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Original Article

Molecular Identification of Clinical *Rothia* Isolates from Human Patients: Proposal of a Novel *Rothia* Species, *Rothia arfidiae* sp. nov.

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Four Gram-positive cocci were isolated from the cerebrospinal fluid or blood of four different patients, but they could not be identified by an automated conventional identification system, so they were identified using cellular fatty acid (CFA) composition analysis and 16S rRNA gene sequencing analysis. Of these, two strains (SMC-A2662 and SMC-A5889), which were previously supposed to be *Rothia dentocariosa* according to the API Coryne system, were identified as *Rothia aeria* by the 16S rRNA gene analysis. SMC-A608, which was unidentified by both the VITEK2 and API Coryne systems, was identified as *Rothia mucilaginosa*. The one remaining SMC-2244^T was distinguished from the other *Rothia* species by its biochemical profile, its CFA composition and its 16S rRNA gene sequence. Phylogenetic analysis showed that it was closely related to *Rothia nasimurium* but the 16S rRNA gene sequence dissimilarity of 1.8% was enough to differentiate it from *R. nasimurium*. Based on both the phenotypic and phylogenetic evidence, we propose a new species name for this bacterium, *Rothia arfidiae* sp. nov. The results of this study show that several *Rothia* species were isolated from human and we have identified them using 16S rRNA gene sequences.

Key Words: *Rothia*; 16S rRNA gene; New species

INTRODUCTION

The genus *Rothia* was firstly proposed in 1967 with designating *Rothia dentocariosa* as the type of species (1). *R. dentocariosa* has long been recognized as the only species of the genus *Rothia*, although intraspecific heterogeneity has been observed (2). However, several novel *Rothia* species were recently described: *Rothia nasimurium*, *Rothia mucilaginosa*, *Rothia amarae*, and *Rothia aeria* (3~5). *R. dentocariosa* is a common colonizer of the human oral cavity, but it has also been reported to be a cause of serious infections such as endocarditis and bacteremia (6, 7). Although *R. aeria* had been recognized as *R. dentocariosa* and designated as *R. dentocariosa* genomovar II, based on a partial 16S rRNA gene sequence and the whole cell protein analysis (8), it was later proposed as being a distinct species (5). At present, correct identification of *Rothia* is difficult, and this is partly because of its rarity. Although chemotaxonomic identification using API Coryne test strips (bioMériuex, Hazelwood, MO, USA) is recommended for *R. dentocariosa*, further confirmation by an alternative

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Table 1. *Rothia* isolates identified in this study

Isolates	Patient (sex/age)	Isolation	Specimen	Underlying disease	Species
SMC-A5889	F / 52	Oct, 2004	Blood	Pituitary adenoma	*Rothia aeria*
SMC-A2662	M / 59	Dec, 2004	Blood	Chronic renal failure	*Rothia aeria*
SMC-A6087	F / 2	Jun, 2004	Blood	Pneumonia	*Rothia mucilaginosa*
SMC-2244^T	F / 22	Feb, 2004	CSF[a]	Pituitary adenoma	*Rothia arfidiae* sp. nov[b]

[a] Cerebrospinal fluid. [b] Novel *Rothia* species proposed in the present study

method is required (9). Moreover, other *Rothia* species could not be identified with using such a chemotaxonomic method.

When attempting to identify microorganisms that were unidentified by conventional methods in our clinical microbiology laboratory using 16S rRNA gene sequencing in 2004, we found four isolates belonging to the genus *Rothia*. In this paper, we identified these four *Rothia* isolates and we propose a novel *Rothia* species, *Rothia arfidiae* sp. nov. based on the phenotypic and phylogenetic analyses.

MATERIALS AND METHODS

Bacterial isolates

The four isolates described in this paper were isolated from four different patients in 2004. The ages and underlying diseases of the patients were variable. The bacterial organisms were isolated from sterile sites such as cerebrospinal fluid or blood (Table 1). They were all gram-positive cocci and grew on blood agar at 37℃. They could not be identified by conventional automated methods such as VITEK2 (bioMérieux) and MicroScan (Dade-Microscan, Sacramento, CA, USA), and so these bacteria were submitted to the API Coryne system (bioMérieux), for cellular fatty acid (CFA) composition analysis and molecular identification with using 16S rRNA gene sequencing.

CFA compositions and 16S rRNA gene sequencing

The CFA compositions were examined using a Hewlett Packard 6890A GC and the MIDI aerobe method (Chem Station ver. 4.02) at MicroID (Seoul, Korea). For performing PCR of the 16S rRNA gene, the genomic DNAs of the four isolates were extracted from bacterial colonies by a simple boiling-lysis method (10). In brief, the colonies were suspended in lysis buffer (100 mM NaCl, 10 mM Tris-HCl, 1 mM EDTA, and 1% Triton X-100), and they were incubated at 90℃ for 10 min. The mixture was then centrifuged for a moment and the aqueous phase was used as a template for PCR. 16S rRNA gene was amplified with universal primers 16S-F3 (5'-CAG GCC TAA CAC ATG CAA GT-3') and 16S-R3 (5'-GGG CGG WGT GTA CAA GGC-3') (11). The template DNA and 20 pmol of each primer were added to a PCR mixture tube (AccuPower PCR PreMix; Bioneer, Daejon, Korea). The reaction mixture was then subjected to 35 cycles for amplification. Each cycle consisted of 30 s at 95℃ for denaturation, 30 s at 60℃ and 1 min at 72℃ for extension; this was followed by a final extension step at 72℃ for 5 min. The amplified PCR product was purified for sequencing with using PCR purification kit (CoreOne, Seoul, Korea). The DNA sequences were determined with an ABI prism Rhodomine terminator cycle sequencing kit (PE Biosystems, Foster City, CA, USA) and an ABI 3710 automated sequencer (PE Biosystems).

Phylogenetic analysis

The 16S rRNA gene sequences of the four bacterial isolates were compared with those of the GenBank database and they were aligned using the CLUSTAL X program. Phylogenetic relationships were determined by the Neighbor-joining (NJ) method. Bootstrap analysis with 1,000 replications was also performed to test the robustness of the groupings.

Nucleotide accession numbers

The 16S rRNA gene sequences of the four isolates

Table 2. Biochemical profiles of *Rothia* isolates[a]

Isolates	Nitrate reduction	Pyrrolidonyl arylamidase	Alkaline phosphatase	α-glucosidase	Catalase
SMC-A5889	+	+	−	+	+
SMC-A2662	+	+	−	+	+
SMC-A6087	−	+	−	−	−
SMC-2244^T	+	−	+	+	+

[a] Positive for esculin hydrolysis and fermented glucose, maltose, and sucrose. Negative for β-glucoronidase, β-galactosidase, N-acetyl-β-glucosaminidase, urea and gelatin hydrolysis, and fermented gelatin, ribose, xylose, mannitol, lactose, and glycogen

determined in this study have been deposited in GenBank under accession nos. DQ673319 to DQ673322.

RESULTS

Biochemical profiles

The biochemical profiles of the four isolates, which were repeatedly determined by using the API Coryne system, are shown in Table 2. Two isolates, SMC-A5889 and SMC-A2662, were identified as *R. denticoriosa* by the API Coryne system. They were positive for nitrate reduction, pyrrolidonyl arylamidase, esculin hydrolysis, α-glucosidase, and catalase, and they were negative for alkaline phosphatase, β-glucoronidase, β-galactosidase, N-acetyl-β-glucosaminidase, urea and gelatin hydrolysis. They fermented glucose, maltose, and sucrose, but not gelatin, ribose, xylose, mannitol, lactose, and glycogen. These characteristics are identical to those of previously described *R. denticoriosa* and *R. aeria* strains (5, 6, 12). The other two isolates could not be identified using the API Coryne system. The isolate SMC-2244^T differed from SMC-A5889 and SMC-A2662 in that it was negative for pyrrolidonyl arylamidase and positive for alkaline phosphatase. Another isolate SMC-A6087 was negative for nitrate reduction, alkaline phosphatase, α-glucosidase, and catalase, but it was positive for pyrrolidonyl arylamidase (Table 2).

Cellular fatty acid composition

The CFA compositions of the four *Rothia* isolates are shown in Table 3. For SMC-A5889 and SMC-A2662, anteiso-C15:0 predominated (47.4% and 51.5%, respectively), and this was followed by iso-C16:0 (15.9% and 20.5%, respectively) and anteiso-C17:0 (10.7% and 13.0%, respectively). The percentages of the compositions of C18:1 w9c and C18:0 were more than 5.0% in SMC-A5889, but not in SMC-A2662. The other two isolates, SMC-6087 and SMC-2244^T, showed similar CFA compositions with each other. Anteiso-C15:0 was also the most predominant in SMC-A6087 and SMC-2244^T (72.6% and 68.4%, respectively), and the percentages of the compositions of the other CFAs were less than 6.0%. Comparisons with the CLIN 40 database indicated that three isolates but SMC-A5889 were not closely related to the *Rothia* species. That is, SMC-2244^T was closely related to *Kocuria varians* (confidence rate: 0.472), SMC-A6087 to *Micrococcus luteus* (confidence rate: 0.434), and SMC-A2662 to *Legionella jordanis* (confidence rate: 0.539). SMC-A5889 was identified as *R. dentoccariosa* with moderate confidence (0.558), when compared to the CLIN 40 database.

Table 3. Fatty acid compositions of four isolates of *Rothia* species[a]

Fatty acid composition	SMC-A5889	SMC-A2662	SMC-A6087	SMC-2244^T
anteiso-C13:0	−	−	2.0	2.0
iso-C14:0	2.9	3.6	2.2	3.1
C14:0	−	1.3	1.2	4.9
iso-C15:0	2.8	3.9	1.2	2.4
anteiso-C15:0	47.4	51.5	72.6	68.4
iso-C16:0	15.9	20.5	3.0	6.0
C16:0	6.4	3.3	5.0	5.3
anteiso-C17:0	10.7	13.0	3.7	5.4
C18:1 w9c	5.4	−	4.4	−
C18:0	5.2	−	2.4	−

[a] Only fatty acid composition of more than 1.0% was shown

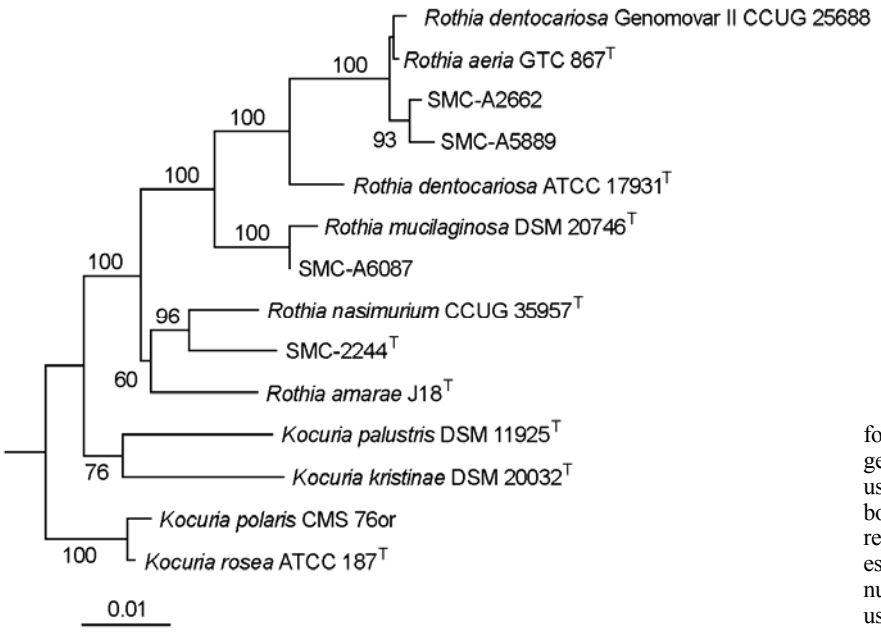

Figure 1. Phylogenetic relationships of four *Rothia* isolates based on 16S rRNA gene sequences. This tree was constructed using the Neighbor-joining method and bootstrap values were evaluated from 1,000 replications. The scale bar indicates the estimated number of substitutions per 100 nucleotides. *Micrococcus lylae* d10 was used as an outlier.

16S rRNA gene analysis

We obtained 1,109 bp to 1,269 bp 16S rRNA gene sequences from the four isolates. Comparison of them with the sequences in the database of GenBank showed the best match to the *Rothia* species. The phylogenetic tree was drawn after retrieving the 16S rRNA gene sequences of different types of strains of five *Rothia* species, i.e., *R. dentocariosa* genomovar II CCUG 25688, and four *Kocuria* species (Fig. 1). SMC-A2662 and SMC-A5889, which showed 0.4% sequence dissimilarity each other, were closely related with *R. aeria* GTC 867^T (0.4% and 0.5% dissimilarities, respectively) and *R. dentocariosa* genomovar II CCUG 25688 (0.3% and 0.4% dissimilarities, respectively). SMC-A6087 showed 0.1% dissimilarity with *R. mucilaginosa* DSM 27046^T. SMC-2244^T was clustered with *R. nasimurium* CCUG 35959^T in the 16S rRNA gene tree, but they showed 1.8% 16S rRNA gene sequence dissimilarity.

DISCUSSION

Accurate identification of bacteria from a clinical sample is critical for administering proper treatment because antibiotic use is finally dependent on the etiologic diagnosis of pathogens. Nowadays, advanced automated systems such as VITEK and MicroScan, which are based on discriminating the biochemical characteristics of bacteria, have been adopted for identifying bacterial isolates by most clinical microbiology laboratories. However, biochemical traits may affect by the environment and deviations among strains may exist. In addition, bacterial species that are only rarely isolated are more difficult to identify as there is no information about them in the commercial identification systems.

Of the several dozens of unidentified isolates that were isolated at Samsung Medical Center (Seoul, Korea), we identified four isolates as *Rothia* species. It has not yet been proved that these isolates caused true infection in patients. However, it is important to identify *Rothia* species because they may often cause serious infections such as endocarditis and bacteremia (6, 7, 13). In this study, two *R. aeria* isolates (SMC-A5889 and SMC-A2662) were identified. The API Coryne system identified SMC-A5889 and SMC-A2662 as *R. dentocariosa*, but the 16S rRNA gene sequence analysis indicated that they belong to *R. aeria* rather than to *R. dentocariosa* (Fig. 1). *R. aeria* is closely related to *R. dentocariosa* as they show similar biochemical characteristics,

and *R. aeria* has been described as *R. dentocariosa* genomovar II (8). CFA composition analysis also could not differentiate *R. aeria* from *R. dentocariosa* (5). However, phylogenetic analysis using the 16S rRNA gene sequence and DNA-DNA hybridization analysis indicated that *R. aeria* is distinct from *R. dentocariosa* (5), which was also confirmed in this study.

One isolate, SMC-A6087, was identified as *R. mucilaginosa* as they showed 99.9% similarity with performing 16S rRNA gene sequencing. *R. mucilaginosa*, previously known as *Stomatococcus mucilaginosa*, is usually a colonizer of the human oral cavity and pharynx (3, 8). However, it is also an opportunistic pathogen that can be responsible for endocarditis, sepsis, peritonitis and infections associated with tissue and organ transplantations (14). According to the biochemical profile, *R. mucilaginosa* can be differentiated from other opportunistic human pathogenic *Rothia* species such as *R. dentocariosa* and *R. aeria* because it is negative for nitrate reduction, α-glucosidase, and catalase (Table 2). The CFA composition of *R. mucilaginosa* was characterized by the relative abundance of iso-C14:0 and iso-C16:0 (3, 5). However, the percent compositions of iso-C14:0 and iso-C16:0 of SMC-A6087 were less than 5.0%, and then comparison with the CLIN 40 database indicated a close relationship with *Micrococcus luteus*; this highlights the difficulty in identifying *Rothia*.

A novel *Rothia* species was identified in this study. An isolate SMC-2244^T, which was isolated from the CSF of a patient with pituitary adenoma, showed 1.8% sequence dissimilarity of the 16S rRNA gene with *R. nasimurium* CCUG 35957^T, a bacterium that was isolated from the nose of a mouse. With considering the 16S rRNA gene sequence dissimilarity between *R. dentocariosa* and *R. aeria* (1.7~2.3%), it is enough to regard SMC-2244^T as a new *Rothia* species. In addition, on the biochemical profile, SMC-2244^T differed from *R. nasimurium* in that it was positive for alkaline phosphatase and it produced lactose (3). In the CFA analysis, iso-C16:0 of SMC-2244^T (6.0%) differed from that of *R. nasimurium* (18.7%), although the overall composition was very similar each other. Thus, based on the biochemical profile, the CFA composition and 16S rRNA gene sequence analysis, the strain SMC-2244^T is proposed as a new *Rothia* species, *Rothia arfidiae* sp. nov.

Description of *Rothia arfidiae* sp. nov.

The species name, *aarfidiae*, stands for ARFID, Asian-Pacific Research Foundation for Infectious Diseases.

It is a gram-positive coccus and it grows well on blood agar. It is positive for nitrate reduction, esculin hydrolysis, α-glucosidase, and catalase, and it's negative for pyrrolidonyl arylamidase, alkaline phosphatase, β-glucoronidase, β-galactosidase, N-acetyl-β-glucosaminidase, urea and gelatin hydrolysis. It can ferment glucose, maltose, lactose, and sucrose, but not gelatin, ribose, xylose, mannitol, and glycogen. It has the cellular fatty acid anteiso-C15:0 as the major fatty acid component, and its 16S rRNA gene sequence showed 1.8% dissimilarity with *Rothia nasimurium*. It was isolated from the cerebrospinal fluid of a patient with pituitary adenoma, but so far, its pathogenic significance remains unknown. The G+C content of the DNA is 56.8 mol%. The type strain of *R. arfidiae* is strain SMC-2244^T.

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