Case Report

'*Neisseria skkuensis*' sp. nov., isolated from the blood of a diabetic patient with a foot ulcer

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A Gram-negative bacterium was isolated from the blood of a patient with diabetes mellitus. However, it could not be identified by conventional microbiological methods, and so was characterized by phenotypic and genotypic analyses. 16S rRNA gene sequence analysis revealed that the strain belonged to the genus *Neisseria*. Based on the phenotypic and genotypic characteristics, we propose that strain SMC-A9199^T (=KCTC 22696^T=JCM 16127^T) should be classified as a novel species, '*Neisseria skkuensis*' sp. nov. The patient was further treated with amoxicillin–clavulanate and ciprofloxacin for 3 weeks.

Introduction

The genus *Neisseria* comprises coccid or rod-shaped Gram-negative bacteria arranged in pairs or short chains (Janda & Gaydos, 2007). It includes a group of closely related Gram-negative bacteria that are primarily commensal inhabitants of the mucus membrane of mammals. At the time our work was undertaken, the genus consisted of 19 species (<http://www.bacterio.cict.fr/>), which are commensal inhabitants of the mucous membrane surfaces of warm-blooded hosts (Janda & Gaydos, 2007). Of these, 15 species are of human origin, with *Neisseria meningitidis* and *Neisseria gonorrhoeae* being important pathogens, and the others being opportunistic pathogens (Han *et al.*, 2006; Vandamme *et al.*, 2006). In this study, we report a novel *Neisseria* species isolated from the blood and wound pus of a diabetic patient with a foot ulcer. The bacteria could be identified as *Neisseria* spp. by conventional methods, such as VITEK (bioMérieux) and MicroScan (Dade-Microscan), in a clinical microbiology lab, but correct identification at the species level could not be obtained. Comparative 16S rRNA gene sequence analysis along with phenotypic analysis showed that the isolate is a novel species of *Neisseria*.

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Abbreviations: CFA, cellular fatty acid; DM, diabetes mellitus.

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA, *argF* and *recA* gene sequences of '*N. skkuensis*' strain SMC-A9199^T are FJ763637, FJ787491 and FJ787492, respectively.

Case report

A 50-year-old man with diabetes mellitus (DM) visited the Emergency Department at Samsung Medical Center, Seoul, Republic of Korea, because of fever and chills. The patient had been suffering from complications of DM, such as right DM foot and chronic renal failure. At the Emergency Department, the patient was treated with amoxicillin–clavulanate. For further management, the patient was admitted to a tertiary-care hospital (Samsung Medical Center, Seoul). On the second hospital day, right foot magnetic resonance imaging was carried out and was found not to be within normal limits. An isolate was identified in blood culture as *Neisseria* and was designated SMC-A9199^T; identification at the species level could not be done. *Neisseria* was isolated from pus culture of the right fifth metatarsophalangeal joint, and designated SMC-G2063, along with *Enterobacter cloacae*. Later, SMC-G2063 was identified as the same strain as SMC-9199^T. On the ninth day of hospitalization, the amoxicillin–clavulanate was replaced by tazobactam–piperacillin. Subsequently, the patient was further treated with amoxicillin–clavulanate and ciprofloxacin for 3 weeks. Ultimately, he was well enough to leave the hospital.

Bacterial isolate

Strain SMC-A9199^T was Gram-negative, when stained at room temperature. It grew well aerobically on sheep blood agar and chocolate agar at 35 °C in an atmosphere of 5 % CO₂ after 24 h incubation. Colonies were round and light grey, and

measured 0.5–1 mm in diameter when incubated for 24 h. For phenotypic characterization, the API 20NE and API 50CHB systems (bioMérieux) were used according to the recommendations of the manufacturer. Routine control organisms (*Pseudomonas aeruginosa* and *Escherichia coli*) were included in the tests. The biochemical reaction results are shown in Table 1 with those of other *Neisseria* species. Strain SMC-A9199^T was oxidase and catalase positive, consistent with most *Neisseria* species (Lawson *et al.*, 2005). API20NE results were positive only for assimilation of glucose. It was negative for the reduction of nitrates to nitrites and nitrites to nitrogen. When tested with API 50CHB, strain SMC-A9199^T could utilize ribose, glucose, fructose, mannitol, sucrose and gluconate, but not the remaining carbohydrates.

For molecular identification, the 16S rRNA gene was amplified using primer sets 16S-F3 (5'-CAG GCC TAA CAC ATG CAA-3') and 16S-R3 (5'-GGG CGG WGT GTA CAA GGC-3') (Shin *et al.*, 2008). A 1415 bp sequence of the 16S rRNA gene (positions 57–1469 in *Escherichia coli*) (GenBank accession no. FJ763637) was obtained from strain SMC-A9199^T. The 16S rRNA gene sequence was compared with those in the EzTaxon public database (<http://www.eztaxon.org/>) (Chun *et al.*, 2007) and the GenBank database (<http://www.ncbi.nlm.nih.gov/blast>) using BLAST searches. The 16S rRNA gene sequence of SMC-A9199^T and those of the type strains of other *Neisseria* species retrieved from the EzTaxon database were aligned using the CLUSTAL_X program. Phylogenetic relationships among them were determined by the neighbour-joining method of the CLUSTAL_X program. The robustness of the branch was assessed by bootstrap analysis from 1000 replications. In addition to the 16S rRNA gene, fragments of *argF* and *recA* of strain SMC-A9199^T were also amplified and sequenced as described by Smith *et al.* (1999). Analysis of the cellular fatty acid (CFA) composition was accomplished using a Hewlett Packard 6890A gas chromatograph

and the MIDI aerobe method (Chem Station ver. 4.02) at MicroID (Daejeon, Republic of Korea). The mol% G+C content was determined via thermal denaturation. *In vitro* antimicrobial susceptibility testing was performed by estimating the MICs to penicillin, cefotaxime, cefoxitin, ceftazidime, tetracycline, ciprofloxacin, erythromycin, clindamycin and trimethoprim–sulfamethoxazole using the broth microdilution method in accordance with the guidelines established by the Clinical and Laboratory Standards Institute (CLSI, 2008). Interpretive criteria for susceptibility were those for *N. gonorrhoeae* (CLSI, 2008).

In phylogenetic analysis, the isolate clustered with *Neisseria animalis* NCTC 10212^T, *Neisseria elongata* ATCC 25295^T and *Neisseria lactamica* NCTC 10617^T, and its grouping was supported by bootstrap analysis (bootstrap value 62 %) (Fig. 1). The 16S rRNA gene sequences of strain SMC-A9199^T showed the highest pairwise similarity (97.5 %) to that of the type strain of *N. animalis* NCTC 10212^T. It exhibited pairwise similarities <97.0 % with other species. Recently, Stackebrandt & Ebers (2006) suggested the 98.7 % guideline, indicating that a strain with a 16S rRNA gene sequence similarity <98.7 % with known species may be placed as a novel species instead of estimating DNA relatedness. Within the genus *Neisseria*, several species present with >97 % 16S rRNA gene sequence similarities. For example, *Neisseria polysaccharea* showed 98.0 and 98.6 % similarities with *Neisseria flavescens* and *N. gonorrhoeae*, respectively. A pairwise similarity of 98.0 % was evident for *N. flavescens* and *Neisseria weaveri*. Accordingly, 97.5 % pairwise similarity between strain SMC-A9199^T and *N. animalis* might be enough to consider SMC-A9199^T as a novel *Neisseria* species. SMC-G2063, an isolate from pus, showed the same 16S rRNA gene sequences with SMC-A9199^T.

The *argF* and *recA* sequences determined for SMC-9199^T (GenBank accession nos FJ787491 and FJ787492, respect-

Table 1. Comparison of biochemical reactions of strain SMC-A9199^T and other *Neisseria* species

The data for other *Neisseria* species were taken from the literature (Han *et al.*, 2006; Janda & Gaydos, 2007; Lawson *et al.*, 2005). *N.*, *Neisseria*.

Species	Acid production from:					Reduction of nitrate	Oxidase	Catalase
	Glucose	Maltose	Lactose	Sucrose	Fructose			
SMC-A9199 ^T	+	–	–	+	+	–	+	+
<i>N. gonorrhoeae</i>	+	–	–	–	–	–	+	+
<i>N. meningitidis</i>	+	+	–	–	–	–	+	+
<i>N. lactamica</i>	+	+	+	–	–	–	+	+
<i>N. cinerea</i>	–	–	–	–	–	–	+	+
<i>N. polysaccharea</i>	+	+	–	±	–	–	+	+
<i>N. subflava</i>	+	+	–	±	±	–	+	+
<i>N. sicca</i>	+	+	–	+	+	–	+	+
<i>N. mucosa</i>	+	+	–	+	+	+	+	+
<i>N. flavescens</i>	–	–	–	–	–	–	+	+
<i>N. elongata</i>	–	–	–	–	–	–	±	±
<i>N. bacilliformis</i>	–	–	–	–	–	±	+	–

Fig. 1. Phylogenetic tree of *'N. skkuensis'* SMC-A9199^T and 18 other *Neisseria* species based on 16S rRNA gene sequences (*Neisseria iguanae* was not included because of the availability of only a small portion of sequence). This tree was constructed by the neighbour-joining method and *Moraxella catarrhalis* ATCC 25238^T (GenBank accession no. U10876) was used as an outgroup. Numbers at branching nodes are percentages of 1000 bootstrap replications. Only values >50 % are shown. Bar, 1 substitution per 100 nucleotides.

ively) were also compared with those in GenBank using BLAST searches. The *argF* sequence of SMC-A9199^T showed the highest similarity with that of *'Neisseria pharynges* var. *flava*' NCTC 4590 (84.4 %), followed by *Neisseria sicca* NRL 30016 (83.5 %). The *recA* sequence of SMC-A9199^T was the most similar to that of *N. mucosa* (86.1 %), followed by *'N. pharynges* var. *flava*' NCTC 4590 (85.9 %) and *N. flavescens* LNP 444 (84.8 %). Of these, *'N. pharynges'* is not a valid named species, although the National Collection of Type Cultures cites this as such. Although there are no guidelines for defining new species for *argF* and *recA* gene similarities, dissimilarities may suggest that SMC-A9199^T is a different species from other *Neisseria* species.

Like other *Neisseria* species, the major CFA components of SMC-A9199^T were C_{16:1}ω7c (35.3 %), C_{16:0} (18.2 %) and C_{18:1}ω7c (10.9 %). The DNA G + C content of strain SMC-A9199^T was 51.2 mol%. Comparatively, strain SMC-A9199^T displayed intermediate resistance against only tetracycline (MIC 0.5 mg l⁻¹), but was susceptible to the other antimicrobial agents such as penicillin, cefotaxime, cefoxitin, ceftazidime, tetracycline and ciprofloxacin. The strain showed relatively high erythromycin and clindamycin MIC values (both 4 mg l⁻¹) and a low trimethoprim-sulfamethoxazole MIC (0.03 mg l⁻¹), but no breakpoints

were provided by the Clinical and Laboratory Standards Institute.

Discussion

Among 19 validated species of the genus *Neisseria*, 15 have been reported as being of human origin. Although *N. meningitidis* and *N. gonorrhoeae* are important pathogens, most human *Neisseria* species are regarded as commensal inhabitants not pathogens (Janda & Gaydos, 2007). However, several *Neisseria* species other than *N. meningitidis* and *N. gonorrhoeae* cause human infections. For example, *N. flavescens* has been linked to an outbreak of meningitis, and *Neisseria cinerea* causes conjunctivitis and has been isolated from patients with endocervical and rectal infections, and with lymphadenitis (Janda & Gaydos, 2007). In addition, *N. lactamica* has been isolated from the urogenital tract, and three subspecies of *N. elongata* cause human diseases such as septicaemia (Hombrouck-Alet *et al.*, 2003). It has been reported that the recently identified species, *Neisseria bacilliformis*, also causes respiratory infections and endocarditis (Han *et al.*, 2006). Human infections by *N. weaveri*, *Neisseria animaloris* and *Neisseria zoodegmatidis* have been reported repeatedly (Capitini *et al.*, 2002; Vandamme *et al.*, 2006). In this study, we report a

novel *Neisseria* species, '*Neisseria skkuensis*', which is associated with human infection (a foot ulcer in a diabetic patient), mainly based on housekeeping gene sequences. This finding may indicate that more *Neisseria* species cause human diseases, although many may be opportunistic pathogens. Like other commensal *Neisseria* species, this organism also shows susceptibility to most antimicrobial agents tested.

Description of '*Neisseria skkuensis*' sp. nov.

'*Neisseria skkuensis*' (skku.en'sis. N.L. fem. adj. *skkuensis* of or belonging to SKKU, an acronym of Sungkyunkwan University, where this study was performed).

Cells are aerobic, Gram-negative, grow well on sheep blood agar and chocolate agar plates at 35 °C with 5% CO₂, and present round and light grey colonies 0.5–1 mm in diameter. Acid is produced from ribose, glucose, fructose, mannitol, sucrose and gluconate, but not from glycerol, erythritol, D-arabinose, L-arabinose, D-xylose, L-xylose, adonitol, β-methyl-D-xyloside, galactose, mannose, sorbose, rhamnose, dulcitol, inositol, sorbitol, α-methyl-D-mannoside, α-methyl-D-glucoside, N-acetylglucosamine, amygdalin, arbutin, aesculin, salicin, cellobiose, maltose, lactose, melibiose, trehalose, inulin, melezitose, raffinose, starch, glycogen, xylitol, gentiobiose, turanose, D-lyxose, D-tagatose, D-fucose, D-arabitol, L-arabitol, 2-keto-gluconate and 5-keto-gluconate. It is negative for the reduction of nitrates to nitrites and nitrites to nitrogen. It does not produce indole, arginine dihydrolase, β-galactosidase, and does not hydrolyse β-glucosidase and protease. Major CFAs are C_{16:1ω7c} (35.3%), C_{16:0} (18.2%) and C_{18:1ω7c} (10.9%). It is susceptible to penicillin, cefotaxime, cefoxitin, ceftazidime, tetracycline and ciprofloxacin, but has intermediate resistance to tetracycline. The original isolate was obtained from a blood and pus sample of a patient with DM. Based on the 16S rRNA gene sequence, it is most closely related with *N. animalis*, but is distinct from other described *Neisseria* species. The DNA G+C content of the type strain is 51.2 mol%. The type strain is strain SMC-A9199^T (=KCTC 22696^T=JCM 16127^T).

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