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Primer Design for the Identification of Ten Oral *Actinomyces* Species Using Multiplex PCR

Taira Kobayashi[1]*, Satoshi Uchibori[1], Osamu Tsuzukibashi[2], Chihiro Uezato[1], Haruhiko Goto[1], Chiho Mashimo[3], Takayuki Nambu[3], Koji Umezawa[4] and Mitsuhiro Ohta[5]

[1]*Department of Crown Bridge Prosthodontics, Nihon University, School of Dentistry at Matsudo, Chiba, Japan*
[2]*Department of Oral Microbiology, Nihon University, School of Dentistry at Matsudo, Chiba, Japan*
[3]*Department of Bacteriology, Osaka Dental University, Osaka, Japan*
[4]*Department of Special Needs Dentistry, Nihon University, School of Dentistry at Matsudo, Chiba, Japan*
[5]*Department of Oral Diagnostics, Nihon University, School of Dentistry at Matsudo, Chiba, Japan*

***Corresponding author:** Taira Kobayashi, Department of Crown Bridge Prosthodontics, Nihon University, School of Dentistry at Matsudo, Chiba, Japan, Tel: +81-47-360-9383; **E-mail:** kobayashi.taira@nihon-u.ac.jp

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Abstract

Background: *Actinomyces* is one of the predominant genera in the oral cavity. Oral *Actinomyces* species play a central role in the initial stages of biofilm formation on teeth; however, limited information is currently available on the distribution of individual species in different sites or clinical conditions. Moreover, a suitable method has yet to be developed to identify oral *Actinomyces* species because of the phenotypic and genetic similarities between these microorganisms.

Objective: *Actinomyces naeslundii*, *A. odontolyticus*, *A. oris*, *A. georgiae, A. gerencseriae, A. graevenitzii, A. dentalis, A. johnsonii, A. israelii*, and *A. meyeri* among the genus *Actinomyces* are regarded as normal human oral *Actinomyces* species. The purpose of the present study was to design primers to identify oral *Actinomyces* species using multiplex PCR.

Methods: Polymerase chain reaction (PCR) primers were designed based on partial sequences of the 16S rDNA genes of the representative oral *Actinomyces* species. The 16S rDNA genes of the representative oral *Actinomyces* species were obtained from the DNA Data Bank of Japan, and a multiple sequence alignment analysis was performed with the CLUSTAL W program. Homology among the primers selected for oral *Actinomyces* species and their respective 16S rRNA sequences was confirmed by a BLAST search.

Results: These primers were able to distinguish each oral *Actinomyces* species and did not display cross-reactivity with representative oral bacteria or other *Actinomyces* species. Moreover, we developed a multiplex PCR method with the ability to identify and differentiate oral *Actinomyces* species (i.e., *A. naeslundii*, *A. johnsonii*, *A. oris*, *A. odontolyticus*, *A. israelii*, *A. georgiae*, *A. dentalis*, *A. graevenitzii*, *A. gerencseriae*, and *A. meyeri*) using only two PCR tubes per sample.

Conclusion: The present results indicate that our multiplex PCR method with these primers is useful for identifying the representative oral *Actinomyces* species. This method is easy because the use of Mighty Amp DNA Polymerase Ver.2 (Takara) means that DNA extraction may be avoided, and species identification using this method only takes approximately 2 hours. Thus, the method described herein will allow the prevalence of oral *Actinomyces* species and their involvement in oral infections to be fully clarified in future studies.

Keywords: Genus *Actinomyces*; Multiplex PCR; Oral cavity; 16S rDNA

Introduction

Although the genus *Actinomyces* was already described in 1919, many new species have recently been discovered. The genus *Actinomyces* currently comprises 47 species and 2 subspecies (http://www.bacterio.net/actinomyces.html). *Actinomyces* consist of Gram-positive, anaerobic, and aero-tolerant, non-spore-forming, non-motile pleomorphic rods with various degrees of branching. *Actinomyces* species are frequently found as members of the normal micro-flora, particularly in the oral cavity of humans; however, they are also etiological agents in infections, such as in classical actinomycosis, human bite wounds and abscesses at different body sites, eye infections, and oral, genital, and urinary tract infections [1,2]. The detection of these microorganisms in clinical specimens is important because it may affect the prognosis and management of patients; however, difficulties are associated with identification by conventional biochemical methods.

Ten species (i.e., *Actinomyces naeslundii*, *A. odontolyticus*, *A. oris*, *A. georgiae*, *A. gerencseriae*, *A. graevenitzii*, *A. dentalis*, *A. johnsonii*, *A. israelii*, and *A. meyeri*) among the genus *Actinomyces* are regarded as normal human oral bacteria [3]. Their distribution in different oral sites and their role in common oral afflictions, particularly periodontal diseases, remains controversial. Previous studies successfully induced destructive periodontitis in gnotobiotic rodents with *A. naeslundii* [4], suggesting a potential role for these organisms in periodontal diseases. However, an elevated level of these microorganisms in inactive lesions has also been reported [5]. Recent studies revealed that *Actinomyces* species are highly prevalent in supra- and sub-gingival plaques in adult periodontitis [6] and gingivitis [7]. Therefore, the data available on the role of *Actinomyces* species in periodontal diseases are equivocal. In addition to periodontal diseases, these microorganisms have been implicated in the pathogenesis of root surface caries and refractory periapical actinomycosis [8]. Therefore, the precise detection and speciation of these microorganisms is a prerequisite for the clarification of their pathogenic potential and possibly therapeutic interventions for the aforementioned disease entities.

The accurate identification and enumeration of *Actinomyces* species are required in order to clarify their role in oral ecology and dental diseases. Although conventional biochemical assays are used to identify *Actinomyces* species, they are often imprecise due to the phenotypic variations displayed by these bacteria. Although a sequence analysis of several target genes is the most reliable method, it is expensive, laborious, and time-consuming. Thus, a simple and more reliable assay for identifying oral *Actinomyces* species is desired. The purpose of the present study was to design primers for the identification of oral *Actinomyces* species using multiplex PCR.

Materials and Methods

Bacterial strains and Culture conditions

The following bacterial strains were used in the present study: *A. naeslundii* ATCC 12104, *A. oris* ATCC 27044, *A. johnsonii* JCM 16129, *A. odontolyticus* ATCC 17929, *A. israelii* ATCC 12102, *A. georgiae* DSM 6843, *A. dentalis* DSM 19115, *A. graevenitzii* DSM 15540, *A. gerencseriae* JCM 12963, *A. meyeri* ATCC 35568, *A. viscosus* ATCC 15987, *Streptococcus oralis* ATCC 10557, *S. salivarius* JCM 5707, *S. anginosus* ATCC 11391, *S. mutans* NCTC 10449, *S. sobrinus* ATCC 33478, *Rothia dentocariosa* JCM 3067, *Rothia mucilaginosa* JCM 10910, *Corynebacterium matruchotii* ATCC 14266, *C. durum* ATCC 33449, *Neisseria sicca* ATCC 29256, *Aggregatibacter actinomycetemcomitans* ATCC 33384, *Staphylococcus aureus* JCM 2874, and *S. epidermidis* ATCC 2414. These strains were maintained by cultivating them in brain heart infusion agar (BHI; Difco Laboratories, Detroit, Mich.) supplemented with 1% yeast extract (BHIY). Bacteria were cultured in BHIY broth at 37 °C for 24 h under anaerobic conditions (85% N_2, 10% H_2, 5% CO_2) for members of the genus *Actinomyces* and in a 5% CO_2 atmosphere for other representative oral bacteria.

Design of species-specific primers for ten oral *Actinomyces* species

The 16S rRNA sequences of *A. naeslundii* (accession no. AB618790), *A. johnsonii* (AB545933), *A. oris* (AB545935), *A. odontolyticus* (AB818950), *A. israelii* (AB849123), *A. georgiae* (X80413), *A. dentalis* (AJ697609), *A. graevenitzii* (AJ540309), *A. gerencseriae* (X80414), and *A. meyeri* (X82451) were obtained from the DNA Data Bank of Japan (DDBJ; Mishima, Japan), and a multiple sequence alignment analysis was performed with the CLUSTAL W program; i.e., the 16S rRNA sequences of ten oral *Actinomyces* species were aligned and analyzed. Homology among the primers selected for oral *Actinomyces* species and their respective 16S rRNA sequences was confirmed by a BLAST search.

Development of a multiplex PCR method using designed primers

In the present study, ten oral *Actinomyces* species were divided into two groups for a multiplex PCR method. Group A consisted of *A. naeslundii*, *A. johnsonii*, *A. oris*, *A. odontolyticus*, and *A. israelii*, and group B consisted of *A. georgiae*, *A. dentalis*, *A. graevenitzii*, *A. gerencseriae*, and *A. meyeri*. Bacterial cells were cultured in BHIY broth for 24 h, and 1-ml samples were then collected in micro centrifuge tubes and resuspended at a density of 1.0 McFarland standard (approximately 107 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 assessed 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 of each group, 10

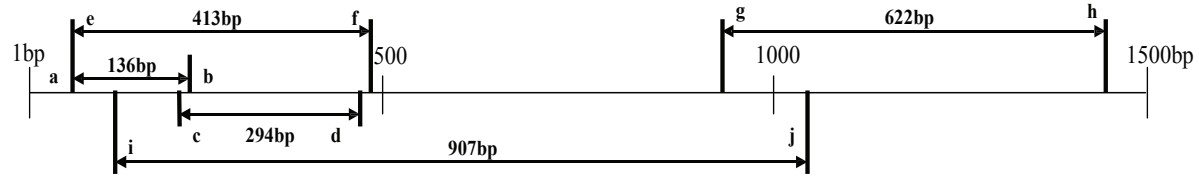
μl of 2 × Mighty Amp Buffer Ver.2 (Takara Bio Inc., Shiga, Japan), 0.4 μl of Mighty Amp DNA Polymerase (Takara), and 5 μl of the template in a final volume of 20 μl. PCR reactions were performed in a DNA thermal cycler (Applied Biosystems 2720 Thermal Cycler; Applied Biosystems, CA, USA). PCR conditions included an initial denaturation step at 98 °C for 2 min, followed by 30 cycles consisting of 98 °C for 10 s and 70 °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.

Results Primer design

Twenty specific primers covering the upstream regions of the 16S rDNA sequences of oral *Actinomyces* species belonging to groups A and B were designed in the present study (Figures 1,2). The specific forward primers of group A were designated as MAJF for *A. johnsonii*, MAORF for *A. oris*, MANF for *A. naeslundii*, MAODF for *A. odontolyticus*, and MAIF for *A.israelii*, whereas the specific reverse primers were designated as MAJR for *A. johnsonii*, MAORR for *A. oris*, MANR for *A. naeslundii*, MAODR for *A. odontolyticus*, and MAIR for *A. israelii*. The amplicon sizes of *A. johnsonii*, *A. oris*, *A. naeslundii*, *A. odontolyticus*, and *A. israelii* were 136 bp, 294 bp, 413 bp, 622 bp, and 907 bp, respectively. The specific forward primers of group B were designated as AGEOF for *A. georgiae*, ADF for *A. dentalis*, AGERF for *A. gerencseriae*, AGRF for *A. graevenitzii*, and AMF for *A. meyeri*, whereas the specific reverse primers were designated as AGEOR for *A. georgiae*, ADR for *A. dentalis*, AGERR for *A. gerencseriae*, AGRR for *A. graevenitzii*, and AMR for *A. meyeri*. The amplicon sizes of *A. georgiae*, *A. dentalis*, *A. gerencseriae*, *A. graevenitzii*, and *A. meyeri* were 328 bp, 464 bp, 583 bp, 837 bp, and 942 bp, respectively.

Multiplex PCR

Detection limit: Our multiplex PCR method for identifying oral *Actinomyces* species successfully amplified DNA fragments of the expected size for each species (Figures 3,4). The detection limit was assessed in the presence of titrated bacterial cells, and



a: Forward primer for *Actinomyces johnsonii*
MAJF : AGCGCTTGCCTTTTTGGTG

	66	84
A. johnsonii	AGCG CTTGCCT TTTT GGTG	
A. oris	AGGGACCGGT TTTT GCTG	
A. naeslundii	AGGGGCCTGC TTTT GTGG	
A. odontolyticus	AG CTCAGC TT GCTGG	
A. israelii	TCTGCCTTGTTTTTTGCGG	

b: Reverse primer for *Actinomyces johnsonii*
MAJR : AACCCGCCATGCGACAGACCCG

	201	180
A. johnsonii	AACCCG CCATGC GACAGACCCG	
A. oris	CACCCA CCATGC AGCAGGCACA	
A. naeslundii	GGCCCA CCATGC GACAGACCCA	
A. odontolyticus	AACCC CCATGCGAAGATCAGT	
A. israelii	GCCACA CCATGC GGCACAGCCAG	

c: Forward primer for *Actinomyces oris*
MAORF : TGCCTGCTGCATGGTGGGTG

	181	200
A. johnsonii	GGTCTGTC GCATGG CGGGTT	
A. oris	TGCCTGCT GCATGG TGGGTG	
A. naeslundii	GGTCTGTC GCATGG TGGGCC	
A. odontolyticus	ACTGATCTTCGCATGG GGGTT	
A. israelii	GGCTGTGCC GCATGG TGTGGC	

d: Reverse primer for *Actinomyces oris*
MAORR : AAAGGGACAGGCCTGCTTC

	474	456
A. johnsonii	ACA GGAGCAG GCCTGCTTC	
A. oris	AAAGGGACAG GCCTGCTTC	
A. naeslundii	AAACGAGGCAG GCCTGCTTC	
A. odontolyticus	TGTTGCGGCTTGACC	
A. israelii	AA AGAGCAGGACCTTCTTC	

e: Forward primer for *Actinomyces naeslundii*
MANF : AGGGGCCTGCTTTTGTGGGTC

	66	86
A. johnsonii	AAGCG CTTGCCT TTTT GGTGGGT	
A. oris	AAGGACCGGT TTTT GCTGGTT	
A. naeslundii	AGGGGCCTGC TTTT GTGGGTC	
A. odontolyticus	AAG CTCAGC TT GCTGGGT	
A. israelii	TCTGCCTTGTTTTTTGCGGGGT	

f: Reverse primer for *Actinomyces naeslundii*
MANR : AAACGAGGCAGGCCTGCTT

	478	460
A. johnsonii	ACA GGAGCAG GCCTGCTT	
A. oris	AAAAGGGACAG GCCTGCTT	
A. naeslundii	AAACGAGGCAG GCCTGCTT	
A. odontolyticus	TGTTGCGGCTTGAC	
A. israelii	AA AGAGCAGGACCTTCTT	

g: Forward primer for *Actinomyces odontolyticus*
MAODF : GCCACCCGTGGTTTCTGCG

	822	840
A. johnsonii	GGCCTTTTCCGGGTTCTTCCGCG	
A. oris	GGCCTTTTCCGGGT CTTCCGCG	
A. naeslundii	GGCCTTTTCCGGGT CTTCCGCG	
A. odontolyticus	G CCACCCGTGGT TTCTGCG	
A. israelii	GGTCTTTCCG GGT CCTCCGCG	

h: Reverse primer for *Actinomyces odontolyticus*
MAODR : GCACAAAGCGGTTAGGCCAT

	1444	1425
A. johnsonii	ATAAGGCCAC	
A. oris	GTAGGGCCAC	
A. naeslundii	GTAGGGCCAC	
A. odontolyticus	GCACAAAGC GGTTAGGCCAT	
A. israelii	ACAGGGCCAC	

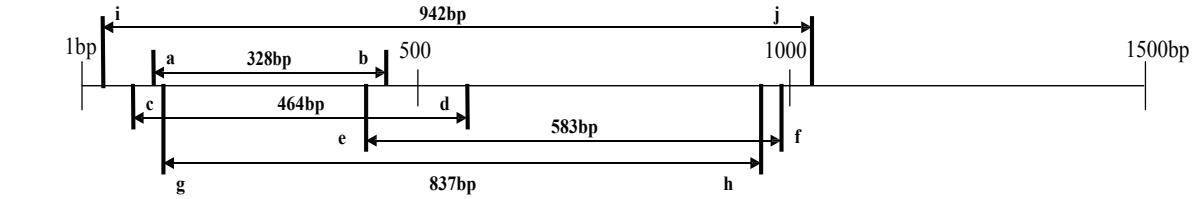
i: Forward primer for *Actinomyces israelii*
MAIF : CTCACTTCTGGATAACCGCT

	129	148
A. johnsonii	TCTTCTTCTGGATAACCTCA	
A. oris	CCTTCTTCTGGATAACCGCA	
A. naeslundii	CCTTCTTCTGGATAACCGCA	
A. odontolyticus	CCTTCTTTGGGATAACGCCC	
A. israelii	CTCACTTCTGGATAACCGCT	

j: Reverse primer for *Actinomyces israelii*
MAIR : GGGAGGCCCCCGTCTCCAGGA

	1035	1015
A. johnsonii	GGAAGGAAACCCCGTCTCC GGA	
A. oris	GG AGGAAACCCCGTCTCC GGA	
A. naeslundii	GG AGGAAACCCCGTCTCC GGA	
A. odontolyticus	TG CCACCACATCTCT GCA	
A. israelii	GGGAGG CCCCCGTCTCC AGGA	

Figure 1: Locations and sequences of species-specific primers for the 16S rDNA of group A oral *Actinomyces* species. The nucleotide sequence of each primer has been underlined.



a: Forward primer for *Actinomyces georgiae*
AGEOF : TCTTCTTCGGGATAACGGTCT

	122	142
A. georgiae	TCTTCTT	CGGGATAACGGTCT
A. dentalis	CCTGCTC	CTGGATAACTGCTT
A. gerencseriae	TTCACTT	CTGGATAACCGCTT
A. graevenitzii	CTTACTTTCCTGGATAACTGCTC	
A. meyeri	TCTTCTT	TGGGATAACGCCCG

b: Reverse primer for *Actinomyces georgiae*
AGEOR : AGTTGCGGCTTGACCACGGG

	449	430
A. georgiae	AGTTGCGGCTTGACCACGGG	
A. dentalis	GACAGGGCCTTCACCACCGG	
A. gerencseriae	GGCAAGGCCTTCCTCACTGG	
A. graevenitzii	AGCAGTGCCTTGTTCCCTAG	
A. meyeri	AGTTGCGGCTTGACCATGAG	

c: Forward primer for *Actinomyces dentalis*
ADF : CCTGCTCCTGGATAACTGCTT

	113	133
A. georgiae	TCTTCTT	CGGGATAACGGTCT
A. dentalis	CCTGCTC	CTGGATAACTGCTT
A. gerencseriae	TTCACTT	CTGGATAACCGCTT
A. graevenitzii	CTTACTTTCCTGGATAACTGCTC	
A. meyeri	TCTTCTT	TGGGATAACGCCCG

d: Reverse primer for *Actinomyces dentalis*
ADR : GGCCGCCTACAAGCCCTTCA

	576	557
A. georgiae	AGCCGCCTACAAGCCCTTTA	
A. dentalis	GGCCGCCTACAAGCCCTTCA	
A. gerencseriae	AACCGCCTACAAGCCCTTTA	
A. graevenitzii	AGCCGCCTACGAGCTCTTTA	
A. meyeri	AACCGCCTACAAGCCCTTTA	

e: Forward primer for *Actinomyces gerencseriae*
AGERF : TGAGGAAGGCCTTGCCCGTG

	414	433
A. georgiae	GGTCAAGCCGCAACT	G
A. dentalis	GGTGAAGGCCCTGTCCG	TTG
A. gerencseriae	TGAGGAAGGCCTTGCCCGT	G
A. graevenitzii	GAACAAGGCACTGCT	TTT
A. meyeri	GGTCAAGCCGCAACT	G

f: Reverse primer for *Actinomyces gerencseriae*
AGERR : GACCCTCCGTCTCCGAAGGA

	996	977
A. georgiae	CGCGCGCGTCTCC	GCGC
A. dentalis	G CGCCGTCTCC	GGCG
A. gerencseriae	GACCCTCCGTCTCCGAAGGA	
A. graevenitzii	TAAACCCTATCTCT	AGAG
A. meyeri	CACGCACGTCTCC	GCAC

g: Forward primer for *Actinomyces graevenitzii*
AGRF : GACCAGCCTGCATGGGTTGG

	146	165
A. georgiae	ACTGGTCCTCGCATGG	GGG
A. dentalis	CGGCTGGTCC GCATGGGCCTG	
A. gerencseriae	TGGGTCTGTC GCATGG	CGGG
A. graevenitzii	G ACCAGCCT GCATGGGTTGG	
A. meyeri	ACTGGTCTTCGCATGG	GG

h: Reverse primer for *Actinomyces graevenitzii*
AGRR : TATCTCTAGAGCCACGCTTAA

	982	962
A. georgiae	CGTCTCC GCGCGCCCGGGGCG	
A. dentalis	CGTCTCC GGCGCCGCCGCCCC	
A. gerencseriae	CGTCTCCGAAGGACAACCG	GCA
A. graevenitzii	TATCTCT AGAGCCACGCTTAA	
A. meyeri	CGTCTCC GCACGCCCAGGGCG	

i: Forward primer for *Actinomyces meyerii*
AMF : CTAGCTTGCTGGGTGGATGA

	63	82
A. georgiae	CTGGC TTTGTGTTGGGT	GGATGA
A. dentalis	CCGCCCCTGTTTTTGGGGGT	GGGTTGA
A. gerencseriae	CTGGCTTTTTTGGTTGGGGTTCTGGGGGTTA	
A. graevenitzii	CAAGC TTG CTTGGTT	GATTA
A. meyeri	CTAGC TT GCTGGGT	GGATGA

j: Reverse primer for *Actinomyces meyerii*
AMR : ACGCACGTCTCCGCACGCCCA

	1004	984
A. georgiae	GCGCGCGTCTCCGCGCGCCCG	
A. dentalis	CGCCGTCTCC GGCGCCGCC	
A. gerencseriae	CCCTCCGTCTCCGAAGGACAACC	
A. graevenitzii	AACCCTATCTCT AGAGCCACG	
A. meyeri	ACGCACGTCTCCGCACGCCCA	

Figure 2: Locations and sequences of species-specific primers for the 16S rDNA of group B oral *Actinomyces* species. The nucleotide sequence of each primer has been underlined.

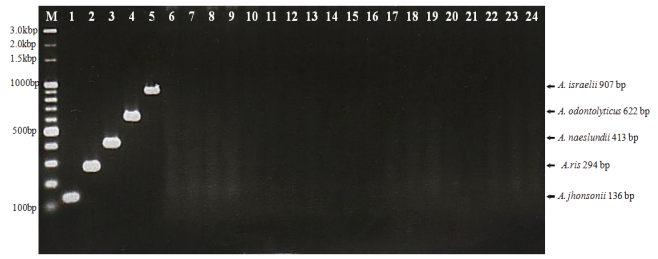


Figure 3: Multiplex PCR assay to detect group A oral *Actinomyces* species.
The primer mixture contained MAJF, MAJR, MAORF, MAORR, MANF, MANR, MAODF, MAODR, MAIF, and MAIR.
Lanes: 1, *A. johnsonii* JCM 16129; 2, *A. oris* ATCC 27044; 3, *A. naeslundii* ATCC 12104; 4, *A. odontolyticus* ATCC 17929; 5, *A. israelii* ATCC 12102; 6, *A. georgiae* DSM 6843; 7, *A. dentalis* DSM 19115; 8, *A. gerencseriae* JCM 12963; 9, *A. graevenitzii* DSM 15540; 10, *A. meyeri* ATCC 35568; 11, *A. viscosus* ATCC 15987; 12, *Streptococcus oralis* ATCC 10557; 13, *S. salivarius* JCM 5707; 14, *S. anginosus* ATCC 11391; 15, *S. mutans* NCTC 10449; 16, *S. sobrinus* ATCC 33478; 17, *Rothia dentocariosa* JCM 3067; 18, *R. mucilaginosa* JCM 10910; 19, *Corynebacterium matruchotii* ATCC 14266; 20, *C. durum* ATCC 33449; 21, *Neisseria sicca* ATCC 29256; 22, *Aggregatibacter actinomycetemcomitans* ATCC 33384; 23, *Staphylococcus aureus* JCM 28744; 24, *S. epidermidis* ATCC 2414. M, molecular size marker (100-bp DNA ladder).

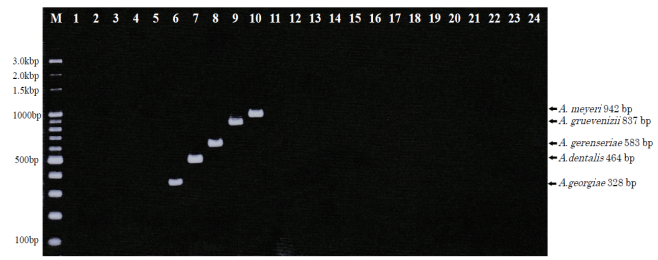


Figure 4: Multiplex PCR assay to detect group B oral *Actinomyces* species.
The primer mixture contained AGEOF, AGEOR, ADF, ADR, AGERF, AGERR, AGRF, AGRR, AMF, and AMR.
Lanes: 1, *A. johnsonii* JCM 16129; 2, *A. oris* ATCC 27044; 3, *A. naeslundii* ATCC 12104; 4, *A. odontolyticus* ATCC 17929; 5, *A. israelii* ATCC 12102; 6, *A. georgiae* DSM 6843; 7, *A. dentalis* DSM 19115; 8, *A. gerencseriae* JCM 12963; 9, *A. graevenitzii* DSM 15540; 10, *A. meyeri* ATCC 35568; 11, *A. viscosus* ATCC 15987; 12, *Streptococcus oralis* ATCC 10557; 13, *S. salivarius* JCM 5707; 14, *S. anginosus* ATCC 11391; 15, *S. mutans* NCTC 10449; 16, *S. sobrinus* ATCC 33478; 17, *Rothia dentocariosa* JCM 3067; 18, *R. mucilaginosa* JCM 10910; 19, *Corynebacterium matruchotii* ATCC 14266; 20, *C. durum* ATCC 33449; 21, *Neisseria sicca* ATCC 29256; 22, *Aggregatibacter actinomycetemcomitans* ATCC 33384; 23, *Staphylococcus aureus* JCM 28744; 24, *S. epidermidis* ATCC 2414. M, molecular size marker (100-bp DNA ladder).

the sensitivity of the PCR assay was between 5×10^3 and 5×10^5 CFU per PCR template (3.6 µl) for the *A. johnsonii*-specific primer set with strain JCM 16129, the *A. oris*-specific primer set with strain ATCC 27044, the *A. naeslundii*-specific primer set with strain ATCC 12104, the *A. odontolyticus*-specific primer set with strain ATCC 17929, the *A. israelii*-specific primer set with strain ATCC 12102, the *A. georgiae*-specific primer set with strain DSM 6843, the *A. dentalis*-specific primer set with strain DSM 19115, the *A. gerencseriae*-specific primer set with strain JCM 12963, the *A. graevenitzii*-specific primer set with strain DSM 15540, and the *A. meyeri*-specific primer set with strain ATCC 35568 (data not shown).

Assay of representative oral bacteria: As representative oral bacteria, some *Streptococcus*, *Corynebacterium*, *Rothia*, *Neisseria*, *Aggregatibacter*, and *Staphylococcus* were subjected to PCR using the designed primer sets. However, no amplicons were produced from any of the representative oral bacteria (Figures 3,4).

Discussion and Conclusion

Actinomyces is one of the predominant genera in the oral cavity. At the age of 2 months, one-third of infants are already colonized with *Actinomyces* [9]. *Actinomyces* species play a central role in the initial stages of biofilm formation on teeth (i.e., dental plaque) both above (supragingival) and below (subgingival) the gum line [10]. Ten species (i.e., *A. naeslundii*, *A. odontolyticus*, *A. oris*, *A. georgiae*, *A. gerencseriae*, *A. graevenitzii*, *A. dentalis*, *A. johnsonii*, *A. israelii*, and *A. meyeri*) among the genus *Actinomyces* are regarded as normal human oral bacteria [3].

Actinomyces species are mainly associated with cervicofacial actinomycosis, oral or cerebral abscesses, caries, root canal infections and periodontitis [11-13]. These microorganisms appear to play a more important role than expected in the pathogenesis of osteoradionecrosis- and bisphosphonate-related osteonecrosis of the jaw [14-16], and may cause lethal infections such as mediastinitis [17]. As a consequence, fast and reliable identification methods for these microorganisms have become increasingly important.

The isolation and identification of *Actinomyces* using conventional methods are often difficult and time-consuming. Previous studies were conducted in order to characterize them using phenotypic [18-20] and molecular [21,22] approaches. Most of the available commercial identification kits do not encompass the majority of new species in their database.

The PCR method is a molecular technique that allows species verification at the genetic level, and possesses high sensitivity and bacterial species specificity. It is also useful for the detection and identification of fungi [23]. Moreover, it may efficiently and accurately detect specific microorganisms in clinical samples, regardless of whether they are alive or dead. Also, it might be an innovative method for detection of microorganisms difficult to culture using traditional microbiological techniques. The 16S rDNA region of the bacterial genome provides an ideal target for species identification using PCR [16]. Moreover, multiplex-PCR is a rapid tool that allows for the simultaneous amplification of more than one sequence of target DNA in a single reaction, thereby saving time and reagents [24]. On the other hand, Hirotaki et al. [25] and we [26,27] developed the multiplex-PCR, which allows for the amplification of one sequence of target DNA in a single reaction containing the plural species-specific primers, for bacterial species identification. Previous studies have used different molecular methodologies to identify and differentiate *Actinomyces* from oral samples after anaerobic cultivation, including PCR-RFLP, chromosomal DNA fingerprinting, 16S rRNA gene sequencing and oligonucleotide-DNA hybridization using universal primers or oligonucletide probes [13,28-30]. Tian et al. reported rapid and sensitive PCR-dipstick DNA chromatography assay that could be read by eye for multiplex and semi quantitative analysis of dental plaque bacteria including *Actinomyces* species [31].

In silico method helps in designing primers, and there are various programs available for PCR primer design [32]. In the present study, we designed species-specific primers with the already mentioned means, for the identification of oral *Actinomyces* species using a PCR method. These primers were able to distinguish each oral *Actinomyces* species and did not display cross-reactivity with representative oral bacteria or other *Actinomyces* species. Moreover, we developed a multiplex PCR method with the ability to identify and differentiate oral *Actinomyces* species (i.e., *A. naeslundii*, *A. johnsonii*, *A. oris*, *A. odontolyticus*, *A. israelii*, *A. georgiae*, *A. dentalis*, *A. graevenitzii*, *A. gerencseriae*, and *A. meyeri*) using only two PCR tubes per sample.

Our multiplex PCR method is easy because the use of Mighty Amp DNA Polymerase Ver.2 (Takara) means that DNA extraction may be avoided, and species identification using this method only takes approximately 2 hours. Thus, the method described herein will allow the prevalence of oral *Actinomyces* species and their involvement in oral infections to be fully clarified in future studies.

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