A Novel Selective Medium for the Isolation of *Rothia aeria* in Oral Cavities

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Abstract

Among the genus *Rothia, Rothia dentocariosa* and *Rothia mucilaginosa* are found in oral cavity and pharynx of humans. Furthermore, there is only one report that *Rothia aeria*, which is capable of causing serious systemic infections, has been also detected in the mouths of healthy individuals. A suitable selective medium for the isolation of *R. aeria* is necessary to assess the veritable prevalence of this organism in the oral cavity. To examine the bacterium population in the oral cavity, a novel selective medium, designated RASM, was developed for the isolation of *R. aeria.* RASM consists of sodium gluconate, tryptone, lablemco powder and aztreonam. Average growth recovery of *R. aeria* on RASM was 96.1% compared with that on BHI-Y agar. Growth of other representative oral bacteria including *R. dentocariosa*, *R. mucilaginosa*, genera *Actinomyces*, *Neisseria*, and *Corynebacterium* were remarkably inhibited on the

selective medium. Though the inhibitions of certain strains of streptococci were not complete, they formed small or pin colony on RASM and could be distinguished from *R. aeria* colony. Clinical efficacy was evaluated by the recovery of *R. aeria* on RASM from the stimulated saliva samples of ten volunteers. *R. aeria* was detected at 3.1 (range: 0.5-14.4) % to total bacteria of 7.0×10^7 CFU/ml on BHI-Y agar in their oral cavities. The new selective medium, RASM, was useful for the isolation of *R. aeria*. Moreover, we examined the antibiotic susceptibility of twenty isolates from four subjects. The bacterium was susceptible to most antibiotics; however *R. aeria* isolates from one subject were highly resistant to erythromycin, lincomycin, and clindamycin.

Introduction

There are actually six species in genus *Rothia*: *Rothia aeria*, *Rothia amarae*, *Rothia dentocariosa*, *Rothia mucilaginosa*, *Rothia nasimurium*, and *Rothia terrae* (1-5). Among the six *Rothia* species, *R. dentocariosa* and *R. mucilaginosa* are found in the oral cavity and pharynx of humans (6-10). Concerning *R. aeria*, it was first isolated from air and condensation water samples from the Russian space station, Mir (1). *R. aeria* is capable of causing serious systemic infections, such as sepsis, bronchitis, pneumonia, and endocarditis (13-17). There is only one report that *R. aeria* has been also detected in the mouths of healthy individuals (18). We previously reported each selective medium for the isolation of *R. dentocariosa* and *R. mucilaginosa* (8, 9). Since both selective media inhibit the growth of *R. aeria*, and this organism has never been detected in our previous studies, we did not know whether *R. aeria* is normal oral flora or

not. Therefore, a suitable selective medium for the isolation of *R. aeria* is necessary to assess the veritable prevalence of this organism in the oral cavity.

According to some previously described case reports on serious systemic infections, *R. aeria* isolates from clinical specimens such as blood is susceptible to many antibiotics (14-16); however, it demonstrates only intermediate susceptibility to clindamycin and vancomycin (14). Resistance to clindamycin (16), aztreonam (16) and ofloxacin (15) determined using disk diffusion tests has also been reported.

The purpose of this study was to develop a new selective medium for the isolation of *R. aeria* and to investigate its distribution in the oral cavity. Moreover, in order to expand the knowledge of *R. aeria*, we examined the antibiotic susceptibility of the isolates from the oral cavities.

Material and Methods

Bacterial strains and culture conditions

All bacterial strains used in this study are listed in Table 1, 2. The strains were maintained by cultivating them in brain heart infusion (BHI; Difco Laboratories, Detroit, Mich.) supplemented with 1% yeast extract (BHI-Y). The bacteria were cultured in BHI-Y broth at 37°C for 24 h in aerobic conditions for members of the genus *Rothia* and in a 5% CO_2 atmosphere for other representative oral bacteria.

Evaluation of the base medium

A base medium was based on RSM (19) with some modifications, and was composed of the following (per liter): 10 g of sodium gluconate (Wako Pure Chemical Ind. Ltd., Tokyo, Japan), 5 g of tryptone (Difco Laboratories, Mich. USA), 5 g of lablemco powder (Oxoid Ltd., Hampshire, England), and 15 g of agar. BHI-Y agar was

used to compare with the base 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 each medium. Plates were cultured at 37°C for 72 h in aerobic conditions and the number of Colony-Forming Units (CFU)/ml was calculated.

Susceptibility tests

The microbroth dilution method was used for susceptibility testing (20). Preliminary studies of antibiotic selection were also performed by disk susceptibility tests (Sensi-Disk, Becton Dickinson Co., MD, USA).

Recovery of R. aeria *and other representative oral bacteria on the selective medium*

Recovery of *R. aeria* and other representative oral bacteria were calculated CFU/ml on the selective medium

compared with those on BHI-Y agar for total cultivable bacteria. Bacteria were pre-incubated in BHI-Y broth at 37°C for 24 h in each condition. 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 BHI-Y agar and the selective medium. The selective medium plates were cultured at 37°C for 72 h in aerobic conditions , BHI-Y agar plates were cultured at 37°C for 48 h in 5% CO_2 atmosphere, and the number of CFU/ml was calculated.

Clinical samples

Clinical specimens were collected from ten volunteers (age 28-66, male 5, female 5). Paraffin-stimulated whole saliva samples were collected into a sterile microcentrifuge tube. Samples were dispersed by sonication for 30 sec in an ice bath (50 W, 20 kHz, Astrason® System model XL 2020, NY., USA). Portions

(100 µl) of appropriate dilutions of these samples were plated, in triplicate, on BHI-Y and the selective medium plates. BHI-Y plates for total bacteria were incubated at 37ºC for 48 h in 5% CO_2 atmosphere, selective medium plates were cultured at 37ºC for 72 h in aerobic conditions, and the number of CFU/ml was calculated for each. This study was approved by the Ethics Committee of Nihon University School of Dentistry at Matsudo, Japan (EC 11-020).

Identification of R. aeria *from clinical samples*

Ten colonies per subject on the selective medium were subcultured to confirm by polymerase chain reaction (PCR) analysis.

PCR analysis

PCR analysis for the identification of *R. aeria* was performed as described previously (21). Briefly, bacterial

cells were cultured in BHI-Y broth for 24 h, and then 1 ml samples were collected in microcentrifuge tubes and resuspended at a density of 1.0 McFarland standard (approximately 10^7 CFU/ml) in 1 ml of sterile distilled water. Finally, 3.6 µl of the suspension were used as a PCR template. The multiplex PCR mixture contained 0.5 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 template in a final volume of 20 µl. The PCR reactions were carried out in a DNA thermal cycler (Applied Biosystems 2720 Thermal Cycler; Applied Biosystems, CA, USA). The PCR conditions included an initial denaturation step at 98°C for 2 min, followed by 25 cycles consisting of 98°C for 10 sec, 66°C for 15 sec and 68°C for 1 min. The PCR products were analyzed by 2.0% agarose gel electrophoresis. A 100 bp DNA ladder (Takara Biomed, Shiga, Japan) was used as a molecular size marker.

Antibiotics susceptibility tests of R. aeria *isolates*

Antibiotics susceptibilities of *R. aeria* isolates were characterized using microdilution method. Antibiotics used in this study are listed in Table 4. They are widely used in the treatment of Gram-positive bacterial infections. Although no Clinical and Laboratory Standards Institute (CLSI) protocols exist for *R. aeria*, we assessed the organism's drug susceptibility utilizing the 2009 CLSI criteria (M100-S19) for staphylococci.

Results

Evaluation of the base medium

A base medium for the growth of *R. aeria* has been studied. *R. aeria* grew well on BHI-Y agar and the test medium, which was a little modified RSM (19), at similar ratios (data not shown). Oral streptococci (*Streptococcus oralis, Streptococcus salivarius*, *and Streptococcus*

anginosus) and oral rothia (*R. dentocariosa* and *R. mucilaginosa*) formed small or pin colonies on the test medium, and genera *Actinomyces*, *Neisseria*, and *Corynebacterium* did not grow on it (data not shown). Finally, a base medium was determined the test medium, because it inhibited the growth of other bacteria and the colony size of *R. aeria* on the test medium was similar to that on BHI-Y agar.

Susceptibility to antibiotics

R. aeria was resistant to aztreonam. The minimal inhibitory concentration (MIC) of aztreonam to *R. aeria*, *R. dentocariosa*, and *R. mucilaginosa* was 250 μg/ml, 100 μg/ml, and 50 μg/ml, respectively.

Composition of new selective medium

The new selective medium, designated *R. aeria* selective medium (RASM), was composed of the following (per

liter): 10 g of sodium gluconate, 5 g of tryptone, 5 g of lablemco powder, 40 mg of aztreonam, and 15 g of agar. The antibiotic was added, after the base medium had been sterilized and cooled to 50°C.

Recovery of R. aeria *and other representative oral bacteria on the selective medium*

Table 1 shows the recovery of *R. aeria* on RASM compared with BHI-Y agar. Recovery of *R. aeria* ranged from 94.5% to 98.9% (average 96.1%) on RASM compared with that on BHI-Y agar. On primary isolation, the colonies of *R. aeria* on RASM commonly exhibited rough, dry, folded and convex appearance (Fig. 1) and adhered to the agar medium such that they were not easily scraped off. The size of colonies on RASM ranged 1.3 to 2.1 mm in diameter after 72 h incubation.

Table 2 shows the inhibition of other representative oral bacteria on RASM compared with BHI-Y agar. RASM

inhibited the growth of them, except for *S. oralis* and *S. salivarius*. Although the growth inhibitions of *S. oralis* and *S. salivarius* were not complete, these organisms formed small or pin colonies, the size of which ranged 0.1 to 0.2 mm on RASM.

Clinical examination

The proportion of *R. aeria* in saliva from ten subjects on BHI-Y agar and RASM is shown in Table 3. The mean number of total bacteria was 0.7×10^8 CFU/ml (range: 0.2×10^8 - 1.6×10^8). The mean number of *R. aeria* was 2.2×10^6 CFU/ml (range: 0.4×10^6 - 6.4×10^6). *R. aeria* accounted for 3.1% of total bacteria, and were detected from saliva samples of all ten subjects.

Antibiotics susceptibility tests of R. aeria *isolates*

Table 4 shows the antibiotics susceptibility tests of *R. aeria* isolates. Reference strains and clinical isolates of *R.*

aeria were susceptible to most antibacterial agents. Among the subjects, the isolates from subject B were highly resistant to erythromycin, lincomycin and clindamycin.

Discussion

R. dentocariosa and *R. mucilaginosa* are part of the normal flora in the human oral cavity and pharynx (6-10).

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). We were unable to find any detailed reports on the localization of *R. aeria* in human oral cavities, although there is only one report of the colonization by *R. aeria* on the tongue of healthy adults (18). Therefore, if *R. aeria* is part of normal flora in the oral cavity, a suitable identification method and selective medium is necessary to assess the

veritable prevalence of *R. aeria* in the oral cavity. To examine the bacterium population in the oral cavity, a novel selective medium, designated RASM, was developed for the isolation of *R. aeria* in this study. RASM was highly selective for *R. aeria*.

On medical 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 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* (25). *R. aeria* is capable of causing serious systemic infections (13-18). Therefore, RASM may contribute to the correct and rapid diagnosis of the infection diseases caused by *R. aeria*.

In this study, *R. aeria* was detected from all subjects, and accounted for 3.1% of total bacteria in saliva. In our previous studies, *R. dentocariosa* and *R. mucilaginosa* accounted for 2.6% and 3.4% of total bacteria in saliva respectively (8, 9). These results indicated that *R. aeria* is part of normal flora in the oral cavity as same as known oral rotiha, *R. dentocariosa* and *R. mucilaginosa*.

In this study, *R. aeria* was susceptible to most antibiotics, however clinical isolates of *R. aeria* from one subject were highly resistant to erythromycin, lincomycin, and clindamycin. Clinical isolates of *R. aeria* in this study were more highly resistant to some antibiotics than the strains of some previously described case reports on serious systemic infections. It might be necessary to monitor the antibiotics resistance of *R. aeria* continually.

We developed a novel selective medium with high selectivity, designated RASM, for the isolation of *R. aeria.* RMSM is useful for determining the distribution and role

of this organism in oral cavities.

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Table 1　Recovery of *R. aeria* on BHI-Y and RASM

Strain		BHI-Y	RASM	
		CFU/ml, $\times 10^8$	CFU/ml, $\times 10^8$	Recovery, %
Rothia aeria				
	JCM 11412	2.1 ± 0.2[a]	2.1 ± 0.2	98.9
	NUM-Ra7006	2.2 ± 0.3	2.1 ± 0.3	96.2
	NUM-Ra7007	1.0 ± 0.1	1.0 ± 0.1	99.6
	NUM-Ra7008	2.1 ± 0.1	1.9 ± 0.2	94.5
	NUM-Ra7009	1.6 ± 0.2	1.5 ± 0.2	95.6

[a] Ave ± SD.

Table 2　Growth of other representative oral bacteria on BHI-Y and RASM

Strain	BHI-Y	RASM
	CFU/ml, $\times 10^8$	Recovery, %
Rothia dentocariosa		
JCM 3067	1.1	<0.0[a]
NUM-Rd6018	1.5	<0.0
NUM-Rd6020	0.9	<0.0
Rothia mucilaginosa		
JCM 10910	2.1	<0.0
NUM-Rm6504	1.2	<0.0
NUM-Rm6505	1.7	<0.0
Streptococcus oralis		
ATCC 10557	1.3	1.5[b]
Streptococcus salivarius		
HHT	3.2	1.9[b]
Streptococcus anginosus		
ATCC 11391	5.3	<0.0
Streptococcus mutans		
NCTC 10449	6.4	<0.0
Actinomyces viscosus		
ATCC 19246	1.3	0
Actinomyces naeslundii		
ATCC 12104	1.1	0
Actinomyces odontolyticus		
NUM-Ao12	0.8	0
Neisseria sicca		
ATCC 2925	2.7	0
Corynebacterium matruchotii		
ATCC 14266	0.8	0

[a] less than 0.1%, [b] small or pin colonies.

Table 3　Proportion of *R. aeria* in saliva samples from 10 subjects on BHI-Y and RASM

Subject	BHI-Y	RASM	Detection ratio
	Total bacteria	*R. aeria*	
	CFU/ml, x 10^8	CFU/ml, x 10^6	%
A	1.6	6.4	3.9
B	0.6	1.2	2.0
C	0.2	2.6	14.4
D	0.3	1.1	3.3
E	1.2	3.6	2.9
F	0.3	2.1	0.8
G	0.9	0.4	0.5
H	0.6	3.3	5.5
I	1.1	1.1	1.0
J	0.2	0.4	2.0
Average	0.7	2.2	3.1

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

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

[a] sensitive, [b] resistant.

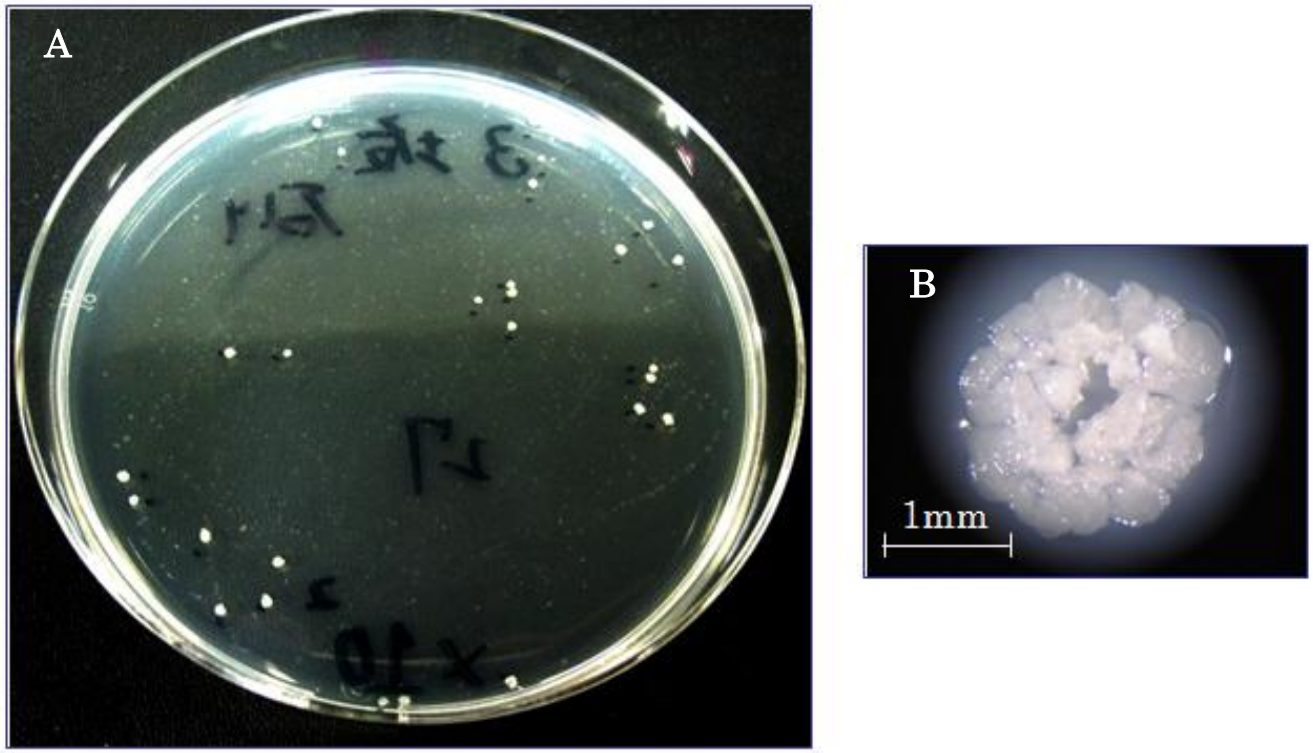

Fig. 1 Appearance of *R. aeria* colonies on RASM
A: *R. aeria* colonies on RASM inoculated with saliva sample
B: Stereomicroscope image of *R. aeria* colony on RASM