rapidly identify bacterial species in clinical samples. In particular, bacterial identification using PCR with the specific primer sets has become established as regards its sensitivity, simplicity and usefulness (31). In the present study, five species-specific primer sets and one genus-specific primer set were used for identification of the alkali-resistant microorganisms.

With this PCR method, 10 strains of *E. faecium*, two strains of *E. faecalis* and three strains of the genus *Staphylococcus* species were identified. It is reported that all bacterial species belonging to genus *Enterococcus* can grow at pH 9.6 (19). Although we used species-specific primer sets for five *Enterococcus* species, only two species, *E. faecium* and *E. faecalis*, were detected as alkali-resistant bacteria in the present study. This finding is consistent with previous reports that enterococci have often been isolated from root canal systems after $Ca(OH)_2$ filling. Also we identified three strains of genus *Staphylococcus,* known to be able to grow at pH 9.3 (17), as alkali-resistant bacteria in infected root canal dentin.

With the 16S rDNA sequence analysis, a total of 15 strains were identified as *E. cancerogenus*, *F. nucleatum*, *K. ornithinolytica*, *L. rhamnozsus*, *S. anginosus*, *S. constellatus* or *S. mitis*. One strain was identified as a *Firmicutes* species. Munson et al. (20) isolated 65 bacterial species or clones from endodontic samples of five patients, comprising over 29 bacterial genera. In the present study, 29 strains were isolated as alkali-resistant bacteria in the infected root canals, and assigned to eight bacterial genera. These bacterial genera were all included in the list of the report by Munson et al. (20) with the exception of genus *Klebsiella.*

Among the alkali-resistant bacteria, the most frequent isolates were enterococci (48.2%), further identified as *E. faecium* and *E. faecalis*. These *Enterococcus* species are known to be able to grow with 6.5% NaCl (19), and to be vancomycin-resistant (6). Our findings suggest that these enterococci may be multiresistant microorganisms. In a recent study these microorganisms represented the third most common cause of hospital-acquired bacteremia (23). The methods used in the present study may be helpful as a first step in isolating the various types of resistant bacteria.

Other bacterial species isolated in the present study are not predominant microorganisms in human oral microflora, and have not previously been known to be alkali resistant. In the previous reports, it was found that only *E. faecium*, *E. faecalis* and *F. nucleatum* remained viable in root canal dentin after relatively extended periods of treatment with $Ca(OH)_2$ paste or $Ca(OH)_2$ with saline solution (25). This is the first report that many bacterial genera and species can be isolated from the infected root canal as alkali resistant.

In conclusion, the present study has demonstrated that alkali-resistant microorganisms in infected root dentin comprise far more diverse bacterial genera and species than previously reported, belonging mainly to the genus *Enterococcus*. These alkali-resistant bacteria may play important etiologic roles in the infection of root canals after $Ca(OH)_2$ filling.

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