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

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Abstract

Rothia dentocariosa and *Rothia mucilaginosa*, which are opportunistic pathogens that are capable of causing serious infections, inhabit the oral cavity. However, there is no suitable method for assessing the prevalence of *Rothia* species in the oral cavity. In this study, four polymerase chain reaction (PCR) primers were designed based on partial sequences of the 16S rDNA genes of the abovementioned oral *Rothia* species. These primers react to the oral *Rothia* species and did not react to other *Rothia* species except *Rothia aeria*. Moreover, representative non-*Rothia* oral bacteria displayed negative reactions to these primers. These results indicate that these primers are useful for identifying *R. dentocariosa* and *R. mucilaginosa*. We also developed a simple multiplex PCR procedure involving the two primer pairs designed in the present study as a rapid and reliable method of identifying known oral *Rothia* species.

Introduction

The genus *Rothia* was proposed by Georg & Brown(1), with *Rothia dentocariosa* being used as the type species. Historically, this taxon was classified within the family *Actinomycetaceae* because of its morphological characteristics(2). However, after performing phylogenetic and other analyses Stackebrandt *et al.* (3) transferred it to the family *Micrococcaceae*. *Rothia dentocariosa* had long been known to display phenotypic heterogeneity(4, 5, 6), and the existence of a second *Rothia* genomovar was proposed by Kronvall *et al.*(7). However, *R. dentocariosa* was considered to be the only species of this genus until Collins *et al.* (8) validly described a novel species (*R. nasimurium*) and reclassified *Stomatococcus mucilaginosus* as *Rothia mucilaginosa*. Currently, the genus *Rothia* encompasses six species with validly published names: *R. aeria*, *R. amarae*, *R. dentocariosa*, *R. mucilaginosa*, *R. nasimurium*, and *R. terrae*. *Rothia* species are Gram-positive, and their cells display a coccoid, coccobacillary, or filamentous appearance. Of the six *Rothia* species, only *R. dentocariosa* and *R. mucilaginosa*,

which inhabit the oral cavity and pharynx, are found in humans(2, 8). *R. dentocariosa* and *R. mucilaginosa* have been identified as opportunistic pathogens and can cause septicemia, endocarditis, and other serious infections(9-14).

Although conventional biochemical assays are used to identify *Rothia* species, they are often imprecise due to the phenotypic variation displayed by these bacteria(4, 5, 6). Sequence analysis of several target genes is the most reliable method. However, this is expensive, laborious, and time-consuming. Thus, a simple and more reliable assay for identifying *Rothia* species is required.

The purpose of the present study was to design primers for identifying oral *Rothia* species using multiplex PCR.

Material and Methods

Bacterial strains and culture conditions

The following bacterial strains were used in this study: *R. dentocariosa* JCM 3067, *R. dentocariosa* NUM-Rd6020, *R. mucilaginosa* JCM 10910, *R. mucilaginosa* NUM-Rm6504, *R. aeria* JCM 11412, *R. terrae* JCM 15158, *R. nasimurium* JCM 10909, *R. amarae* JCM 11375, *Streptococcus oralis* ATCC 10557, *S. salivarius* JCM 5707, *S. anginosus* ATCC 11391, *S. mutans* NCTC 10449, *S. sobrinus* ATCC 33478, *Actinomyces*

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viscosus ATCC 19246, *A. naeslundii* ATCC 12104, *A. odontolyticus* NUM-Ao12, *Neisseria sicca* ATCC 29256, *Aggregatibacter actinomycetemcomitans* ATCC 33384, *Staphylococcus aureus* JCM 2874, and *S. epidermidis* ATCC 2414. The strains were maintained by cultivating them in brain heart infusion agar (BHI; Difco Laboratories, Detroit, Mich.) supplemented with 1% yeast extract (BHIY).

The bacteria were cultured in BHIY broth at 37°C for 24 h in aerobic conditions for members of the genus *Rothia* and in a 5% CO₂ atmosphere for other representative oral bacteria.

Design of species-specific primers for R. dentocariosa and R. mucilaginosa

The 16S rRNA sequences of *R. aeria* (accession no. AB071952), *R. amarae* (AY043359), *R. dentocariosa* (M59055), *R. mucilaginosa* (X87758), *R. nasimurium* (AJ131121), and *R. terrae* (DQ822568) were obtained from the DNA Data Bank of Japan (DDBJ; Mishima, Japan), and a multiple sequence alignment analysis was carried out with the CLUSTAL W program; i.e., the 16S rRNA sequences of the *Rothia* species were aligned and analyzed. The homology between the primers selected for *R. dentocariosa* and *R. mucilaginosa* and their respective 16S rRNA sequences was confirmed by a BLAST search.

Development of a multiplex PCR method using the designed primers

Bacterial cells were cultured in BHIY 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 10⁷ colony-forming units (CFU)/ml) in 1 ml of sterile distilled water. Finally, 3.6 µl of the suspension were used as a PCR template. The detection limit of the PCR was determined by serially diluting known numbers of bacterial cells in sterile distilled water and then subjecting each suspension to PCR. The multiplex PCR mixture contained 0.75 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 5 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 30 cycles consisting of 98 °C for 10 s, 66 °C for 15 s and 68 °C for 1 min. The 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

In the present study, four specific primers covering the upstream regions of the 16S rDNA sequences of oral *Rothia* species were designed (Fig. 1). The specific forward primers were designated as RDF for *R. dentocariosa* and as RMF for *R. mucilaginosa*, whereas the specific reverse primers were designated as RDR for *R. dentocariosa* and as RMR for *R. mucilaginosa*. The amplicon sizes of *R. dentocariosa* and *R. mucilaginosa* were 202 bp and 360 bp, respectively.

Multiplex PCR

1) Detection limit

Our multiplex PCR method for identifying oral *Rothia* species successfully amplified DNA fragments of the expected size for each species (Fig. 2). The detection limit was determined in the presence of titrated bacterial cells, and the sensitivity of the PCR assay was found to be between 5 × 10⁵ and 5 × 10⁶ CFU per ml for the *R. dentocariosa* specific primer set with strain JCM 3067 and between 5 × 10³ and 5 × 10⁴ CFU per ml for the *R. mucilaginosa* specific primer set with strain JCM 10910.

2) Assay of non-oral Rothia species

The two primer sets did not produce any amplicons from the non-oral *Rothia* species except *R. aeria* (Fig. 3).

3) Assay of representative oral bacteria

The *R. dentocariosa* and *R. mucilaginosa* strains both produced positive bands. As representative oral bacteria, some streptococci, *Actinomyces*, *Neisseria*, *Aggregatibacter*, and staphylococci were subjected to PCR using the designed primer sets. However, no amplicons were produced from any of the representative oral bacteria (Fig. 4).

Discussion

Members of the genus *Rothia* are non-sporulating, non-motile, facultative anaerobic gram-positive cocci that form irregularly arranged cell groups as well as tetrads, diploids, or short chains of cells. Their colonies display a smooth, convex, white, or transparent appearance, and each colony is

Fig. 1 Locations and sequences of species-specific primers for the 16S rDNA of oral *Rothia* species. The nucleotide sequence of each primer has been underlined.

Fig. 2 Sensitivity of the multiplex PCR assay for detecting *R. dentocariosa* and *R. mucilaginosa*. The primer mixture contained RDF, RDR, RMF, and RMR. Lanes 1-8, *R. mucilaginosa* JCM 10910; Lanes 9-16, *R. dentocariosa* JCM 3067. The following numbers of cells were added: 5×10^8 (lanes 1 and 9), 5×10^7 (lanes 2 and 10), 5×10^6 (lanes 3 and 11), 5×10^5 (lanes 4 and 12), 5×10^4 (lanes 5 and 13), 5×10^3 (lanes 6 and 14), 5×10^2 (lanes 7 and 15), 5×10 (lanes 8 and 16). M, molecular size marker (100-bp DNA ladder).

Fig. 3 Multiplex PCR assay for detecting oral *Rothia* species. The primer mixture contained RDF, RDR, RMF, and RMR. Lanes: 1, *R. dentocariosa* JCM 3067; 2, *R. mucilaginosa* JCM 10910; 3, *R. aeria* JCM 11412; 4, *R. terrae* JCM 15158; 5, *R. nasimurium* JCM 10909; 6, *R. amarae* JCM 11375; 7, clinical isolate *R. dentocariosa* NUM-Rd6018; 8, clinical isolate *R. dentocariosa* NUM-Rd6020; 9, clinical isolate *R. mucilaginosa* NUM-Rm6504; 10, clinical isolate *R. mucilaginosa* NUM-Rm6505. M, molecular size marker (100-bp DNA ladder).

surrounded by a diffuse halo. *R. dentocariosa* and *R. mucilaginosa* are part of the normal flora in the human oral cavity and pharynx(2,8). Although these species are considered to have low virulence, immunocompromised patients are often infected by these pathogens, leading to endocarditis, sepsis, peritonitis, meningitis, and infections associated with tissue and organ transplantation(9-16). The involvement of these organisms in oral infections has attracted little attention, and the prevalence of oral *Rothia* species in the oral cavity has not been fully clarified because of the difficulty of identifying them.

PCR is a molecular technique that allows species verification at the genetic level, and it possesses high sensitivity and bacterial species-specificity. Moreover, it can efficiently and accurately detect specific microorganisms in clinical samples, regardless of whether they are alive or dead. The 16S rDNA region of the bacterial genome provides an ideal target for species identification using PCR.

In this study, we designed species-specific primers for detecting oral *Rothia* species using a multiplex PCR method. These primers were able to distinguish each oral *Rothia*

Fig. 4 Specificity of the multiplex PCR assay for representative oral bacteria. The primer mixture contained RDF, RDR, RMF, and RMR. Lanes: 1, *R. dentocariosa* JCM 3067; 2, *R. mucilaginosa* JCM 10910; 3, *S. oralis* ATCC 10557; 4, *S. salivarius* JCM 5707; 5, *S. anginosus* ATCC 11391; 6, *S. mutans* NCTC 10449; 7, *S. sobrinus* ATCC 33478; 8, *A. viscosus* ATCC 19246; 9, *A. naeslundii* ATCC 12104; 10, *A. odontolyticus* NUM-Ao12; 11, *N. sicca* ATCC 29256; 12, *A. actinomycetemcomitans* ATCC 33384; 13, *S. aureus* JCM 2874; 14, *S. epidermidis* ATCC 14990. M, molecular size marker (100-bp DNA ladder).

species and did not display cross-reactivity with representative oral bacteria or other *Rothia* species except *R. aeria*. Moreover, our multiplex PCR method is easy, since the use of MightyAmp DNA Polymerase Ver.2 (Takara) means that DNA extraction can be avoided, and species identification using this method only takes about 2 hours. Thus, we hope that our method will allow the prevalence of oral *Rothia* species and their involvement in oral infections to be fully clarified in future studies.

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References

- George LK, Brown JM: *Rothia*, gen. nov., an aerobic genus of the family *Actinomycetaceae*. *Int J Syst Bacteriol*, 17: 79-88, 1967.
- Schaal KP: The genera *Actinomyces*, *Arrcanobacterium*, and *Rothia*. In *The Prokaryotes*, 2nd ed, eds. Balows A, Truper HG, Dworkin M, Harder W, Schleifer KH, 850-905, 1992. New York: Springer.
- Stachebrandt E, Rainey FA, Ward-Rainey NL: Proposal for a new hierarchic classification system, *Actinobacteria* classis nov. *Int J Syst Bacteriol*, 47: 479-491, 1997.
- Leshner RJ, Gerencser MA, Gerencser VF: Morphological, biochemical, and serological characterization of *Rothia dentocariosa*. *Int J Syst Bacteriol*, 24: 154-159, 1974.
- Schofield GM, Shaal KP: A numerical taxonomic study of members of the *Actinomycetaceae* and related taxa. *J Gen Microbiol*, 127: 237-259, 1981.
- Fotos PG, Gerencser MA, Yelton DB: Strain differentiation of *Rothia dentocariosa* and related isolates by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. *Int J Syst Bacteriol*, 34: 102-106, 1984.
- Kronvall G, Lannér-Sljöberg M, von Stedingk LV, Hanson HS, Petterson B, Falsen E: Whole cell protein and partial 16S rRNA gene sequence analysis suggest the existence of second *Rothia* species. *Clin Microbiol Infect*, 4: 255-263, 1998.
- Collins MD, Hutson RA, Båverud V, Falsen E: Characterization of a *Rothia*-like organism from a mouse: description of *Rothia nasimurium* sp. nov. and reclassification of *Stomatococcus mucilaginosus* as *Rothia mucilaginosa* comb. nov. *Int Syst Evol Microbiol*, 50: 1247-1251, 2000.
- Schafer FJ, Wing EJ, Norden CW: Infectious endocarditis caused by *Rothia dentocariosa*. *Ann Intern Med*, 91: 747-748, 1979.
- Pape J, Singer C, Krehn TE, Lee BJ, Armstrong D: Infective endocarditis caused by *R. dentocariosa*. *Ann Intern Med*, 91: 746-747, 1979.
- Pers C, Kristiansen JE, Joensson V, Hansen NE: *Rothia dentocariosa* septicemia in a patient with chronic lymphocytic leukaemia and toxic granulocytopenia. *Dan Med Bull*, 34: 322-323.
- Schiff MJ, Kaplan MH: *Rothia dentocariosa* pneumonia in an immunocompromised patient. *Lung*, 165: 279-282, 1987.
- Pinsky RL, Piscitelli V, Patterson JE: Endocarditis caused by relatively penicillin-resistant *Stomatococcus mucilaginosus*. *J Clin Microbiol*, 27: 215-216, 1989.
- Poirier LP, Gaudereau CL: *Stomatococcus mucilaginosus* catheter-associated infection with septicemia. *J Clin Microbiol*, 27: 1125-1126, 1989.
- Pinsky RL, Piscitelli V, Patterson JE: Endocarditis caused by related penicillin-resistant *stomatococcus mucilaginosus*. *J Clin Microbiol*, 27: 215-216, 1989.
- Plag J, Kjoller E, Espersen F: *Stomatococcus mucilaginosus* endocarditis. *Eur J Clin Microbiol*, 4: 422-424, 1985.