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Novel Selective Medium for the Isolation of *Rothia aeria*, Which Is an Inhabitant of the Human Oral Cavity

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Abstract

Roth aeria, *Rothia dentocariosa*, and *Rothia mucilaginosa* are isolated from the human oral cavity. Among them, *R. aeria* can cause severe systemic infection diseases (e.g., bronchitis, endocarditis, pneumonia, and sepsis). However, the veritable prevalence of this organism in the human oral cavity has not ever been known. Thus, the selective medium for the isolation and quantification of *R. aeria* is necessary to assess the prevalence of this organism, and to diagnose various *R. aeria* infection diseases.

To investigate *R. aeria* distribution in oral cavities, a novel selective medium (RAESM) was developed for isolating and quantifying *R. aeria*. RAESM consists of sodium gluconate, tryptone, meat extract, sodium fluoride, acriflavin neutral, fosfomycin, lincomycin, colistin, aztreonam, and agar. Polymerase chain reaction (PCR) primers were designed based on partial sequences of the 16S rDNA genes of *R. aeria*. The percentage of *R. aeria* in saliva samples collected from 20 subjects was examined. Moreover, we examined the antibiotic susceptibility of thirty isolates from six subjects.

The average growth recovery of *R. aeria* on RAESM was 96.6% compared with that on Brain Heart Infusion supplemented with Yeast Extract (BHI-Y) agar. Growth of other representative oral bacteria, including other *Rothia* species, was remarkably inhibited on the selective medium. The PCR primers reacted to *R. aeria* and did not react to other *Rothia* species or representative oral bacteria. *R. aeria* was detected as 1.0% of the total bacteria, 5.9×10^7CFU/ml, on BHI-Y agar in the oral cavities of all subjects. *R. aeria* isolates obtained in this study were susceptible to most antibiotics; however *R. aeria* isolates from one subject were highly resistant to erythromycin, lincomycin, and clindamycin. *R. aeria* may be a part of the normal flora in the human oral cavities. A novel selective medium, RAESM, was useful for isolating *R. aeria*. Moreover, it was indicated that RAESM was useful for diagnosing *R. aeria* infections.

Key words

Rothia aeria; Genus *Rothia*; Selective medium; Oral cavity; PCR

Introduction

The genus *Rothia* comprises 8 species (www.bacterio.net/rothia.html), *Rothia aeria* [1], *Rothiaamarae* [2], *Rothia dentocariosa* [3], *Rothia mucilaginosa* [4], *Rothia nasimurium* [4], *Rothia terrae* [5], *Rothia endophytica* [6], and *Rothia saerolata* [7], which were isolated from an air sample, sludge of a foul water sewer, a human oral cavity, a human pharynx, the nose of a healthy mouse, subtropical fields, plant tissues, and pig barn respectively. Among the genus *Rothia*, *R. dentocariosa*, *R. mucilaginosa*, and *R. aeria* are found in the oral cavity and pharynx of humans [8-11]. Concerning *R. aeria*, it was first isolated from air and condensation water samples from the Russian space station Mir. Initially, it was known as *R. dentocariosa* genomovar II [1]. *R. aeria* is capable of causing serious systemic infections, such as sepsis, bronchitis,pneumonia,and endocarditis [12-17]. *R. aeria* is difficult to identify because of its similar morphology and colony appearance to those of *Nocardia* species. Therefore, 16S rRNA sequencing is required to distinguish *R. aeria* from *Nocardia* species [18].

R. aeria was detected in the mouths of healthy individuals [11,19]. We have previously reported selective media for the isolation of *R. dentocariosa and R. Mucilaginous*, respectively

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[9,10]. Moreover, we have also previously reported selective media for the simultaneous isolation of three oral *Rothia* species (*R. dentocariosa, R. mucilaginosa, and S. aeria*) [11]. However, there has never been reported the selective medium for the isolation of *R. aeria* only. Therefore, a suitable selective medium for the isolation of *R. aeria* is necessary to assess the accurate prevalence of this organism in the human oral cavity.

The aim of the present study was to develop a selective medium for the isolation of *R. aeria* and reveal its distribution in the human oral cavity.

Materials and Methods

Bacterial strains and culture conditions

Table 1 shows all bacterial strains used in this study. All strains were subcultured on brain heart Infusion (BHI, CM1135, Oxoid, UK) supplemented with 0.5% yeast extract (Oxoid, UK) and 1.5% agar (BHI-Y agar, Difco agar, BD, USA). Genus *Rothia* were cultivated at 37°C in the air for up to 24 hours. Representative oral bacteria strains except genus *Rothia* were cultivated at 37°C in 5% CO_2 for up to 24 hours.

Development of the selective medium

ORSM [11] was chosen as a base medium for the selective medium. Disk susceptibility tests were used for antibiotic selection. (KB-Disk, EIKEN CHEMICAL CO., LTD., Tokyo, Japan). After choosing appropriate antibiotics, the microbroth dilution method was performed [20,21].

Recovery of representative *Rothia* species

The recovery of Rothia species reference strains and isolates from the human oral cavity in our previous studies [11] were calculated as colony-forming units (CFU) /mL on the selective medium compared with those on BHI-Y agar for total cultivable bacteria.

Rothia species were cultivated at 37°C in the air for up to 24 hours. *Rothia* reference strains and isolates were suspended at three different concentrations (10^5 , 10^6, and 10^7 CFU/mL), and then 0.1 mL Tris-HCl buffer (0.05 M, pH 7.2) of each suspension was inoculated in triplicates onto BHI and the selective medium. After the culture, CFU/ml was calculated.

Clinical samples

Clinical samples were obtained from twenty volunteers (age 22-58, male 9, female 11). Slaiva samples stimulated with paraffin from each volunteer were collected in sterile vials containing 0.5 mL of 0.05 M Tris-HCl buffer (pH 7.2). This study was approved by the Ethics Committee of Nihon University School of Dentistry at Matsudo, Japan (EC 15- 025). Samples were processed as described previously [11].

Identification of *R. aeria* isolated from clinical samples

Ten colonies (which appeared to be *R. Erie* based on colony morphology), per subject were subcultured to confirm the presence of *R. aeria*. Pure cultures of each isolate were identified by: (i) gram staining; and (ii) polymerase chain reaction (PCR) analyses.

Design of species-specific primers for representative *Rothia* species and PCR method procedure

The 16S rRNA sequences of *R. aeria* (accession no. AB071952) were obtained from the DNA Data Bank of Japan (DDBJ; Mishima, Japan). Design of species-specific primers and the procedure of PCR method were performed as described previously [11].

Antibiotics susceptibility tests of *R. aeria* isolates

Antimicrobial susceptibility testing of *R. aeria* isolates were evaluated using the microdilution method. Table 4 shows antibiotics used in this study. They are widely used in the treatment of Gram-positive infections. Because there is not Clinical and Laboratory Standards Institute (CLSI) protocols for *R. aeria,* the organism's drug susceptibility utilizing the 2016 CLSI criteria (M100-S27) for staphylococci was substituted in this study.

Results

Development of the selective medium

R. Dentocariosa, R. mucilaginosa, and R. aeria grew well and at similar ratios on a base medium, i.e. ORSM [11]. The minimal inhibitory concentration (MIC) of aztreonam for *R. mucilaginosa and R. aeria* were 100 µg/mL. *R. dentocariosa* was sensitive to 30 µg/ml of aztreonam. Moreover, *R. aeria* was more resistant to fosfomycin than *R. mucilaginosa*. The MIC of fosfomycin for *R. aeria* was 80 µg/mL. *R. Mucilaginous* was sensitive to 3 µg/ml of fosfomycin.

The selective medium for the isolation of *R. aeria* (RAESM) was composed of the following (per liter): 5 g meat extract (Sigma-Aldrich Co. LLC., Tokyo, Japan), 1 g tryptone (Sigma-Aldrich), 10 g sodium gluconate (Tokyo Chemical Industry Co., Ltd., Tokyo, Japan), 125 mg sodium fluoride (Sigma-Aldrich), 15 g agar (Difco agar, BD, USA), 3 mg acriflavine neutral (Sigma-Aldrich), 10 mg colistin sulfate salt (Sigma-Aldrich), 0.2 mg lincomycin hydrochloride (Tokyo Chemical Industry), 3 mg fosfomycin disodium salt (Tokyo Chemical Industry), and 30 mg aztreonam (Tokyo Chemical Industry). When the mixture except of the antibiotics was cooled to 50ºC, colistin sulfate salt, lincomycin hydrochloride, fosfomycin disodium salt, and aztreonam were added aseptically.

PCR analyses

Table 2 shows specific primer sets covering the upstream regions of the 16S rDNA sequences of *R. aeria*, and the amplicon size of this organism was 918 bp. The PCR method used to identify *R. aeria* produced positive bands from *R. aeria (Figure 1*) and did not produce any amplicons from other *Rothia* species or any of the representative oral bacteria, i.e. some *Streptococcus, Actinomyces, Neisseria, and Corynebacterium species.*

Recovery of *R. aeria* on the selective medium

Table 1 shows the recovery of *R. aeria* and isolates on RAESM relative to BHI-Y agar. The growth recoveries of *R. aeria* reference strains and the isolates ranged from 95.1% to 97.7% (average 96.6%) on RAESM relative to that on BHI-Y agar. The growth of *R. dentocariosa and R. mucilaginosa* was markedly inhibited on the selective medium.

Clinical examination

The percentage of *R. aeria* in saliva from the twenty subjects on BHI-Y and RAESM is shown in Table 3. The mean number of total cultivable bacteria was 5.9×10^7 CFU/ml (range: 2.1 × 107 - 9.8×10^7). The mean number of *R. aeria* was 5.6×10^5 CFU/ml (range: 0.3×10^5 - 27×10^5). *R. aeria* accounted for 1.0% of the total cultivable bacteria number on the BHI-Y medium and was detected in all twenty subjects.

In the first isolation, *R. aeria* colonies on RAESM commonly exhibited a rough, dry, folded, and convex appearance (Figure 2) and

Strain	BHI-Y CFU/ml, × 10^8	RAESM CFU/ml, × 10^8	Recovery, %
R. aeria			
JCM 11412	1.1 ± 0.2a	1.0 ± 0.2	96.7
GTC 02043	1.7 ± 0.1	1.7 ± 0.1	95.1
NUM-Ra 7006	1.0 ± 0.1	1.0 ± 0.1	96.3
NUM-Ra 7007	1.2 ± 0.2	1.2 ± 0.2	97.3
NUMRa7008	1.0 ± 0.2	1.0 ± 0.2	97.7
R. dentocariosa			
JCM 3067	1.4	0.0001	0.01
NUM-Rd 6018	1.9	0	0
NUM-Rd 6020	1.0	0	0
R. mucilaginosa			
JCM 10910	1.0	0	0
NUM-Rm 6504	2.1	0	0
NUM-Rm 6505	1.6	0	0

[a]Ave ± SD

Table 1: Recovery of representative *Rothia species* on BHI-Y and RAESM

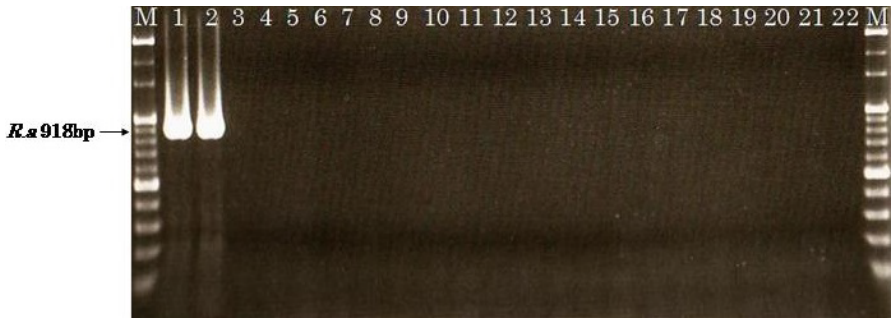

Figure 1: Specificity of multiplex PCR assays. Primers are a mixture of RAF and RAR

Lanes: 1, *R. aeria* JCM 11412; 2, *R. aeria* GTC 02043; 3, *R. dentocariosa* JCM 3067; 4, *R. mucilaginosa* JCM 10910; 5, *R. terrae* JCM 15158; 6, *R. amarae* JCM 11375; 7, *R. nasimurium* JCM 10909; 8, *R. endophytica* JCM 18541; 9, *Streptococcus mitis* JCM 12971; 10, *S. oralis* JCM 12977; 11, *S. gordonii* JCM 12995; 12, *S. sanguinis* JCM 5708; 13. *S. salivarius* ATCC 7073; 14, *S. anginosus* JCM 12993; 15, *S. mutans* JCM 5705; 16, *S. sobrinus* DSM 20742; 17, *Actinomyces naeslundii* JCM 8349; 18, *A. oris* JCM 16131; 19. *A, odontolyticus* JCM 14871; 20, *Neisseria sicca* CCUG 23929; 21, *Corynebacterium matruchotii* JCM 9386; 22, *C. durum* JCM 11948.M, molecular size marker (100-bp DNA ladder).

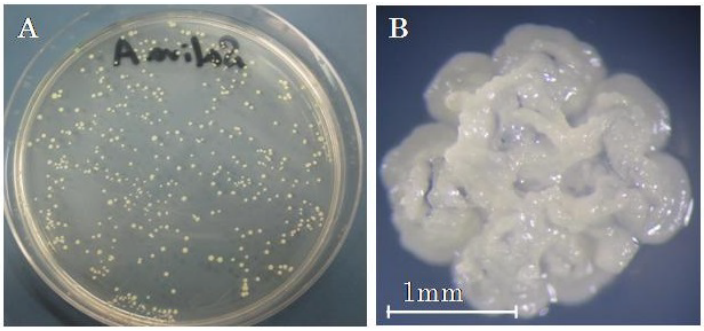

Figure 2: Appearance of *R. aeria* colonies on RAESM

A: *R. aeria* colonies on RAESM inoculated with saliva sample.

B: Stereomicroscope image of *R. aeria* colony on RAESM.

Species	Primer	Sequence	Product size (bp)	Position	number
R. aeria	RAF	GTGCTTGCACGTGGATTAGTGG	918	28-49	AB071952
	RAR	TGACGCGATCTAATGCATGTCAAG		945-922	

Table 2: Locations and sequences of species-specific primers for the 16S rDNA of *R. aeria*

Subject	Total bacteria	*R. aeria*	Detection rate
	BHI-Y CFU/ml, × 10^7	*RAESM* CFU/ml, × 10^5	
A	3.3	2.0	0.6
B	2.7	0.3	0.1
C	2.1	0.7	0.3
D	5.8	27	4.7
E	4.5	1.0	0.2
F	6.2	4.8	0.8
G	11	6.0	0.5
H	2.3	3.2	1.4
I	4.1	1.5	0.4
J	3.8	15	4.0
K	6.3	4.0	0.6
L	7.2	2.5	0.4
M	11	3.3	0.3
N	3.4	1.1	0.3
O	9.8	3.0	0.3
P	6.5	5.7	0.9
Q	8.8	8.0	0.9
R	8.7	8.4	1.0
S	5.2	9.0	1.7
T	4.6	4.8	1.0
Average	5.9	5.6	1.0

Table 3: Percentage o*f R. aeria* in saliva samples from 20 subjects

Antimicrobial agent	CLSI Standards (μg/ml)		MIC (μg/ml) *R. aeria* JCM 11412	Range of MIC (μg/ml) Clinical isolates of *R. aeria* (No. of isolates) Subject					
Oxacillin	S ≦ 0.25	R ≧ 0.5	0.06	A (n=5)	B (n=5)	C (n=5)	D (n=5)	E (n=5)	F (n=5)
Erythromycin	S ≦ 0.5	R ≧ 8	0.25	0.03-0.06	0.03-0.06	0.03-0.06	0.03-0.06	0.03-0.06	0.03-0.06
Lincomycin	S ≦ 0.5	R ≧ 8	2	0.05-0.125	64-128	0.05-0.125	0.05-0.125	0.125-0.5	0.05-0.125
Clindamycin	S ≦ 0.5	R ≧ 8	1	2-4	64-128	1-2	1-2	2-4	1-2
Gentamycin	S ≦ 4	R ≧ 16	8	4-8	4-8	32	4-8	4-8	4-8
Teikoplanin	S ≦ 8	R ≧ 32	0.5	1-2	1-2	0.5-2	0.5-1	1-2	0.5-2
Vancomycin	S ≦ 4	R ≧ 32	2	1-2	1-2	1-2	1-2	1-2	2

Table 4: Antibiogram of *R. aeria* reference strains and clinical isolates

adhered to the agar medium such that they were not easily scraped off. The average colony size of *R. aeria* on RAESM was 1.8 mm in diameter.

Antibiotics susceptibility tests of *R. aeria* isolates

R. aeria reference strains and isolates from subject A, C, D, E, and F were susceptible to most antibiotics (Table 4). On the other hand, *R. aeria* isolates from subject B were highly resistant to erythromycin, lincomycin and clindamycin.

Discussion

R. dentocariosa, R. mucilaginosa, and R. aeria are part of the normal flora in the human oral cavity and pharynx [8-11]. *R. aeria* was first isolated from air and condensation water samples from the Russian space station Mir [1]. *R. aeria* was originally classified as *R. dentocariosa* genomovar II before the report of Li *et al*. [1]. *R. aeria* is capable of causing serious systemic infections, such as sepsis, bronchitis, pneumonia, and endocarditis [12-17]. However, there has never been reported the selective medium for the isolation of *R. aeria* only. Therefore, a suitable selective medium for the isolation of *R. aeria* is necessary to assess the veritable prevalence of this organism in the human oral cavity and to diagnose *R. aeria* infections rapidly. To examine the bacterium population in the oral cavity, a novel selective medium, designated RAESM, was developed for the isolation of *aeria* in this study. RAESM was highly selective for *R. aeria.*

On clinical microbiological examination, *Rothia species* can be mistaken for bacteria such as *Dermabacter hominis, Actinomyces viscosus, Propionibacterium avidum, Corynebacterium matruchotii, and Nocardia* species because many laboratories are unfamiliar with these organisms, which may be difficult to culture due to having the same gram positive rods and to their variable aero-tolerance [22-24]. Moreover, the colonies of *Nocardia* species are similar to those of *R. aeria* [18]. *R. aeria* is capable of causing serious systemic infections [15-20]. Therefore, RAESM may contribute to the correct and rapid diagnosis of the infectious diseases caused by *R. aeria.*

In this study, *R. aeria* was detected in all subjects and accounted for 1.0% of total bacteria in saliva. These results indicated that *R. aeria* is a part of the normal flora in the oral cavity of humans and is not a microorganism peculiar to a specific environment, such as the space station. In our previous studies, *R. dentocariosa and R. mucilaginosa* accounted for 2.6% and 3.4% of total bacteria in saliva, respectively [9,10]. Consequently, it was indicated that *R. aeria* is a part of the normal flora in the human oral cavity, and the genus *Rothia* includes three species that inhabit the human oral cavity. *aeria* can be mistaken for *Nocardia* spp due to the morphological similarities, and discrimination between *R. aeria* and *Nocardia* spp needs further analyses, such as 16S rRNA sequencing [18]. Microorganisms of the genus *Nocardia* are branching and partially acid-alcohol-fast gram-positive bacilli, and they belong to the order of *Actinomycetales.* Numerous species have been described and are being reclassified continuously thanks to the use of molecular biology techniques. Nocardiosis is caused by various species of the genus *Nocardia*. It can cause lung disease, skin disease, or systemic disorders by involving the central nervous system, but it can also colonize the airways asymptomatically. Saraya *et al*. [25] has reported that *R. aeria* should be considered in the differential diagnosis of Nocardia spp, especially in immunocompromised patients who are vulnerable to Nocardia infections. Selective medium for the isolation of *R. aeria*, i.e. RAESM, and the PCR analysis developed in this study may help us rapidly diagnose the infectious diseases caused by *R. aeria* or *Nocardia* spp.

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