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Isolation and Identification Methods for *Actinomyces israelii* Involved in Actinomycosis

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Abstract

Purpose: *Actinomyces israelii* is known as the key species to cause classical actinomycosis. Although *A. israelii* is frequently isolated from human oral cavities, the distribution of this microorganism has been little reported. The purpose of the present study was to develop selective media (AISM) for the isolation of *A. israelii* and to assess the prevalence of this organism in the oral cavity. **Methods**: To examine the bacterial population in the oral cavity, a novel selective medium (AISM) was developed for isolating *A. israelii*. AISM consists of BHI, yeast extract, agar, ofloxacin, fosfomycin, colistin, and sodium fluoride. **Results**: *A. israelii* strains grew well on AISM. *A. israelii* was detected in all dental plaque samples collected from 20 subjects and the mean number of this organism in the samples was 7.9×10^4 CFU/ml. **Conclusion**: These results indicated that the selective medium was useful for the isolation of *A. israelii* and this organism was a part of the normal flora in the human oral cavity.

Keywords

Actinomyces israelii, Selective Medium, Oral Cavity, Actinomycosis

1. Introduction

Although the genus *Actinomyces* was already described in 1919, a lot of new species have been found recently. At present, the genus *Actinomyces* comprises 235 species and 7 subspecies (http://www.bacterio.net/actinomyces.html). *Actinomyces* consists of gram-positive, anaerobic, and aero-tolerant, non-spore-forming, non-motile pleomorphic rods with various degrees of branching. *Acti-*

nomyces species are frequently found as members of the normal micro-flora, especially in the oral cavity of humans; however, they are also found to be etiologic 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 *Actinomyces* species in clinical specimens is important, as it may affect the prognosis and patient management, but identification by conventional biochemical methods can be difficult.

Actinomycosis is a chronic disease characterized by inflammatory lesions of the lymph nodes, abscess formation, tissue fibrosis, and a discharge of sulfur granules. It most commonly affects healthy individuals, but may occur in persons with diminished host defenses, and generally has few systemic signs. Cervicofacial tissues are the most commonly involved in actinomycosis, followed by thoracic, abdominopelvic regions, and central nervous systems [3]. The portal of entry is usually through a disruption of the mucosal barrier after trauma or surgery [4].

Actinomycosis is most commonly caused by *Actinomyces israelii* [5] [6] [7]. However, it currently remains unclear whether this organism is part of the normal oral flora. Thus, a suitable selective medium is needed to assess the prevalence of *A. israelii* involved in actinomycosis as well as eye infection, such as keratitis and canaliculitis, dental caries, endodontic infections, osteomyelitis of the sternum, and infective endocarditis [8]-[13].

The accurate identification and enumeration of *Actinomyces* species are required to determine their role in oral ecology, dental disease, and also systemic diseases such as actinomycosis. Although conventional biochemical assays are used to identify *Actinomyces* species, they are often imprecise due to the phenotypic variations displayed by these bacteria. Although 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 required.

The purpose of the present study was to develop selective media for the isolation of *A. israelii*, a simple and more reliable assay for identifying it, and also to assess the prevalence of this organism in the oral cavity.

2. Materials and Methods

2.1. Bacterial Strains and Culture Conditions

All bacterial strains used in the present study are listed in **Table 1**. The anaerobic bacteria (*i.e.*, *A. israelii*, and *Actinomyces meyeri*) 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 for 48 h in an anaerobic jar with a gas pack system (AnaeroPack®, Mitsubishi Gas Chemical Co., Inc., Tokyo, Japan). *A. israelii* isolates (NUM-Ai 7071, NUM-Ai 7072, and NUM-Ai 7074) were obtained with a non-selective medium, *i.e.*, BHI agar, from the human oral cavity in our previous studies.

Table 1. Recovery of *A. israelii* and other bacteria on BHI agar and AISM.

Strain	BHI-Y	AISM	Recovery, %
	CFU/ml, $\times 10^7$	CFU/ml, $\times 10^7$	
Actinomyces israelii			
ATCC 12102	2.5 ± 0.2[a]	2.5 ± 0.3	98.5
NUM-Ai 7071	2.6 ± 0.3	2.5 ± 0.3	97.8
NUM-Ai 7072	2.1 ± 0.1	1.9 ± 0.1	94.5
NUM-Ai 7074	1.1 ± 0.1	1.1 ± 0.2	99.6
Actinomyces viscosus			
ATCC 15987	2.6	0	0
Actinomyces naeslundii			
ATCC 12104	1.3	0	0
Actinomyces johnsonii			
JCM 16129	0.8	0	0
Actinomyces oris			
ATCC 27044	1.1	0	0
Actinomyces odontolyticus			
ATCC 17929	0.5	0	0
Actinomyces georgiae			
DSM 6843	0.7	0	0
Actinomyces dentalis			
CCUG 48064	0.5	0	0
Actinomyces graevenitzii			
CCUG 27294	0.7	0	0
Actinomyces gerencseriae			
JCM 12963	0.8	0	0
Actinomyces meyeri			
JCM 3067	1.3	0	0
Streptococcus mitis			
ATCC 49456	2.1	0	0
Streptococcus gordonii			
ATCC 10558	1.8	0	0
Streptococcus oralis			
ATCC 10557	3.3	0	0
Streptococcus salivarius			
JCM 5707	2.2	0	0
Streptococcus anginosus			
ATCC 33397	5.1	0	0
Streptococcus mutans			
NCTC 10449	6.6	0	0

Continued

Rothia dentocariosa JCM 3067	1.3	0	0
Rothia mucilaginosa JCM 10910	2.5	0	0
Corynebacterium matruchotii ATCC 14266	1.4	0	0
Corynebacterium durum ATCC 33449	1.9	0	0
Neisseria sicca ATCC 29256	5.5	0	0

[a]Ave ± SD.

Strains other than anaerobic bacteria were maintained by cultivating them also on BHI agar. These organisms were cultured at 37˚C overnight in an atmosphere of 5% CO_2 in a CO_2 incubator (NAPCO® Model 5400; Precision Scientific, Chicago, IL, USA).

2.2. Development of the New Selective Medium

2.2.1. Evaluation of the Base Medium

BHI supplemented with 1% yeast extract (BHI-Y), BHI-Y supplemented with 5% sheep blood (BHI-Y blood), and anaerobic blood agar (CDC) were examined as the base medium in the selective medium. CDC composed of a Tryptic soy agar (Becton, Dickinson and Co., Sparks, MD, USA) base supplemented with vitamin K_1 (10 μg/ml), hemin (5 μg/ml), L-cysteine (800 μg/ml), 0.5% yeast extract, and 5% sheep blood. 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 on which bacteria, except anaerobic bacteria, were inoculated were cultured at 37˚C for 72 h in an atmosphere of 5% CO_2 in a CO_2 incubator, and the plates on which anaerobic bacteria were inoculated were cultured at 37˚C for 72 h under anaerobic conditions. 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 [14].

2.3. Recovery of *A. israelii* and Other Representative Oral Bacteria

The recoveries of the *A. israelii* reference strain, *A. israelii* isolates, and other representative oral bacteria were calculated as CFU/ml on a selective medium and compared with those on CDC for total cultivable bacteria. All bacterial strains used in the present study are listed in **Table 1**.

All bacterial strains, except anaerobic bacteria, were pre-incubated in BHI broth at 37˚C overnight in an atmosphere of 5% CO_2 in a CO_2 incubator. Anaerobic bacteria were pre-incubated in tryptic soy broth (Becton, Dickinson and Co., Sparks, MD, USA) supplemented with vitamin K_1 (10 μg/ml), hemin (5 μg/ml), and 0.5% yeast extract at 37˚C overnight under anaerobic conditions. 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 on which bacteria, except anaerobic bacteria, were inoculated were cultured at 37˚C for 72 h in an atmosphere of 5% CO_2 in a CO_2 incubator, and those on which anaerobic bacteria were inoculated were cultured at 37˚C for 72 h under anaerobic conditions. After cultivation, the number of CFU/ml was counted.

2.4. Clinical Samples

Twenty volunteers (9 men, 11 women; mean age 42 years, range 18 - 61 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.

Dental plaque samples on the buccal surfaces of the first permanent molars of all subjects were obtained using sterile micro-applicators (Benda Micro, Centrix, Inc., Shelton, CT, USA) and placed in a sterile microcentrifuge tube containing 1.0 ml of Tris-HCl buffer (0.05 M, pH 7.2). Samples were dispersed, and 0.1 ml of each was diluted and inoculated on BHI-Y and selective medium plates according to a previously described procedure [17]. BHI-Y plates for total cultivable bacteria and selective medium plates were cultured at 37˚C for 3 days under anaerobic conditions. After cultivation, CFU/ml in each sample was calculated. The present study was approved by the Ethics Committee of Nihon University School of Dentistry at Matsudo, Japan (EC 18-17-012-1). Informed consent was obtained from all volunteers.

2.5. Identification of *A. israelii* 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 PCR analysis.

2.6. Design of Species-Specific Primers for *A. israelii*

The design of species-specific primers for *A. israelii* was performed as follows. 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 multiple sequence alignment analyses were performed using the CLUSTAL W program; *i.e.*, the 16S rRNA sequences of four species were aligned and analyzed. Homologies

among the primers selected for *A. israelii* were confirmed by a BLAST search.

2.7. Development of a PCR Method for Identifying *A. israelii* Using Designed Primers

A PCR method for identifying *A. israelii* using the designed primers was developed as follows. Bacterial cells were cultured in a BHI broth overnight, and 1 ml of the sample was then collected in a microcentrifuge tube and resuspended at a density of 1.0 McFarland standard (approximately 10^7 CFU 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 for PCR was assessed by serially diluting known numbers of bacterial cells in sterile distilled water and then subjecting each suspension to PCR. The PCR mixture contained 2 µM of each primer, 10 µl of 2 × MightyAmp Buffer Ver.2 (Takara Bio Inc., Shiga, Japan), 0.4 µl of MightyAmp DNA Polymerase (Takara), and 3.6 µl of the template in a final volume of 20 µl. PCR was performed in a DNA thermal cycler (Applied Biosystems 2720 Thermal Cycler; Applied Biosystems, Carlsbad, CA). 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.

3. Results

3.1. Development of Selective Medium

3.1.1. Selection of the Base Medium

The selection of a base medium for the growth of *A. israelii* was performed. *A. israelii* grew well on BHI-Y as same as BHI-Y blood and CDC (data not shown). Because of the low cost, BHI-Y without blood was ultimately selected as the base medium.

3.1.2. Susceptibility to Antibiotics

A. israelii was more resistant to colistin than oral Gram-negative cocci, such as *Neisseria* and *Veillonella* species. The minimal inhibitory concentration (MIC) of colistin for *A. israelii* was 1000 µg/ml. Oral Gram-negative cocci were sensitive to 10 µg/ml of colistin. *A. israelii* was more resistant to ofloxacin than oral Gram-negative rods, such as *Aggregatibacter*, *Fusobacterium*, *Porphyromonas*, and *Prevotella* species. The MIC of ofloxacin for *A. israelii* was 10 µg/ml. Oral Gram-negative bacteria were sensitive to 4 µg/ml of ofloxacin. *A. israelii* was more resistant to fosfomycin than oral *Rothia* species. The MIC of fosfomycin for *A. israelii* was 10 µg/ml. Oral *Rothia* species were sensitive to 5 µg/ml of fosfomycin. *A. israelii* was more resistant to sodium fluoride than oral *Corynebacterium* species and oral *Actinomyces* species. The MIC of sodium fluoride for *A. israelii* was 800 µg/ml. Oral *Corynebacterium* species and oral *Actinomyces* species except *A. israelii* were sensitive to 50 µg/ml of sodium fluoride.

3.1.3. Composition of the New Selective Medium

The new selective medium, designated *A. israelii* selective medium (AISM), was composed of the following (per liter): 37 g of BHI, 10 g of yeast extract, 15 g of agar, 4 mg of ofloxacin, 5 mg of fosfomycin, 20 mg of colistin, and 150 mg of sodium fluoride. Antibiotics, *i.e.*, ofloxacin, fosfomycin, and colistin, were added after the base medium had been sterilized and cooled to 50˚C.

3.2. PCR Method for Identifying *A. israelii*

3.2.1. Primer Design

The specific primer set covering the upstream region of the 16S rDNA sequence of *A. israelii* was designed in the present study (Table 2). The amplicon size of *A. israelii* was 907 bp.

3.2.2. Detection Limit

A PCR method was used to identify the *A. israelii*-amplified DNA fragment of the expected size for this organism (Figure 1). The detection limit was assessed in the presence of titrated bacterial cells, and the detection sensitivity of the PCR assay was 50 - 100 CFU per PCR template (5.6 µl) for the *A. israelii*-specific primer set with the ATCC 12102 strain (data not shown).

Table 2. Locations and sequences of species-specific primers for the16S rDNA of *A. israelii*.

Species	Primer	Sequence	Product size (bp)	Position	Accession number
A. israelii	AIF	CTCACTTCTGGATAACCGCT	907	129 - 148	AB849123
	AIR	GGGAGGCCCCCGTCTCCAGGA		1035 - 1015	

Figure 1. Specificity of PCR assay. Primers are a mixture of AIF and AIR. Lanes: 1, *A. israelii* ATCC 12102; 2, *A. viscosus* ATCC 15987; 3, *A. naeslundii* ATCC 12104; 4, *A. johnsonii* JCM 16129; 5, *A. oris* ATCC 27044; 6, *A. odontolyticus* ATCC 17929; 7, *A. georgiae* DSM 6843; 8, 8, *A. dentalis* CCUG 48064; 9, *A. graevenitzii* CCUG 27294; 10, *A. gerencseriae* JCM 12963; 11, *A. meyeri* ATCC 35568; 12, *Streptococcus mitis* ATCC 49456; 13, *S. goldonii* ATCC 10558; 14, *S. oralis* ATCC 10557; 15, *S. sanguinis* ATCC 10556; 16, *S. salivarius* JCM 5707; 17, *S. anginosus* ATCC 33397; 18, *S. mutans* NCTC 10449; 19, *Rothia dentocariosa* JCM 3067; 20, *R. mucilaginosa* JCM 10910; 21, *Corynebacterium matruchotii* ATCC 14266; 22, *C. durum* ATCC 33449; 23, *Neisseria sicca* ATCC 29256; M, molecular size marker (100-bp DNA ladder).

3.2.3. Assay of *A. israelii* and Representative Oral Bacteria

The PCR method used to identify *A. israelii* produced positive bands from the *A. israelii* reference strain ATCC 12102 (Figure 1). Some *Streptococci* and *Actinomyces* except *A. israelii*, *Neisseria*, *Corynebacterium*, and *Rothia* species were used as representative oral bacteria in PCR using the designed primer set. No amplicons were produced from any of the representative oral bacteria (Figure 1).

3.3. Recovery of *A. israelii* and Inhibition of Other Representative Oral Bacteria on Selective Medium

Table 1 shows the recovery of the *A. israelii* reference strain ATCC 12102 and isolates on AISM relative to BHI-Y. The growth recoveries of the *S. moorei* reference strains and isolates on AISM were between 94.5% and 99.6% (average 97.6%) that on BHI-Y.

Table 1 also shows the inhibition of other representative oral bacteria on AISM relative to BHI-Y. The growth of other representative oral bacteria was markedly inhibited on the selective medium.

3.4. Clinical Examination

The detection frequencies of *A. israelii* in dental plaque samples from twenty healthy subjects are shown in Table 3. *A. israelii* was detected in all samples from the subjects. The mean numbers of total bacteria and *A. israelii* in all healthy subjects were 1.7×10^7 CFU/ml (range: 0.2×10^7 - 4.6×10^7) and 7.9×10^4 CFU/ml (range: 1.9×10^4 - 8.1×10^5), respectively.

In the first isolation, *A. israelii* colonies on AISM commonly had a rough, dry, folded and convex appearance, and adhered to the agar medium such that they were not easily scraped off. The colony color and average colony size of *A. israelii* on AISM were light white and 1.4 mm in diameter, respectively (Figure 2).

Table 3. Proportion of *A. israelii* in dental plaque samples.

Sublect (n = 20)	BHI-Y	AISM	Detection ratio
	Total bacteria CFU/ml, $\times 10^7$	*A. israelii* CFU/ml, $\times 10^4$	%
Average	1.7	7.9	0.46

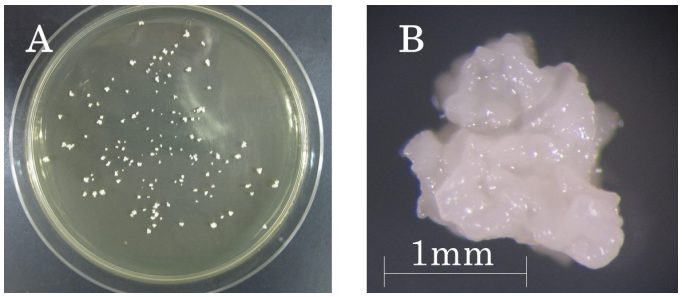

Figure 2. Appearance of *A. israelii* colonies on AISM. A: *A. israelii* colonies on AISM inoculated with a dental plaque sample. B: Stereomicroscope image of *A. israelii* colony on AISM.

4. Discussion

The genus *Actinomyces* consists of species that are Gram-positive, pleomorphic ore filamentous, non-spore-forming, and anaerobic or microaerophilic bacteria. Some *Actinomyces* species have been related to the diseases of humans. These organisms include the strict anaerobic bacteria such as *A. israelii*, and the facultative anaerobic species such as *A. naeslundii*, *A. odontolyticus*, *A. viscosus*, *A. meyeri*, and *A. gerencseriae* (formerly *A. israelii* serotype II) [2]. *Actinomyces* are commensals and normal inhabitants of the oropharynx, gastrointestinal tract, and female genital tract of humans.

A. israelii has long been recognized as a causative agent of actinomycosis. During the past 3 decades, a large number of novel *Actinomyces* species have been found. The detection and identification of these organisms in clinical microbiology laboratories and also clinical settings have been attempted. With the introduction of advanced molecular methods, knowledge about their clinical relevance is gradually increasing, and the spectrum of diseases associated with *Actinomyces* and *Actinomyces*-like organisms is widening accordingly; for example, *Actinomyces meyeri*, *Actinomyces neuii*, and *Actinomyces turicensis* as well as *Actinotignum* (formerly Actinobaculum) *schaalii* are emerging as important causes of specific infections as various body sites.

Human actinomycosis, a chronic, granulomatous infectious disease, has been recognized for a long time, and its causative agent, originally named *Streptothrix israeli* (currently *A. israelii*) was described in 1896 by Kruse [15]. However, it currently remains unclear whether this organism is part of the normal oral flora. Moreover, difficulties are associated with the isolation of *A. israelii* due to the differential exhibition of phenotypic characteristics. Thus, a suitable selective medium and reliable identification method are needed in order to assess the prevalence of *A. israelii* involved in actinomycosis as well as eye infection such as keratitis and canaliculitis, dental caries, endodontic infections, osteomyelitis of the sternum, and infective endocarditis [8]-[13].

In the present study, we designed species-specific primers to identify *A. israelii* using a PCR method. These primers were able to distinguish *A. israelii* and did not react with representative oral bacteria other than this organism. 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 *A. israelii* may contribute to the correct and rapid diagnosis of infectious diseases caused by this organism. However, a selective medium that is useful for the isolation of *A. israelii* has not ever been developed. In the present study, *A. israelii* strains were more resistant to sodium fluoride, ofloxacin, fosfomycin, and colistin than other representative oral bacteria. The growth of oral bacteria detected in the oral cavity was inhibited by the addition of 4 mg/L ofloxacin, 5 mg/L fosfomycin, 20 mg/L colistin, and 150 mg/L sodium fluoride to BHI-Y agar. All of the *A. israelii* reference

strains and isolates tested grew well on the new selective medium, designated as AISM, while the growth of other bacteria was markedly inhibited (**Table 1**). Moreover, AISM allowed for the identification of *A. israelii* by its characteristic colony morphology.

The distribution of *A. israelii* in the oral cavity of humans has not yet been reported in detail. In the present study, *A. israelii* was detected in all samples from healthy subjects by a culture method using the selective medium, *i.e.*, AISM. Moreover, *A. israelii* was detected at 0.46% to total bacteria of 1.7×10^7 CFU/ml on BHI-Y agar in their oral cavities. These results indicated that *A. israelii* was a part of the normal oral flora.

We developed a selective medium, designated AISM, to isolate *A. israelii* in the oral cavity of humans. Since AISM is highly selective for *A. israelii*, it will be useful for assessing the distribution and role of this organism at various locations in humans. Actinomycosis is usually diagnosed by recovering the organism in culture. Labs are unable to recover the organisms from the culture in over 50% of cases. This is likely due to the overgrowth of concomitant organisms, inadequate anaerobic transport and culture techniques [3]. The isolation method for *A. israelii* using AISM herein might help to overcome such problems.

5. Conclusion

The selective medium (AISM) and our PCR method as isolation and identification methods, respectively, for *A. israelii*, 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.

Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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