Gemella parahaemolysans sp. nov. and Gemella taiwanensis sp. nov., isolated from human clinical specimens

Wei-Chun Hung,1 Hsiao-Jan Chen,1 Jui-Chang Tsai,2,3 Sung-Pin Tseng,4 Tai-Fen Lee,5 Po-Ren Hsueh,5 Wung Yang Shieh6 and Lee-Jene Teng1,5

1Department of Clinical Laboratory Sciences and Medical Biotechnology, National Taiwan University College of Medicine, Taipei, Taiwan, ROC
2Center for Optoelectronic Medicine, National Taiwan University College of Medicine, Taipei, Taiwan, ROC
3Division of Neurosurgery, Department of Surgery, National Taiwan University Hospital, Taipei, Taiwan, ROC
4Department of Medical Laboratory Science and Biotechnology, Kaohsiung Medical University, Kaohsiung, Taiwan, ROC
5Department of Laboratory Medicine, National Taiwan University Hospital, Taipei, Taiwan, ROC
6Institute of Oceanography, National Taiwan University, PO Box 23-13, Taipei, Taiwan, ROC

Four Gram-staining-positive, catalase-negative, coccoid isolates, designated NTUH_1465T, NTUH_2196, NTUH_4957 and NTUH_5572T, were isolated from human specimens. The four isolates displayed more than 99.6 % 16S rRNA gene sequence similarity with Gemella haemolysans ATCC 10379T, and 96.7 to 98.6 % similarity with Gemella sanguinis ATCC 700632T, Gemella morbillorum ATCC 27824T or Gemella cuniculi CCUG 42726T. However, phylogenetic analysis of concatenated sequences of three housekeeping genes, groEL, rpoB and recA, suggested that the four isolates were distinct from G. haemolysans ATCC 10379T and other species. Isolates NTUH_2196, NTUH_4957 and NTUH_5572T clustered together and formed a stable monophyletic clade. DNA–DNA hybridization values among strains NTUH_1465T and NTUH_5572T and their phylogenetically related neighbours were all lower than 49 %. The four isolates could be distinguished from G. haemolysans and other species by phenotypic characteristics. Based on the phylogenetic and phenotypic results, two novel species Gemella parahaemolysans sp. nov. (type strain NTUH_1465T = BCRC 80365T = JCM 18067T) and Gemella taiwanensis sp. nov. (type strain NTUH_5572T = BCRC 80366T = JCM 18066T) are proposed.

Members of the genus Gemella comprise facultatively anaerobic, catalase-negative cocci arranged in pairs, tetrads, short chains or small, irregular clusters. Cells are usually Gram-staining-positive but easily decolorized (Collins, 2006). Thus, Gemella haemolysans, the first described species of the genus Gemella, was originally classified as a species of the Gram-staining-negative genus Neisseria (Thjotta & Boe, 1938). Species of the genus Gemella grow slowly and can be cultured aerobically, except for some strains that require strictly anaerobic conditions for primary isolation (Kilpper-Bälz & Schleifer, 1988; Ulger-Toprak et al., 2010). Seven species of the genus Gemella with validly published names have been described at the time of writing, including Gemella asaccharolytica (Ulger-Toprak et al., 2010), G.bergeri (Collins et al., 1998b), G. cuniculi (Hoyles et al., 2000), G. haemolysans (Berger, 1961; type species of the genus), G. morbillorum (Kilpper-Bälz & Schleifer, 1988), G. palatianus (Collins et al., 1999) and G. sanguinis (Collins et al., 1998a). Among these species, G. haemolysans and G.
Two novel species of the genus Gemella

morbillorum are commensals of the mucous membranes in humans and could lead to opportunistic infections, such as bacteraemia (Malik et al., 2010; Vossen et al., 2012), meningitis (Anil et al., 2007) and endocarditis (Brouqui & Raoult, 2001). G. saccharolytica, G. bergeri and G. sanguinis were isolated from human clinical specimens and are most likely components of human normal microbiota (Collins et al., 1998a, b; Ulger-Toprak et al., 2010). G. palatinus was isolated from the oral cavity of a dog, and G. cuniculi was isolated from a submandibular abscess of a rabbit (Collins et al., 1999; Hoyles et al., 2000). The DNA G+C content of species of the genus Gemella is between 30 and 33.5 mol% (Collins, 2006).

Identification of species of the genus Gemella in clinical laboratories is usually difficult and problematic. Commercial systems, like API rapid ID 32 Strep system, are usually used for identification of these bacteria (Collins, 2006). However, some studies displayed poor identification of species of the genus Gemella in 6 or 13.3% of the endocarditis cases caused by viridians streptococci (Durack et al., 1983; La Scola & Raoult, 1998).

Information about the chemotaxonomic characters in species of the genus Gemella is very limited. For example, cell-wall murein was only determined in G. morbillorum and was of the Lys-Ala1-3 type (Kilpper-Bälz & Schleifer, 1988). Fatty acid composition was only reported in G. haemolysans in early days when it was classified as a species of the genus Neisseria (Brooks et al., 1971; Yamakawa & Ueta, 1964).

In our previous study, we found high heterogeneity of the groES, groEL and rpoB sequences in four G. haemolysans-like strains (Hung et al., 2010). These G. haemolysans-like strains were further characterized in the present study by multilocus sequence analysis (MLSA), phenotypic tests, fatty acid analysis and DNA–DNA hybridization. Data from the polyphasic characterization indicated that these strains could be classified as two novel species of the genus Gemella.

The four G. haemolysans-like strains, designated NTUH_1465T, NTUH_2196, NTUH_4957 and NTUH_5572T, as well as one G. haemolysans isolate, designated NTUH_6138, were isolated from blood cultures of five different patients in the Bacteriology Laboratory of the National Taiwan University Hospital between 1997 and 2008. NTUH_1465T was obtained from an 80-year-old female with hepatocellular carcinoma and liver cirrhosis related to chronic hepatitis C. NTUH_2196 was obtained from a 65-year-old male with diabetes mellitus (DM). NTUH_4957 was obtained from a 69-year-old female with nasopharyngeal carcinoma and DM who also had end-stage renal disease and had received a renal transplantation. NTUH_5572T was obtained from a 56-year-old male with DM and hepatocellular carcinoma. All these isolates produced pinpoint colonies on tryptase soy agar supplemented with 5% sheep blood (BD BBL Stacker Plate; Becton Dickinson) after incubation for 24 h at 37 °C under aerobic conditions, and were preliminarily identified by commercial identification systems, BD Phoenix Automatic Microbiology System (BD Diagnostic systems), API 20 Strep system or rapid ID 32 Strep system (bioMérieux), G. haemolysans ATCC 10379T, G. sanguinis ATCC 700632T, G. morbillorum ATCC 27824T and G. cuniculi ATCC BAA-287 (=CCUG 44726) were obtained from the American Type Culture Collection (ATCC) and used as reference strains. Two strains of G. morbillorum and four strains of G. sanguinis isolated at the National Taiwan University Hospital were also included for 16S rRNA gene sequence analysis and MLSA.

Genomic DNA was isolated and purified with a DNA isolation kit (Puregene; Gentra Systems) according to the manufacturer’s instructions. Nucleotide sequences of 16S rRNA, groEL and rpoB genes were determined by the method described previously (Hung et al., 2010). The recA and gyrB genes were amplified and sequenced using primer pairs 5′-GGWCCWGRWSHTCWGGTAA-3′ and 5′-GARATDCCCTSWCCRWACAT-3′ (recA-F and recA-R) and 5′-GHTYTWAGGGWYTVGAACG-3′ and 5′-AABCNCN-NYTTCACTGVTWC-3′ (gyrB-F and gyrB-R), respectively. The DNA sequences were aligned using the Gene-Works software (IntelliGenetics). Distance matrices for the aligned sequences were calculated using Kimura’s two-parameter model. Phylogenetic relationships among bacterial strains were analysed by the neighbour-joining method embedded in the MEGA4 analytical software package (Tamura et al., 2007). Bootstrap values were obtained for 1000 randomly generated trees. The topology of neighbour-joining tree was evaluated and confirmed by maximum-parsimony analysis.

On the basis of 16S rRNA gene sequence analysis, NTUH_1465T, NTUH_2196, NTUH_4957 and NTUH_5572T displayed more than 99.6% similarity to G. haemolysans ATCC 10379T, and were closely related to G. sanguinis ATCC 700632T (98.4 to 98.6%), G. morbillorum ATCC 27824T (98.2 to 98.5%) and G. cuniculi CCUG 44726 (96.7 to 96.9%). These isolates, together with a clinical isolate of G. haemolysans, NTUH_6138, formed a cluster with 78% bootstrap values in the 16S rRNA gene-based neighbour-joining tree (Fig. 1). This cluster further clustered with strains of G. morbillorum and G. sanguinis in the phylogenetic tree with 99% bootstrap support.

MLSA results revealed that the four G. haemolysans-like isolates shared variable sequence similarity in the housekeeping genes groEL (85.7–92.1%), gyrB (90.0–97.7%), recA (85.6–92.0%) and rpoB (95.2–97.0%) with G. haemolysans strains ATCC 10379T and NTUH_6138. Among the above genes, recA has been considered an alternative phylogenetic marker for the family Vibrionaceae, since members of the same species in this family displayed more than 94% recA sequence similarity (Thompson et al., 2004, 2005). In the present study, NTUH_2196, NTUH_4957 and NTUH_5572T, shared 95.0–100% recA sequence similarity to each other, whereas they shared only 85.1–85.2% recA sequence similarity with NTUH_1465T. MLSA has been considered a promising approach for species delineation (Gevers et al., 2005; Hanage et al., 2006; Stackebrandt et al., 2002). The housekeeping genes
used for this purpose should be widely distributed, found in a single copy and present at diverse chromosomal loci. In addition, examination of at least three housekeeping genes is suggested to avoid a misleading phylogenetic signal and get much more accurate reconstruction (Konstantinidis et al., 2006). gyrB was not included in the MLSA since this gene was found closely located to rpoB according to whole-genome shotgun sequences of G. haemolysans ATCC 10379T (GenBank accession number NZ_ACDZ02000000). The phylogenetic tree based on concatenated sequences of groEL, recA and rpoB indicated that the four G. haemolysans-like isolates were phylogenetically separated from G. haemolysans and other species (Fig. 2). Isolates NTUH_2196, NTUH_4597, and NTUH_5572T clustered together and formed a stable monoclonal clade (bootstrap value 100 %) located next to a sister clade (bootstrap value 100 %) in which NTUH_1465T was an outgroup of the subclade (bootstrap value 100 %) containing G. haemolysans strains ATCC 10379T and NTUH_6138 (bootstrap value 100 %) (Fig. 2).

Physiological characterization of the strains was carried out in duplicate by commercially available biochemical kits, API rapid ID 32 Strep and API ZYM systems according to the manufacturer’s instructions, and some of the results were confirmed by conventional tests. Acid production from D-glucose, maltose, D-mannitol, raffinose, D-sorbitol and trehalose was tested in phenol red broth (Difco) supplemented with 1 % yeast extract, together with casein (4 g l⁻¹), DNA (2 g l⁻¹), gelatin (5 g l⁻¹) or starch (5 g l⁻¹), were used to test the hydrolysis of these substrates. Hydrolysis of Tween was tested using the yeast extract-supplemented BHI containing Tween 80 (1 %, v/v) and CaCl₂ (0.1 g l⁻¹). All test cultures were incubated aerobically at 37 °C in the dark for 7 days.

Cells grow on trypticase soy agar (Difco) at 37 °C for 2 days were used for analysis of DNA G+C content and cellular fatty acid composition, which were conducted at the Biore source Collection and Research Center (BCRC), Food Industry Research and Development Institute, Taiwan, by using the methods described by Jean et al. (2009).

The four G. haemolysans-like isolates shared many physiological characteristics with G. haemolysans. However, NTUH_1465T was differentiated from G. haemolysans ATCC 10379T by a positive reaction for the Voges–Proskauer test and a negative reaction for hydrolysis of Tween 80, while NTUH_2196, NTUH_4597 and NTUH_5572T were distinguished from NTUH_1465T and G. haemolysans ATCC 10379T by production of leucine acrylamidase and acid from d-mannitol and d-sorbitol. The four G. haemolysans-like strains were also distinguished from other species of the genus Gemella as listed in Table 1.

The four G. haemolysans-like strains exhibited rather similar fatty acid composition. These strains and G. haemolysans strains ATCC 10379T and NTUH_6138 all contained C_{16:0}
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as the most abundant fatty acid (53.0–67.3 %). However, the fatty acids of the G. haemolysans-like strains were quantitatively distinguishable from those of G. haemolysans strains ATCC 10379T and NTUH_6138 (Table S1 available in the online Supplementary Material). For example, the G. haemolysans-like strains contained higher levels of C16:0 (64.2–67.3 % vs 53.0–54.1 %) and C18:1ω9c (6.9–8.2 % vs 3.0–3.3 %) and lower levels of C10:0 (1.1–2.1 % vs 7.7–8.0 %) and C12:0 (1.1–1.4 % vs 4.6–6.2 %). Moreover, the G. haemolysans-like strains contained C18:0 (1.4–2.0 %) and summed feature 5 (1.7–2.0 %), which were not detected in G. haemolysans strains ATCC 10379T and NTUH_6138. The DNA G+C contents of NTUH_1465T and NTUH_5572T were 30.9 and 30.2 mol%, respectively, which were similar to those determined in G. haemolysans ATCC 10379T and other species of the genus Gemella (Table 1).

DNA–DNA hybridization among NTUH_1465T, NTUH_5572T and their phylogenetically related neighbours G. haemolysans ATCC 10379T, G. sanguinis ATCC 700632T, G. morbillorum ATCC 27824T and G. cuniculi CCUG 42726T was performed as described by Jean et al. (2009). Reciprocal reactions (i.e. A × B and B × A) among NTUH_1465T, NTUH_5572T and G. haemolysans ATCC 10379T were carried out. The results revealed low DNA–DNA relatedness values ranging from 23 to 49 % among the indicated strains (Table S2). These data indicated that NTUH_5572T and NTUH_1465T could be classified as representing two novel species of the genus Gemella.

Antimicrobial susceptibility tests were performed by the standard agar dilution method according to the CLSI guidelines (CLSI, 2010). Because there are no defined MIC breakpoints for species of the genus Gemella, the results of the susceptibility tests were interpreted by the criteria for viridans group streptococci, according to Cerdà Zolezzi et al. (2004) and Woo et al. (2004). Streptococcus pneumoniae ATCC 49619 was used as the reference organism. NTUH_1465T, NTUH_2196 and strains of G. haemolysans strains (n=2), G. morbillorum (n=2) and G. sanguinis (n=5) were all susceptible to chloramphenicol, erythromycin, penicillin G and tetracycline. However, NTUH_4957 was resistant to erythromycin (MIC=2 μg ml⁻¹) and intermediate resistant to penicillin G (MIC=0.5 μg ml⁻¹) and tetracycline (MIC=4 μg ml⁻¹), and NTUH_5572T was intermediate resistant to penicillin G (MIC=0.5 μg ml⁻¹). The erythromycin-resistant strain NTUH_4957 contained the mef gene as detected by PCR and sequencing (data not shown). The mef gene is known to be responsible for resistance to macrolides in many Gram-staining-positive cocci including species of the genera Enterococcus and Streptococcus, and has previously been found in erythromycin-resistant G. haemolysans (Cerdà Zolezzi et al., 2004; Woo et al., 2004).

The polyphasic characterization data obtained in this study support the establishment of two novel species in the genus Gemella. Therefore, NTUH_1465T and NTUH_5572T are proposed as the type strains of Gemella parahaemolysans sp. nov. and Gemella taiwanensis sp. nov., respectively.

**Description of Gemella parahaemolysans sp. nov.**

Gemella parahaemolysans [pa.ra.hae.mo.ly’sans. Gr. prep. para resembling; N.L. part adj. haemolysans specific epithet
The DNA G+C content (mol%) of Gemella haemolysans is 30.9. Cells are non-endospore-forming, Gram-staining-positive cocci (0.6–1 μm in diameter) but easily decolorized during Gram staining. Colonies produced on trypticase soy agar supplemented with 5% sheep blood at 37 °C for 1 day are pinpoint-sized and non-haemolytic. Facultatively anaerobic. Catalase- and oxidase-negative. Positive result in Voges–Proskauer test, but negative for nitrate reduction. Casein, DNA, gelatin, hippurate, starch and Tween 80 are not hydrolysed. H2S and indole are not produced. Aerobic acid production is detected from D-glucose, sucrose, and maltose, but not from raffinose, D-ribose, D-mannitol, D-sorbitol, lactose, trehalose, L-arabinose, D-arabitol, α-cyclodextrin, glycogen, pullulan, melibiose, melezitose, methyl β-D-glucopyranoside or D-tagatose. Activities of alkaline phosphatase, acid phosphatase, esterase (C4), esterase lipase (C8) and naphthol-AS-BI-phosphohydrolase are present in API ZYM tests. Major cellular fatty acids are C16:0 and C14:0.

**Table 1. Characteristics useful for differentiating Gemella parahaemolysans sp. nov. and Gemella taiwanensis sp. nov. from recognized species of the genus Gemella**

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*Data from this study.
†Determined for strain NTUH_5572T only.
‡Data from Collins (2006).

**Description of Gemella taiwanensis sp. nov.**

Gemella taiwanensis (Tai.wan.en’sis. N.L. fem. adj. taiwanensis pertaining to Taiwan, where the type strain was isolated).

Cells are non-endospore-forming, Gram-staining-positive cocci (0.8–1.2 μm in diameter) but easily decolorized during Gram staining. Colonies produced on trypticase soy agar supplemented with 5% sheep blood at 37 °C for 1 day are pinpoint-sized and non-haemolytic. Facultatively anaerobic. Catalase- and oxidase-negative. Casein, DNA, gelatin, hippurate, starch and Tween 80 are not hydrolysed. Variable reactions are observed in the Voges–Proskauer test (NTUH_2196 is positive, NTUH_5572T and NTUH_4957 are negative). Negative for nitrate reduction. H2S and indole are not produced. Aerobic acid production is detected from D-glucose, D-mannitol, D-sorbitol, sucrose and maltose, but not from raffinose, D-ribose, lactose, trehalose, L-arabinose, D-arabitol, α-cyclodextrin, glycogen, pullulan, melibiose, melezitose, methyl β-D-glucopyranoside or D-tagatose. Activities of alkaline phosphatase, acid phosphatase, leucine arylamidase, esterase (C4), esterase lipase (C8) and naphthol-AS-BI-phosphohydrolase are present in API ZYM tests. Major cellular fatty acids are C16:0 and C14:0.
The type strain is NTUH_5572T (=BCRC 80366T=JCM 18066T), isolated from blood cultures in the Bacteriology Laboratory of the National Taiwan University Hospital. The DNA G+C content of the type strain is 30.2 mol%. Two additional strains of the species are NTUH_4957 and NTUH_2196.

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References


