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Novel Isolation and Identification Methods for *Enterococcus* Species and Their Distribution in the Human Oral Cavity

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Abstract

Purpose: In recent years, vancomycin-resistant *Enterococcus* (VRE) has spread worldwide, and the acquisition of resistance to antibiotics other than vancomycin has become a significant problem. While *Enterococcus* species have been considered part of the normal flora of the digestive tract, including the oral cavity, it remains unclear whether they are actually present in all human mouths and which species are detected. Therefore, this study aimed to develop selective media for reliable detection of *Enterococcus* species from various oral samples and to establish a highly accurate identification and detection method using multiplex PCR targeting five *Enterococcus* species. Using this method, we conducted a detailed investigation of the distribution of these organisms in the human oral cavity. **Methods:** Antimicrobial susceptibility testing using antibiotic discs was performed to develop selective media for the highly accurate detection of *Enterococcus* species in human oral samples. Additionally, *Enterococcus* species-specific primers for multiplex PCR identification were designed. Furthermore, the developed selective media and multiplex PCR method were used to investigate the distribution of *Enterococcus* species in 30 subjects and the emergence of vancomycin resistance in isolated strains. **Conclusion:** *Enterococcus* species were detected in 8 subjects (26.7%). Multiplex PCR-based bacterial species identification revealed that the *Enterococcus* isolates were *E. faecalis*, *E. hirrae*, *E. casseliflavus*, and *E. durans*. No VRE was detected.

Keywords

Enterococcus Species, Selective Medium, Oral Cavity

1. Introduction

By 2025, the genus *Enterococcus* comprised a large taxonomic group consisting of 90 species and 3 subspecies (https://lpsn.dsmz.de/genus/*enterococcus*). Enterococci, ubiquitous in nature, are Gram-positive, catalase-negative, facultative anaerobic cocci belonging to the lactobacilli group. Enterococci are important not only because they are a leading cause of nosocomial infections, but also because they may have a significant role in dissemination and persistence of antimicrobial resistance [1] [2]. The widespread use and misuse of antimicrobials such as glycopeptides and aminoglycosides in human and livestock have resulted in the rapid increase of vancomycin and high-level gentamicin-resistance in *Enterococcus* strains [3]. Vancomycin-resistant enterococci (VRE) were first isolated from patients in 1988 in the United Kingdom and France [4]. Since then, VRE have spread to many other countries including Malaysia, through meat from livestock and humans [5] [6]. Vancomycin resistance in enterococci has been classified based on the gene sequence and resistance characteristics. The vanA-type strains are resistant to high levels of both vancomycin and teicoplanin antimicrobials (MIC $\geq$ 64 μg/ml and >16 μg/ml, respectively). vanB-type strains are resistant to a wide range of vancomycin concentrations (MIC between 4 and $\geq$1024 μg/ml) and are susceptible to teicoplanin. vanD-type strains are resistant to moderate levels of vancomycin (MIC 128 μg/mL) and susceptible to teicoplanin, while vanC, vanE, and vanG-type strains exhibit low-level resistance to vancomycin [4] [7].

Accurate isolation and identification methods are essential for monitoring which species cause disease for therapeutic purposes. It is currently unclear whether *Enterococcus* species are part of the normal oral microbiota. Therefore, appropriate selective media are necessary to evaluate the oral distribution of *Enterococcus* species involved in nosocomial infections. Several selective media for these organisms have been developed for their isolation [8] [9]. Commercially available Enterococcosel agar (Becton Dickinson) is also usable. Selective media developed to date exhibit high selectivity for enterococci but may allow growth of some staphylococci, potentially leading to false positives. Most selective media use sodium azide as the selective agent; however, this substance is highly toxic to humans and can adversely affect health. Therefore, using these selective media in routine clinical testing and research may not be appropriate from both human health and environmental perspectives. Presently, the standard method for identification of enterococci is phenotypic characterization, primarily using biochemical tests [10]. Tests are usually performed in test tubes and may require significant amounts of time for preparation and interpretation of results. Furthermore, processing of large numbers of samples is inhibited by phenotypic characterization,

as 10 or more tests may be necessary for differentiation of the species. Commercial identification kits, such as the API Rapid ID 32 Strep and BBL Crystal identification gram-positive ID kits, and automated identification systems, such as the VITEK gram positive identification system, are available for identifying enterococci to the species level [11]-[13]. These methods have been developed to allow rapid identification of enterococci based upon reactions to panels of biochemicals. Although the kits are cost-effective and results can be obtained in less than 24 h, there are concerns about the reliability of the kits [11] [14].

Previous studies [15] [16] have detected five *Enterococcus* species (*E. faecium*, *E. faecalis*, *E. casseliflavus*, *E. hirrae* and *E. durans*) from the human oral cavity. However, the detailed distribution of these *Enterococcus* species remains unclear. Furthermore, the vancomycin resistance status among oral isolates also remains unknown.

The objective of this study is to develop a novel selective medium for isolating *Enterococcus* species, establish a simple and more reliable testing method for identification at species level using multiplex PCR targeting five *Enterococcus* species detected in the human oral cavity, and evaluate the distribution of this microorganism in the oral cavity and its development of resistance to vancomycin.

2. Materials and Methods

2.1. Bacterial Strains and Culture Conditions

Bacterial strains were obtained from Japan Collection of Microorganisms (JCM; Japan), Center for Conservation of Microbial Genetic Resource, Gifu University (GTC; Japan), and American Type Culture Collection (ATCC; America). All bacterial strains used in the present study are listed in **Table 1**. Bacterial strains used in the present study were maintained by cultivating them on Bact™ Brain Heart Infusion (BHI, Becton, Dickinson and Co., Sparks, MD, USA) and 1.5% agar (BHI agar). These organisms were cultured at 37˚C overnight under an aerobic condition.

Table 1. Recovery of *Enterococcus* species and other bacteria on BHI agar and OESM.

Species	Strain	BHI-Y CFU/ml, $\times 10^8$	OESM CFU/ml, $\times 10^8$	Recovery (%)
E. faecalis	JCM 5803^T	4.5 ± 0.2a	4.4 ± 0.3	97.8
E. faecium	JCM 5804^T	6.9 ± 0.2	6.8 ± 0.3	98.1
E. casseliflavus	JCM 8723^T	4.5 ± 0.2	4.4 ± 0.3	99.1
E. durans	JCM 8725^T	1.5 ± 0.2	1.5 ± 0.3	98.7
E. hirrae	JCM 8729^T	5.1 ± 0.2	5.0 ± 0.3	98.1
E. avium	JCM 8722^T	1.3 ± 0.2	1.3 ± 0.3	98.8
E. gallinarum	JCM 8728^T	3.1 ± 0.2	3.0 ± 0.3	98.6

Continued

E. lactis	JCM 30200^{T}	6.9 ± 0.2	6.8 ± 0.3	97.7
Staphylococcus aureus	JCM 20624^{T}	8.2	0	0
Staphylococcus epidermidis	JCM 2414^{T}	8.6	0	0
Streptococcus oralis	ATCC 35037^{T}	3.8	0	0
Streptococcus salivarius	ATCC 10557^{T}	1.3	0	0
Actinomyces naeslundii	ATCC 12104^{T}	0.6	0	0
Corynebacterium matruchotii	ATCC 14266^{T}	0.7	0	0
Corynebacterium durum	ATCC 33449^{T}	0.8	0	0
Rothia dentocariosa	JCM 3067^{T}	0.5	0	0
Rothia mucilaginosa	JCM 10910^{T}	0.5	0	0
N. sicca	ATCC 29256^{T}	0.3	0	0

[a]Ave ± SD.

2.2. Development of New Selective Medium

2.2.1. Evaluation of Base Medium

BHI agar supplemented with 1% yeast extract (BHI-Y), BHI-Y supplemented with 5% sheep blood (BHI-Y blood), and Mitis Salivarius agar (MS agar, Becton, Dickinson and Co., Sparks, MD, USA) were examined as the base medium in the selective medium. Ten-fold dilutions of cultures were made in 0.9 ml of Tris-HCl buffer (0.05 M, pH 7.2) and aliquots of 0.1 ml were spread onto the test media. The plates inoculated with bacteria were cultured at 37˚C for 48 h under an aerobic condition. After cultivation, the number of colony-forming units (CFU)/ml was counted.

2.2.2. Susceptibility Tests

Preliminary studies of antibiotic selection were also performed using disk susceptibility tests (Sensi-Disk, Becton Dickinson Co., MD, USA). The microbroth dilution method was used for susceptibility testing [17].

2.3. Recovery of *Enterococcus* Species and Other Representative Bacteria

The recoveries of the *Enterococcus* reference strains and other representative bacteria were calculated as CFU/ml on selective medium and compared with those on BHI agar for total cultivable bacteria. All bacterial strains used in the present study are listed in Table 1.

All bacterial strains were pre-incubated in BHI broth at 37˚C overnight in an atmosphere of 5% CO_2 in a CO_2 incubator. Ten-fold dilutions of cultures were made in 0.9 ml of Tris-HCl buffer (0.05 M, pH 7.2) and aliquots of 0.1 ml were spread onto the test media. The plates inoculated with bacteria were cultured at 37˚C for 48 h under an aerobic condition. After cultivation, the number of CFU/ml was counted.

2.4. Clinical Samples

Thirty volunteers (17 men, 13 women; mean age 35 years, range 18 - 75 years) participated in the present study. They had no systemic disease and received no antibiotic therapy for at least 3 months. All participants were asked not to brush, rinse, or smoke immediately prior to the assessment and not to eat or drink for at least 2 h beforehand. In this study, participants' oral health status (e.g., periodontal disease status, presence of active caries, history of endodontic treatment, denture use, smoking status) was not included as an analytical factor.

Paraffin-stimulated whole saliva samples were collected in a sterile microcentrifuge tube. All samples were dispersed by sonication for 30 s in an ice bath (50 W, 20 kHz, Astrason® System model XL 2020, NY, USA), and 0.1 ml of each was diluted and inoculated on BHI-Y and selective medium plates. The plates were cultured at 37˚C for 2 days in an atmosphere of 5% CO_2 in a CO_2 incubator. After cultivation, CFU/ml in each sample was calculated. The present study was conducted in accordance with the principles of the Declaration of Helsinki, and was approved by the Ethics Committee of Nihon University School of Dentistry at Matsudo, Japan (EC23-012). Participant consent was obtained by the researchers themselves verbally explaining the study details to participants and having them sign a consent form.

2.5. Species Identification of *Enterococcus* Strains Isolated from Clinical Samples

Twenty-four of the approximately 50 colonies that grew on the selective medium plate per subject were randomly isolated and subcultured, and their identity was then confirmed by a PCR analysis.

2.6. Design of Species-Specific Primers for *Enterococcus* Species

Design of species-specific primers for *Enterococcus* species was performed as described previously [18]. Briefly, the *atpA* gene sequences of *E. faecium* (accession no. AB594765), *E. faecalis* (AJ843301), *E. casseliflavus* (EU153590), *E. hirrae* (AJ843484) and *E. durans* (AJ843269) were obtained from the DNA Data Bank of Japan (DDBJ; https://www.ddbj.nig.ac.jp/services.html, Mishima, Japan), and a multiple sequence alignment analysis was performed with the CLUSTAL W program; *i.e.*, the *atpA* gene sequences of five *Enterococcus* species were aligned and analyzed, respectively. Homology among the primers selected for each *Enterococcus* species and their *atpA* gene sequences was confirmed by a BLAST search.

2.7. Development of PCR Method Using Designed Primers

Bacterial cells were cultured in BHI supplemented with 0.5% yeast extract for 24 h, and 1 ml of the samples were then collected in microcentrifuge tubes and resuspended at a density of 1.0 McFarland standard (approximately 10^7 colony-forming units (CFU)/ml) in 1 ml of sterile distilled water. A total of 3.6 µl of the suspension was then used as a PCR template. The detection limit of PCR was as-

sessed by serially diluting known numbers of bacterial cells in sterile distilled water and then subjecting each suspension to PCR. The multiplex PCR mixture contained 0.2 μM of each primer, 10 μl of 2 × MightyAmp Buffer Ver.3 (Takara Bio Inc., Shiga, Japan), 0.4 μl of MightyAmp DNA Polymerase (Takara), and 5 μl of the template in a final volume of 20 μl. PCR reactions were performed in a DNA thermal cycler (Applied Biosystems 2720 Thermal Cycler; Applied Biosystems, CA, USA). PCR conditions included an initial denaturation step at 98˚C for 2 min, followed by 30 cycles consisting of 98˚C for 10 s and 68˚C for 1 min. PCR products were analyzed by 2.0% agarose gel electrophoresis before being visualized by electrophoresis in 1× Tris-borate-EDTA on a 2% agarose gel stained with ethidium bromide. A 100-bp DNA ladder (Takara Biomed, Shiga, Japan) was used as a molecular size marker. All experiments were performed in triplicate.

2.8. Antimicrobial Susceptibility of Isolated *Enterococcus* Strains

The screening of antimicrobial susceptibility of isolated *Enterococcus* strains was performed by disk diffusion method according to the European Committee on Antimicrobial Susceptibility Testing (EUCAST) [19].

3. Results

3.1. Development of Selective Medium

3.1.1. Selection of Base Medium

The selection of a base medium for the growth of *Enterococcus species* was performed. *Enterococcus species* grew well on MS agar as same as BHI-Y and BHI-Y blood (data not shown). To inhibit the growth of Gram-positive bacteria except genera *Enterococcus* and *Streptococcus*, and Gram-negative bacteria, MS agar was ultimately selected as the base medium.

3.1.2. Susceptibility to Antibiotics

Enterococcus species exhibited resistance to sodium chloride, trimethoprim-sulfamethoxazole combination (ST), 2,3,5-Triphenyltetrazolium Chloride (TTC), and oxacillin. The minimal inhibitory concentrations (MICs) of sodium chloride, ST, TTC, and oxacillin for *Enterococcus* were more than 90 mg/ml, 1000 μg/ml, 1000 μg/ml, and 2 μg/ml, respectively.

3.1.3. Composition of New Selective Medium

The new selective medium, designated oral *Enterococcus* selective medium (OESM), was composed of the following (per liter): 90 g of MS agar, 30 g of sodium chloride, 10 mg of colistin, 500 mg of ST, 180 mg of TTC, and 0.5 mg of oxacillin. Antibiotics, *i.e.*, colistin, ST, TTC, and oxacillin were added after the base medium had been sterilized and cooled to 50˚C.

3.1.4. Recovery of *Enterococcus* Species and Inhibition of Other Representative Bacteria on Selective Medium

Table 1 shows the recovery of some *Enterococcus* reference strains on OESM relative to BHI-Y. The growth recoveries of the *Enterococcus* reference strains on

ABSM were between 97.7% and 99.1% (average 98.4%) that on BHI-Y.

Table 1 also shows the inhibition of other representative bacteria except *Enterococcus* species on OESM relative to BHI-Y. The growth of other representative bacteria was markedly inhibited on the selective medium.

3.2. PCR Method for Identifying Five *Enterococcus* Species

3.2.1. Primer Design

The specific primer set covering the upstream region of the *atpA* gene sequence of five *Enterococcus* species was designed in the present study (Table 2). The amplicon size of *E. faecium*, *E. faecalis*, *E. casseliflavus*, *E. hirrae* and *E. durans* was 169 bp, 255 bp, 345 bp, 563 bp and 718 bp, respectively.

Table 2. Locations and sequences of species-specific primers for the *atpA* gene of five *Enterococcus* species.

Species	Primer name	Sequence	Product size (bp)
E. faecium	EFCIF	AGAGGCCTTGATCGGACGGG	169 bp
	EFCIR	AAGGGCGTCGATCGCTTTTAGC	
E. faecalis	EFCAF	GGAAGCAACAGCTCCCGGTGTTA	255 bp
	EFCAR	ACGAAGTGTCTCTACTTGGTTACGA	
E. casseliflavus	ECF	CGGCGATGCATTAATTGGCCG	345 bp
	ECR	CGGCGTAATGTTTCTACTTGTGC	
E. hirrae	EHF	TGGGTTAGGAGAAATCGTTACAGAT	563 bp
	EHR	GTTTTGCCGCACGTTCTAGTAAACG	
E. durans	EDF	TGCAACAGACAAGGCTCGTCC	718 bp
	EDR	CCCAGCGTCAACAGCTGGTC	

3.2.2. PCR Condition

A multiplex PCR method for identifying five *Enterococcus* species successfully amplified DNA fragments of each expected size (Figure 1). The detection limit was assessed in the presence of titrated bacterial cells, and the sensitivity of the PCR assay was between 5 × 1 and 5 × 10 CFU per PCR template (5.0 µl) for the *E. faecalis*-specific primer set with strain JCM 5803 (data not shown).

3.3. Clinical Examination

The detection frequencies of *Enterococcus* species in the saliva samples from thirty healthy subjects are shown in Table 3. *Enterococcus* species were detected in eight saliva samples (26.7%). In positive samples, the mean number of this microorganism and its proportion relative to the total bacterial number were 7.4×10^2 CFU/ml and 0.0003%, respectively. VRE was not detected in any of the samples. Table 4 shows the distribution of detected *Enterococcus* species at species level. *E. faecalis* was the most common, followed by *E. hirrae*, *E. durans*, and *E. casseliflavus* in that order.

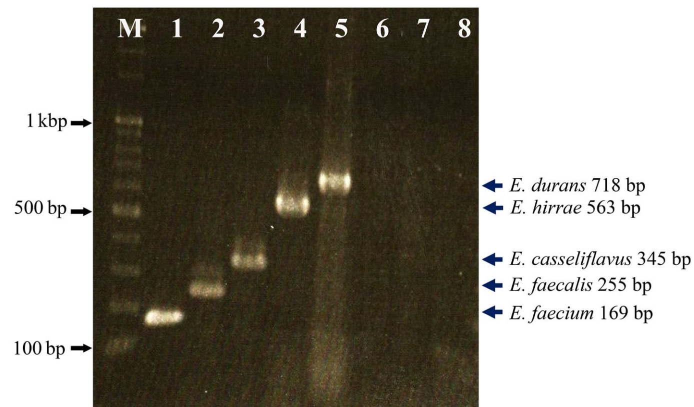

Figure 1. Multiplex PCR assay for detecting five *Enterococcus* species. The primer mixture contained EFCIF, EFCIR, EFCAF, EFCAR, ECF, ECR, EHF, EHR, EDF, and EDR. Lanes: 1, *E. faecium* JCM 5804; 2, *E. faecalis* JCM 5803; 3, *E. casseliflavus* JCM 8723; 4, *E. hirrae* JCM 8729; 5, *E. durans* JCM 8725; 6, *E. avium* JCM 8722; 7, *E. gallinarum* JCM 8728; 8, *E. lactis* JCM 30200. M, molecular size marker (100-bp DNA ladder).

Table 3. Detection frequency of *Enterococcus* species in the saliva samples.

No. of *Enterococcus* positive samples (%, frequency) n = 30	No. of VRE positive samples (%, frequency) n = 30	No. of total bacteria (CFU/ml)	No. of *Enterococcus* (CFU/ml)	*Enterococcus*/total bacteria (%)
8 (26.7)	0 (0)	2.1×10^8	7.4×10^2	0.0003

Table 4. Distribution of detected *Enterococcus* species at species level.

No. of *Enterococci* positive samples n = 30	*E. faecalis*	*E. faecium*	*E. casseliflavus*	*E. durans*	*E. hirrae*
8	6	0	1	2	4

In the initial isolation, *Enterococcus* genus colonies on OESM generally exhibited a circular, smooth appearance. The colony color was reddish-purple. Therefore, on OESM, they could be distinguished from other bacteria based on colony morphology. The average colony size of *Enterococcus* species on OESM was 1.2 mm in diameter (**Figure 2**).

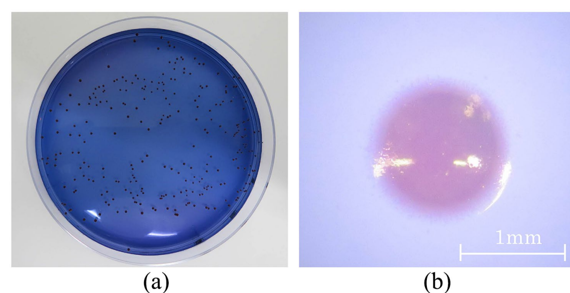

Figure 2. Appearance of *Enterococcus* colonies on OESM. (a) *E. faecalis* colonies on OESM inoculated with a saliva sample. (b) Stereomicroscope image of *E. faecalis* colony on OESM.

4. Discussion

Enterococci are increasingly recognized as a cause of nosocomial infections such as endocarditis, bacteremia, urinary tract infections, and neonatal sepsis [20]. While enterococci are used as an indicator of fecal contamination, they have also been shown to cause human infections, particularly in hospital-associated patients [21]. Furthermore, the widespread use and misuse of antimicrobial agents, such as glycopeptides and aminoglycosides, in humans and livestock has led to a rapid increase in *Enterococcus* strains exhibiting vancomycin resistance and high levels of gentamicin resistance. Some studies have confirmed that *E. faecalis* is the dominant species in teeth with failed root canal treatment [22] [23]. Endodontic infections are polymicrobial, consisting of obligate anaerobes and facultative anaerobes [24]. However, few studies have properly investigated the distribution of *Enterococcus* species and VRE in the human oral cavity. This is due to the lack of established methods for reliably isolating *Enterococcus* species from human oral samples and for accurately identifying them at the species level. Therefore, this study aimed to develop a novel selective medium for isolating *Enterococcus* species, establish a simple and reliable identification method using multiplex PCR, and investigate the distribution of these microorganisms in the oral cavity and their resistance to vancomycin.

In the present study, we designed species-specific primers with the already mentioned means, for the identification at the species level of *Enterococcus* species with a PCR method. These primers were able to distinguish *Enterococcus* species at the species level and did not display cross-reactivity with each other. Moreover, we developed a multiplex PCR method with the ability to *Enterococcus* identify and differentiate species at the species level using only each one PCR tubes per sample. Species-specific primers for five *Enterococcus* species were designed based on the sequences of *atpA* gene. Moreover, the PCR method in the present study directly uses bacterial cells with MightyAmp DNA Polymerase Ver.3 (Takara) and is completed within approximately 2 hours.

A useful selective medium for isolating *Enterococcus* species may contribute to the correct and rapid diagnosis of infectious diseases caused by this microorganism. Several selective media for *Enterococcus* species have been developed [9] [10]. Commercially available Enterococcosel agar (Becton Dickinson) is also usable. Some selective media cannot completely inhibit the growth of fungi or Gram-positive cocci such as staphylococci other than enterococci, significantly inhibit the growth of certain *Enterococcus* species, and are difficult to prepare. Additionally, Enterococcosel agar medium is less selective for human saliva specimens than for fecal specimens. The genus *Enterococcus* was formerly classified within the genus *Streptococcus*, and *Enterococcus* species grow well on MS agar. The novel selective medium OESM uses MS agar as its base medium. In the present study, *Enterococcus* species were more resistant to 30 g of sodium chloride, colistin, ST, TTC, and oxacillin than other representative microorganisms. The growth of other representative bacteria and fungi was inhibited by the addition of 30 g of

sodium chloride, 10 mg of colistin, 500 mg of ST, 180 mg of TTC, and 0.5 mg of oxacillin to MS agar. All of the *Enterococcus* reference strains and isolates tested grew well on the new selective medium, designated as OESM, while the growth of other bacteria was markedly inhibited (**Table 1**). Moreover, OESM allowed for the identification of *Enterococcus* species by its characteristic colony morphology. OESM exhibits high selectivity for enterococci, eliminating the possibility of false positives or false negatives.

In the previous study, the prevalence of enterococci was 18% in diluted saliva samples [25]. In this study, *Enterococcus* species were detected in 8 of 30 saliva samples (26.7%), which was similar to previous study result. Furthermore, in the results of this case, the proportion of *Enterococcus* species within the total bacterial count was significantly low (0.0003%). This result indicate that *Enterococcus* species were found at a very low level in the oral cavity and this organism in oral cavity is probably of exogenous origin. The source of the enterococci found in the oral cavity is thus still unclear. Among the genus *Enterococcus*, *E. faecalis* and *E. faecium* represent approximately 90% of clinical isolates belonging to this genus [2]. In this study, the most frequently isolated species was *E. faecalis*, followed by *E. hirrae*, *E. durans*, and *E. casseliflavus*; however, *E. faecium* was not isolated. The distribution of *Enterococcus* species in the human oral cavity may be highly diverse. Furthermore, no VRE was detected in any of the specimens in this study. These results may be due to the fact that this study involved healthy subjects, utilized saliva samples, or that the number of isolates examined was small.

We developed the selective medium OESM to isolate *Enterococcus* species from various specimens. OESM exhibits high selectivity for *Enterococcus* species and is useful for evaluating the distribution and role of this microorganism in humans and various animals, as well as its antimicrobial resistance.

5. Conclusion

The novel selective medium (OESM) and our PCR method as isolation and identification methods, respectively, for *Enterococcus* species may contribute to the diagnosis of actinomycosis as well as eye infection such as keratitis and canaliculitis, dental caries, endodontic infections, osteomyelitis of the sternum, and infective endocarditis, which are caused by this organism.

Authors' Contributions

Tsuzukibashi O, Fukatsu A, Tayama T, Idei K, Usuda K, Uchibori S, Umezawa K, Iizuka Y and Asano T corrected the data. Tsuzukibashi O, Fukatsu A, Wakami M, Murakami H, Kobayashi T and Fukumoto M drafted and wrote the manuscript. The concept of this manuscript was devised by Tsuzukibashi O. All authors read and approved the final manuscript.

Conflicts of Interest

The authors declare that there is no conflict of interest.

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