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Isolation and Identification of *Rothia nasimurium* from a Giant Panda (*Ailuropoda melanoleuca*)

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Abstract: A Gram-positive coccus isolated from a Giant Panda was identified by 16S ribosomal RNA (rRNA) gene sequencing analysis. Phylogenetic analysis showed that the organism was a member of the family Rothia. The *16S rRNA* gene sequence of the organism showed 100% similarity with *R. nasimurium* strain CCUG 35957. Animal experiments were conducted to study the pathogenicity of *R. nasimurium* SCGP. This is the first reported isolation of *R. nasimurium* from the upper respiratory tracta of Giant Panda.

Key words: *Ailuropoda melanoleuca*, Giant Panda, *Rothia nasimurium*, 16S rDNA, Gram-positive

INTRODUCTION

The genus *Rothia* which belongs to the phylum Actinobacteria and family Micrococcaceae, consists of aerobic, Gram-positive coccoid, coccobacillary or filamentous, non-acid-fast, non-spore-forming, non-hemolytic and non-motile bacteria (Michon *et al*., 2010; Georg and Brown, 1967) proposed the genus *Rothia* and characterized the species *Rothia dentocariosa*.

The genus *Rothia* encompasses six species: *R. aeria*, *R. amarae*, *R. dentocariosa*, *R. mucilaginosa* (formerly *Stomatococcus mucilaginosus*), *R. nasimurium* and *R. terrae*.

The species *R. aeria* was isolated from an air sample at the Russian space station Mir (Li *et al*., 2004) and was previously identified as an opportunistic pathogen (Michon *et al*., 2010; Monju *et al*., 2009). *Rothia amarae* was isolated from a sludge sample obtained from a foul water sewer in Shanghai Jiao Tong University, China. This organism is capable of deodorizing dung and sewage (Fan *et al*., 2002). *Rothia dentocariosa* was isolated from the human oral cavity and pharynx of a man where it was part of the normal microflora (Collins *et al*., 2000; Schaal, 1992). *Rothia dentocariosa* which is frequently isolated from patients with bacteremia, endocarditis and other serious infections is an opportunistic pathogen (Boudewijns *et al*., 2003; Minato and Abiko, 1984; Pape *et al*., 1979; Pers *et al*., 1987; Schafer *et al*., 1979; Schiff and Kaplan, 1987). *Rothia mucilaginosa*, previously known as *Stomatococcus mucilaginosus* is part of the normal flora of the human oral cavity and upper respiratory tract but has been isolated from other human body sites including the duodenum (Collins *et al*., 2000; Kazor *et al*., 2003; Ou *et al*., 2009; Zamakhchari *et al*., 2011; Zaura *et al*., 2009). *Rothia nasimurium* was first isolated from the nose of a healthy mouse (Collins *et al*., 2000) and is part of the normal flora found in the upper respiratory tract of pigs (Baele *et al*., 2001). *Rothia terrae* was isolated from a soil sample collected in Tainan County, Taiwan (Chou *et al*., 2008). Researchers recently carried out a study to characterize the normal flora of the upper respiratory tract in giant pandas to help clarify the pathogenesis of upper respiratory tract infections in this animal. In this study, a Gram-positive coccus was isolated and identified at the species level using 16S ribosomal RNA (*rRNA*) gene sequencing analysis.

MATERIALS AND METHODS

Bacterial isolation: Nasal swabs of apparently healthy giant pandas were collected from Bifeng Xia Giant Panda protection base in Ya'an, Sichuan Province, Southwest China. The samples were cultured aerobically and anaerobically on tryptic soy agar supplemented with 5% fetal calf serum (Gibco) at 37°C. Bacteria were isolated and distinguished by colony morphology and microscopic appearance. A Gram-positive, ovoid coccus (named strain SCGP) was picked and subcultured to obtain a pure culture on Luria-Bertani plates supplemented with 5% fetal calf serum.

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Preparation of bacterial DNA for PCR: Genomic DNA was prepared from a pure culture of strain SCGP. A single colony was suspended in 200 μL sterilize double-distilled water. The supension was boiled for 10 min, centrifuged at 12000 rpm for 5 min and used as a template for PCR.

PCR amplification of the *16S rDNA* gene: To identify the isolate, the 16S *rDNA* gene was amplified by PCR using the following universal primers (Tian *et al*., 2010): F: 5'-AGAGTTTGATCCTGGCTCAG-3; R: 5'-AAGGAGGTGATCCAACCGCA-3'. *Escherichia coli* DNA was used as the positive control and distilled water as the negative control. Touchdown PCR was performed in a total volume of 20 μL containing 1 μL template DNA, 0.5 μL each primer (20 pmol μL^{-1}), 10 μL 2×Taq PCR Master Mix (TakaRa) and nuclease-free PCR-grade water. The cycling conditions consisted of initial denaturation at 95°C for 6 min 12 cycles of denaturation at 95°C for 40 sec, annealing for 40 sec (starting at 62°C and decreasing 0.5°C every cycle) and extension at 72°C for 90 sec 23 cycles of denaturation at 95°C for 40 sec, annealing at 55°C for 40 sec and extension at 72°C for 90 sec and a final extension at 72°C for 7 min.

Sequence analysis of the 16S rDNA fragment: The PCR product was separated by agarose gel electrophoresis (10 g L^{-1} agarose in Tris-borate-EDTA buffer gel containing 0.5 mg mL^{-1} ethidium bromide). A band of the expected size (approximately 1500 bp) was purified using a Universal DNA purification kit (TIANGEN Biotech) and the recovered DNA was ligated into a pMD®18-T vector (TakaRa). The ligation mixture was then transformed into *E. coli* DH5a competent cells. The recombinant plasmid DNA was sequenced by Takara. Researchers used BLAST to compare the *16S rRNA* gene of SCGP1 with nucleotide sequences in the GenBank database. A phylogenetic tree was constructed by using the Neighbor-Joining Method (MEGA 5.0 Software).

Animal experiments: To determine whether strain SCGP is an opportunistic pathogen, 12 healthy mice (mean weight, approximately 20 g) were selected and divided into four groups (each group, n = 3). Group A was challenged with 100 μL (10^8 colony-forming units/mL) strain SCGP by intranasal instillation; group B (negative control) was challenged with 100 μL phosphate buffered saline (pH 7.4) by intranasal instillation; group C was challenged with 200 μL strain SCGP by intraperitoneal injection and group D (negative control) was challenged with 200 μL phosphate buffered saline by intraperitoneal injection. The groups were housed separately and observed carefully for a week. Infection or death after the challenge was taken as evidence that the isolate may be pathogenic.

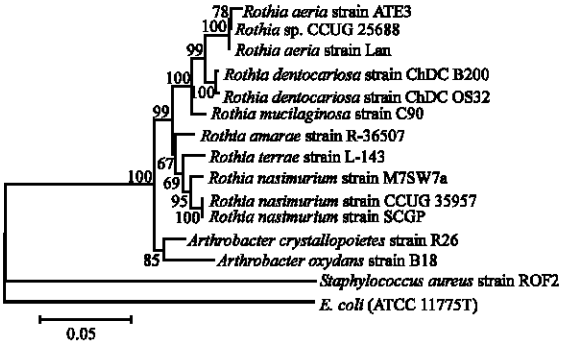

Fig. 1: Phylogenetic neighbor-joining tree based on nucleotide sequences of the partial *16S rRNA* gene. *E. coli* was used as an outgroup. Gene sequences were aligned using the ClustalW Method and the phylogenetic tree was performed by the Neighbour Joining Method using 1000 bootstrap

RESULTS AND DISCUSSION

After growth on Luria-Bertani plates supplemented with 5% fetal calf serum at 37°C for 24 h under aerobic conditions, the isolated bacterial strain SCGP was observed with the naked eye and examined microscopically. Microscopic analysis showed that cells were Gram-positive, ovoid, non-motile, non-spore forming cocci, occurring singly or in pairs, tetrads or small clusters. The strain formed cream-white colonies (1-2 mm diameter) that were dry with scalloped edges. The colony surface was cerebriform or convoluted. The isolate showed 100% similarity with *R. nasimurium* strain CCUG 35957 and 98.8% identity with *Rothia nasimurium* strain M7SW7a but lower similarity with other Rothia species. Phylogenetic analysis of the *16S rRNA* gene revealed that the isolate SCGP represented a species in the genus *Rothia* that belonged on the same branch as *R. nasimurium* (Fig. 1).

CONCLUSION

Results of the animal experiments revealed that one mouse died 24 h after intraperitoneal injection of SCGP. Bacteria recovered from the liver of the dead mouse showed a pure heavy growth on Luria-Bertani plates supplemented with 5% fetal calf serum and was identified as *R. nasimurium*. Other mice appeared normal and nothing unusual was observed during the study period. Like *Actinomyces* species, species belonging to the *Rothia* genus are considered to be part of the resident flora of the oral cavity and pharynx in humans. Although, these species rarely cause severe infections, some of them

may be opportunistic pathogens in immunocompromised patients (Michon *et al*., 2010). To date, no studies have reported *R. nasimurium* as an opportunistic pathogen, suggesting that the mouse may have died because of stress caused by the experimental procedures. Therefore, the ability to culture the isolate SCGP from the mouse liver may be evidence of colonization rather than infection. Additional studies are needed to clarify the pathogenicity of *R. nasimurium*.

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