Detection of Identical Isolates of *Enterococcus faecalis*

from the Blood and Oral Mucosa in a Patient with Infective Endocarditis

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Abstract

The detection of infective endocarditis (IE) of oral origin has been previously discussed. However, there are few reports confirming this infection using molecular biological techniques. We herein describe the case of a 67-year-old man who developed IE. Blood culture samples and strains obtained from the gingival and buccal mucosa showed 100% identity to *Enterococcus faecalis* JCM 5803 on sequencing of 16S rRNA gene fragments. A random amplification of polymorphic DNA (RAPD) analysis showed the same pattern for these samples, thus confirming the identity of *E. faecalis* isolates in the blood and oral mucosa. Our observations provide novel information regarding the level of identity between IE pathogens and oral bacteria.

Key words: causative pathogen, identity, infective endocarditis, oral bacteria

Introduction

Infective endocarditis (IE) of oral origin has been frequently discussed in the literature. In the majority of retrospective studies published during the 1990's, the oral cavity was identified to be the portal of entry of the causative microbial agent in 14% – 20% of cases of bacterial endocarditis (1 – 3), with the prevalence of bacterial endocarditis of possible oral origin in recently published series being similar to that reported in these older series (4 – 6).

However, reports confirming the direct origination of the IE pathogen from the oral cavity are very limited. In 1995, the oral origin of two cases of bacterial endocarditis was demonstrated using molecular biological techniques, as complete concurrence of identity was observed between isolates from the blood and oral cavity (7). Subsequently, a case of IE caused by *Granulicatella elegans* originating in the oral cavity was reported (8). The genotype of the oral *G. elegans* strain was indistinguishable from that of a blood-derived isolate that caused IE, indicating that the bacteria were derived from the same clone (8). On the other hand, Nomura et al. (9) isolated *Streptococcus mutans* from the heart valve and dental plaque specimens of a patient with bacterial endocarditis using the application of broad-range polymerase chain reaction (PCR) with DNA sequencing. A DNA fingerprinting analysis indicated that all of the oral isolates of *S. mutans* had a similar pattern, which

differed from that of the isolates obtained from the infected heart valve. These observations indicate that the *S. mutans* strains isolated from the infected valve were distinct from typical oral strains. There have been few studies of the similarity of blood and oral isolates evaluated using molecular biological techniques, although there are many reports of oral bacteria species detected as IE-causing pathogens according to culture methods.

In this report, we examined the similarity of blood and oral isolates from an IE patient using molecular biological techniques. The clinical results for the causative pathogen (blood isolates) obtained according to the culture method identified *G. elegans* or *Enterococcus faecalis*, while the results of a 16S rRNA gene analysis found the causative pathogen (blood isolates) to be *E. faecalis*. We subsequently isolated *E. faecalis* from the patient's oral buccal and gingival mucosa and performed a random amplification of polymorphic DNA (RAPD) analysis, the results of which showed the blood and oral isolates to be the same. Notably, the patient had no remarkable oral problems, i.e., he had a good periodontal status and oral hygiene.

We herein present this case along with the results of the above analyses and discuss the role of oral bacteria as IE-causing pathogens.

Case Report

The patient, who provided informed consent for the publication of this case report, was a 67-year-old Japanese man with arrhythmia controlled by a pacemaker for the past five years; the device had been inserted at another hospital. Prior to admission, he had experienced a recurrent fever for three months despite receiving antibiotic treatment. He was subsequently referred to Okayama University Hospital, where aortic valve vegetation measuring 6 mm in diameter was observed on echocardiography. He was therefore admitted to the Department of Cardiovascular Medicine. The clinical results for the causative pathogen (blood isolates) obtained using the automated VITEK system (SYSMEX bioMérieux, Tokyo, Japan) identified *G. elegans*, which was detected in independent blood cultures performed on three consecutive days (Table), and a diagnosis of IE was made. No signs of infection suspected to be related to IE were observed, including in the urinary, intestinal or biliary tract. As *G. elegans* is a component of the oral mucosal flora (10), four days after admission, the patient was referred by his physician to the Division of Hospital Dentistry for an oral examination and treatment, if necessary.

Generally, periodontitis is diagnosed by measuring the pocket depth and degree of loss of attachment using a periodontal probe supplemented by radiographic findings (11, 12). The radiographs indicated that the patient had previously suffered from mild chronic

periodontitis; however, it had been well controlled with good plaque control. The gingiva did not show any inflammatory redness (Fig. 1), and a periodontal pocket examination (6 points per tooth) indicated that the patient did not have any deep periodontal pockets (range: from 1 to 3 mm, which is within the normal range) or bleeding on probing, indicating a lack of inflammation in the gingiva. The patient performed daily brushing with an ultrasonic toothbrush and had a high level of awareness of oral hygiene; there was no visible dental plaque. An oral bacterial sample was obtained by swabbing the buccal and gingival mucosa over as large an area as possible in order to confirm the presence of identical isolates of the IE pathogen. An examination of the similarity of the bacteria identified in the blood and oral cavity was performed as described in the Materials and Methods section.

The IE was initially treated with antibiotics on the following schedule (shown in days after admission): days 1 – 3, VCM 0.5 g × 4 + GM 60 mg × 3; days 4 – 7, ABPC/SBT 3 g × 4 + GM 60 mg × 3; days 8 – 26: ABPC 2 g × 4 + GM 90 mg × 2.

The pacemaker was removed on day 8 after admission. *Enterococcus faecium* was isolated from the right ventricular (RV) pacing lead according to the proliferation culture method, whereas no bacteria were detected on the right atrium pacing lead or in the pacemaker pocket (Table). Although the pacemaker had been inserted five years previously, the lead was easily removed. *E. faecium* was detected only once and not identified in any of

the other cultures (Table).

Blood cultures were positive on days 1, 2, 3, 7 and 10 after admission. All bacteria detected using the automated VITEK system (SYSMEX bioMérieux) were found to be *G. elegans*, and all isolates showed similar antibiotic sensitivity. After day 11, all blood cultures were negative. No antibiotics were used after day 27, and no signs of infection were subsequently observed. However, despite four weeks of antibiotic administration, aortic valve vegetation continued to be detected on echocardiography, and aortic valve replacement surgery was thus performed on day 56. An area of vegetation measuring 6 mm in diameter was identified attached to the right coronary cusp with perforation, with a small area of vegetation attached to the noncoronary cusp. Modified bilateral maze and permanent pacemaker implantation were additionally performed. The surgery was successful, and the patient was discharged from the hospital on day 79 after admission (postoperative day 23).

Clinical bacterial samples and culture conditions

The blood culture samples BC1 and BC2 obtained from the first and fourth blood cultures performed on days 1 and 7 after admission, respectively (Table), were evaluated at the Clinical Laboratory of Okayama University Hospital. The colonies in both samples were identified using the automated VITEK system (SYSMEX bioMérieux), according to the manufacturer's instructions; the results indicated that both isolates were *G. elegans*.

An oral mucosal bacteria sample was obtained by swabbing the patient's buccal and gingival mucosa on day 4 after admission and subsequently plated and cultured for four days on chocolate agar plates (Kyokuto Pharmaceutical Industrial, Tokyo, Japan) at 37°C under microaerobic conditions (CO_2: 5% – 8%, O_2: 6% – 12%). Twelve colonies (OK1 – 12), with characteristics similar to those of BC1 and BC2, were carefully isolated from among the multiple large and small colonies formed on the chocolate agar and subjected to subsequent analyses.

DNA extraction

The InstaGene Matrix (Bio-Rad, Hercules, CA) was used for DNA extraction from

cultivated bacterial colonies according to the manufacturer's instructions. Aliquots of the

obtained DNA samples were used as templates for subsequent molecular biological analyses.

Sequence analysis of 16S rRNA gene fragments

16S rRNA gene fragments of approximately 600 bp were amplified via PCR using

the primers 341f (5'-CCT ACG GGA GGC AGC AG-3') (13) and 907r (5'-CCG TCA ATT

CMT TTG AGT TT-3') (14). PCR was performed with an initial denaturation step of 95°C for

five minutes followed by 25 cycles of 95°C for one minute, 55°C for one minute and 72°C

for one minute, with a final extension step of 72°C for five minutes. The amplified DNA

fragments were cloned using a TOPO TA Cloning® Kit (Invitrogen, Carlsbad, CA) according

to the manufacturer's instructions, and the insert DNAs were sequenced. DNA sequencing

was performed using a BigDye® cycle sequencing kit (Applied Biosystems, Foster City, CA)

and an automated DNA sequencer (3130xl Genetic Analyzer; Applied Biosystems). The

sequence data were analyzed using the BLAST sequence homology search program at

GenBank.

Random amplification of polymorphic DNA (RAPD)

A RAPD analysis for oral and blood isolates was performed using Ready-To-Go™ RAPD Analysis Beads (GE Healthcare UK Ltd., Little Chalfont, Buckinghamshire, UK) according to the manufacturer's instructions. DNA extracts from the blood isolates and oral isolates were subjected to RAPD analyses. DNA extracted from *E. faecalis* NBRC100481 was also used as a control to confirm the ability to identify polymorphisms. RAPD-PCR was performed by adding 50 pmol of primers, 50 ng of template DNA and distilled water to a final volume of 25 μL to one Ready-To-Go™ RAPD Analysis bead (GE Healthcare UK Ltd). The following primers supplemented with Ready-To-Go™ RAPD Analysis beads (GE Healthcare UK Ltd) were used: primer 1, 5'-GGT GCG GGA A-3'; primer 2, 5'-GTT TCG CTC C-3'; primer 3, 5'-GTA GAC CCG T-3'. PCR was performed with an initial denaturation step of 95°C for five minutes followed by 45 cycles of 95°C for one minute, 36°C for one minute and 72°C for two minutes. The products were electrophoresed on 2% agarose gels, and the band patterns were evaluated visually using staining with ethidium bromide.

Matrix Assisted Laser Desorption/Ionization (MALDI)-Time of Flight Mass Spectrometry

(TOFMS) analysis of the blood isolates

The blood isolates identified to be *G. elegans* according to the VITEK system
(SYSMEX bioMérieux) did not grow well. We therefore performed a Matrix Assisted Laser
Desorption/Ionization (MALDI)-Time of Flight Mass Spectrometry (TOFMS) analysis of
these bacteria using a MALDI-TOF Microflex mass spectrometer (Bruker Daltonik, Bremen,
DE), according to the manufacturer's instructions. The bacteria were identified by matching
the fragmentation patterns in the mass spectra with the libraries using the maldiBioTyper 2.0
software program (Bruker Daltonik).

Results

Sequence analysis of the 16S rRNA gene fragments obtained from the blood isolates

The blood isolates BC1 and BC2 had identical sequences to each other, and the obtained sequences of 589 bp showed 100% identity to *E. faecalis* strain JCM 5803 16S ribosomal RNA. The sequences also showed 97% similarity to *Granulicatella balaenopterae* strain M1975/96/1, 96% similarity to *Granulicatella adiacens* strain GIFU 12706 and 95% similarity to *G. elegans* ATCC 700633 strain B1333. The clinical results for BC1 and BC2 obtained according to the culture method identified both isolates as *G. elegans*, and their level of genetic similarity was very high. In contrast, the results of the molecular analysis indicated that BC1 and BC2 were both *E. faecalis* rather than *G. elegans*.

Sequence analysis of the 16S rRNA gene fragments obtained from the oral isolates

A sequence analysis of the 16S rRNA gene fragments was also performed for the oral isolates OK1 – 12. Two strains, OK5 and OK10, showed 100% identity to *E. faecalis* strain JCM 5803 16S ribosomal RNA, the same as BC1 and BC2.

RAPD analysis of the oral and blood isolates

The results of the RAPD analysis of the blood and oral isolates (BC1, BC2, OK5 and OK10) and *E. faecalis* NBRC100481 are shown in Figure 2. Three random primers were used, and the RAPD pattern with two of three primers identified the clinical isolates and *E. faecalis* NBRC100481; the clinical isolates showed the same pattern. One of the three primers showed the same pattern in all samples, likely because all strains were *E. faecalis,* and this primer could not distinguish between minor genetic differences.

MALDI-TOFMS analysis of the blood isolates

The results of a MALDI-TOFMS analysis of five blood isolates identified to be *G. elegans* according to the VITEK system (SYSMEX bioMérieux) indicated the bacteria to be *E. faecalis* rather than *G. elegans*.

Discussion

As *E. faecalis* is considered to be a component of the human intestinal flora, although it is sometimes detected in the oral mucosa, we did not expect to identify this organism as a causative pathogen of IE in the oral cavity. However, the results of the 16S rRNA gene and MALDI-TOFMS analyses indicated the causative pathogen (blood isolates) in this case to be *E. faecalis*. Because the growth of the bacteria was poor, the VITEK system possibly misidentified the bacteria as *G. elegans* instead of *E. faecalis*. Indeed, *E. faecalis* was not dominant in the oral mucosal samples. The main bacteria comprising the oral mucosal flora were streptococci, and it was very difficult to find colonies visibly similar to those of the IE pathogen. Unexpectedly, however, complete identity was observed between the *E. faecalis* isolated from blood and the oral cavity. As the current patient did not have any remarkable oral problems, the reason for this finding is unclear.

RAPD analyses are a technique for rapidly detecting genomic polymorphisms (15). These techniques differ with regard to their DNA amplification conditions, the length of the primers used and the resolution of the products obtained. Arbitrary primers are usually not more than 7 – 10 bases in length, with an arbitrary sequence not directed toward any known target sequence of the bacterial genome. Therefore, the amplification of random segments of genomic DNA is directed by a single oligonucleotide primer of arbitrary sequence (AP-PCR),

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thus generating a characteristic spectrum of short DNA products of various complexities. Polymorphisms can be detected by variations in the length of the obtained amplified sequences, commonly known as RAPD, which can be used to compare bacterial strains (16). RAPD-PCR analyses are a widely accepted and reliable tool for differentiating and identifying enterococci, as described in a previous review article (16). For example, Tyrrell et al. (17), Quednau et al. (18) and Monstein et al. (19) described its reliability for differentiating and identifying enterococci. Furthermore, in the current case, a sequence analysis of the 16S rRNA gene fragments of 589 bp showed 100% identity between the blood isolates and oral isolates. Therefore, the results of the RAPD and sequence analyses indicated the blood and oral isolates to be the same.

The patient's had no remarkable oral problems, and his oral hygiene was good at the time of IE onset. Que and Moreillon (20) assessed the theoretical cumulative development of bacteremia resulting from daily oral activities, such as brushing the teeth or chewing, and concluded that the 1-year daily risk of bacteremia is 6×10^6 times greater than that associated with tooth extraction. We agree that maintaining optimal oral health and hygiene may reduce the incidence of bacteremia from daily activities and the risk of IE. On the other hand, the present results suggest that there some cases of bacteremia are not realistically preventable, even with good oral hygiene, as bacteremia originating from the oral cavity can occur

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continuously even if the patient's oral condition is good.

Another explanation for isolation of the same *E. faecalis* strain from the blood and oral mucosa is that the origin of the IE pathogen, *E. faecalis*, may not have been the oral cavity. The pathogen may have originated from other sites, e.g., the intestinal flora, and subsequently caused IE, with detection in the oral cavity via transfer from the blood. However, this possibility is unlikely in this case because the bacterial count in the blood was low, even though the patient exhibited bacteremia. This phenomenon may confound discussions regarding the role of oral bacteria as IE pathogens. However, even streptococci have been reported as IE pathogens; in one study, a DNA fingerprinting analysis indicated that all of 18 oral isolates of *S. mutans* had a similar pattern, which subsequently differed from that of the isolates from the infected heart valve (9). The authors indicated that the properties of the *S. mutans* strains isolated from the infected valve were different from those of typical oral strains (9). It is very difficult to confirm whether oral bacteria are actually IE pathogens.

It is interesting that *E. faecium* was isolated once from the pacemaker RV lead, while the findings of the blood isolates were completely different (initially considered to be *G. elegans* and later shown to be *E. faecalis* on the 16S rRNA gene analysis). This phenomenon again confounds the identification of the causative pathogen. The current patient may have

suffered from a mixed infection. Notably, five independent blood isolates obtained at different times showed a similar pattern of antibiotic sensitivity. Furthermore, the results of the RAPD and 16S rRNA gene analyses were identical for all of the oral isolates. Evidence suggesting that transient bacteremia associated with dental, gastrointestinal and/or genitourinary procedures may result in the infection of cardiovascular implantable electronic devices is lacking (21).

In conclusion, the same *E. faecalis* isolates were identified in the blood and oral mucosa in a patient with IE. This observation suggests that there is a route between the focus of infection with *E. faecalis* IE and the oral mucosa. Non-dominant bacteria of the oral flora, such as *E. faecalis*, may cause IE despite good oral hygiene. On the other hand, the pathogen may have originated from other sites, e.g., the intestinal flora, and caused IE in this case, with detection in the oral cavity. It is very difficult to clarify whether oral bacteria are actually responsible pathogens for IE.

Acknowledgments

Conflicts of interest

The authors have no conflicts of interest related to this study.

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Table

Table. Results of the bacterial examinations

Day after admission (day)	Sample	Identified bacteria
1	blood (BC1)	*E. faecalis*[†]
	urine	negative
2	blood	*E. faecalis*[†]
3	blood	*E. faecalis*[†]
7	blood (BC2)	*E. faecalis*[†]
8	pacemaker RV lead*	*E. faecium*
	pacemaker RA lead*	negative
	pacemaker pocket (upper)*	negative
	pacemaker pocket (lower)*	negative
10	blood	*E. faecalis*[†]
11	stool	Enterobacteriaceae (GNR)
12	blood	negative
19	blood	negative
26	blood	negative
37	blood	negative
56	vegetation*	negative

*Proliferation culture method.

[†]The pathogens were identified as *G. elegans* according to the VITEK system (SYSMEX bioMérieux); however, the results of the MALDI-TOFMS analysis of the isolates indicated

the bacteria to be *E. faecalis* rather than *G. elegans.*

Figure legends

Fig. 1

Gingival appearance in the present case. The patient had mild chronic periodontitis, although it was well controlled with good plaque control. The gingiva did not show any inflammatory redness. A periodontal pocket examination (6 points per tooth) indicated that the patient had periodontal pockets ranging from 1 to 3 mm in depth, which was within the normal range, with no bleeding observed on probing, indicating a lack of inflammation in the gingiva.

Fig. 2

Results of the RAPD analysis of the blood and oral isolates (BC1, BC2, OK5 and OK10) and type strain (TS): *E. faecalis* NBRC100481.

Three random primers were used. The RAPD patterns of primers 1 and 2 identified the clinical isolates and *E. faecalis* NBRC100481; the clinical isolates showed the same pattern. The RAPD pattern obtained with primer 3 was the same in all samples, likely because all of the strains were *E. faecalis,* and this primer could not distinguish between minor genetic differences. M: 100-bp DNA ladder marker.

Fig. 1

Fig. 2.