

## Characterization of Blood Culture Isolates of *Streptococcus dysgalactiae* subsp. *equisimilis* Possessing Lancefield's Group A Antigen

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**For three human blood culture isolates of beta-hemolytic streptococci with Lancefield's serogroup A antigen, phylogenetic analysis of the 16S rRNA genes confirmed biochemical identification as *Streptococcus dysgalactiae* subsp. *equisimilis*. Genes encoding M or M-like proteins, which are considered to be major virulence determinants in streptococci, were detected in all of these strains. Our data clearly demonstrate that for beta-hemolytic streptococci, the species assignment should not be based on the results of serogrouping alone.**

Streptococci were first serogrouped by Rebecca C. Lancefield in 1933 on the basis of specific cell wall-associated carbohydrates (16). The term group A streptococcus has been used generally as a synonym for *Streptococcus pyogenes* despite the fact that minute-colony-forming species of the "*Streptococcus milleri*" group may also rarely possess Lancefield's serogroup A antigen (12, 18). Moreover, Bert and Lambert-Zechovsky (3) have recently published a case report of recurrent bacteremia due to a biochemically identified strain of *Streptococcus dysgalactiae* subsp. *equisimilis* exhibiting Lancefield's serogroup A antigen. This clinical isolate was kindly provided by Bert and Lambert-Zechovsky for further investigation and assigned streptococcal culture collection no. AC-2074. The present report describes the phylogenetic analysis and molecular characterization of blood culture isolates of *S. dysgalactiae* subsp. *equisimilis* belonging to Lancefield's serogroup A.

(This work has been presented in part at the 9th European Congress of Clinical Microbiology and Infectious Diseases, Berlin, Germany, 21 to 24 March 1999 [4a].)

**Case 1.** A 78-year-old man presented to the hospital with acute onset of headache, fever of 40°C, and chills. His medical history included liver cirrhosis due to chronic hepatitis C, with signs of portal hypertension. On physical examination, local erythema and tenderness of his right foot were noted and the diagnosis of an erysipelas was made. A minor skin lesion acquired 4 days prior to admission probably served as the site of entry for bacteria. Blood cultures were positive for beta-hemolytic streptococci, and antimicrobial therapy with a standard dose of intravenous amoxicillin was started. The patient's condition improved, and he was discharged 6 days later on an oral regimen to complete a 10-day course of amoxicillin. One blood culture isolate was referred to the National Reference Center for Streptococci, Aachen, Germany, and assigned streptococcal culture collection no. AC-2713.

**Case 2.** A 72-year-old woman who was undergoing diagnostic workup for an indeterminate paraspinal mass (chronic inflammatory lesion versus osteochondroma versus osteosar-

coma) was referred for symptoms consistent with acute tonsillitis. Blood was obtained, and blood cultures grew beta-hemolytic streptococci. The patient was treated according to the results for in vitro susceptibility testing with cefuroxime. Twenty-four days later, the patient had fully recovered. This clinical isolate was assigned streptococcal culture collection no. AC-2832.

**Methods.** Lancefield's group-specific streptococcal carbohydrates were extracted from an overnight culture in Todd-Hewitt broth according to the hot-formamide technique of Fuller (8). Following extraction, serogroup was determined by the Ouchterlony immunodiffusion technique with rabbit antisera against the antigens A, C, G, and A-variant (14, 17). Antigen-antibody precipitation lines were visualized by Coomassie blue staining. In addition, trypsinized bacterial suspensions were tested for agglutination with the lectin from *Dolichos biflorus* (Biotrend, Cologne, Germany) in order to detect the group C streptococcal antigen (15, 20, 23). The isolates were biochemically identified with the Rapid ID 32 Strep API system (bioMérieux, Marcy-l'Étoile, France).

Streptococcal genomic DNA was prepared according to standard protocols (2). The genomes of the strains were hybridized with an oligonucleotide probe specific for *S. pyogenes* (Accuprobe; Gen-Probe Incorporated, San Diego, Calif.). Nearly the entire 16S rRNA gene was amplified by PCR, and the resulting products were purified with the Quiaquick PCR purification kit (Qiagen, Heiden, Germany) and sequenced on an ABI 373 automated DNA sequencer (Applied Biosystems, Weiterstadt, Germany) by following the protocol of Hiraishi (10). The sequence editor DCSE, version 2.54 (5), was used for editing and concatenation and for multiple alignment of the three strains' 16S ribosomal DNA (rDNA) sequences with those of the following strains deposited in the EMBL database: *S. pyogenes* ATCC 12344 (accession no. AB002521); *S. agalactiae* ATCC 13813 (accession no. U02908); *Streptococcus dysgalactiae* subsp. *dysgalactiae* ATCC 43078, serogroup C (accession no. AB002485), and ATCC 27957, serogroup C (accession no. AB002484); *S. dysgalactiae* subsp. *equisimilis* LMG 16026, serogroup C (accession no. AB008926); and *S. pneumoniae* MAFF 911410 (accession no. AB002522). The 16S rDNA sequences of the three strains were also aligned with 16S rDNA sequences obtained in our laboratory from the following strains of the National Reference Center's collection: *S. pyo-*

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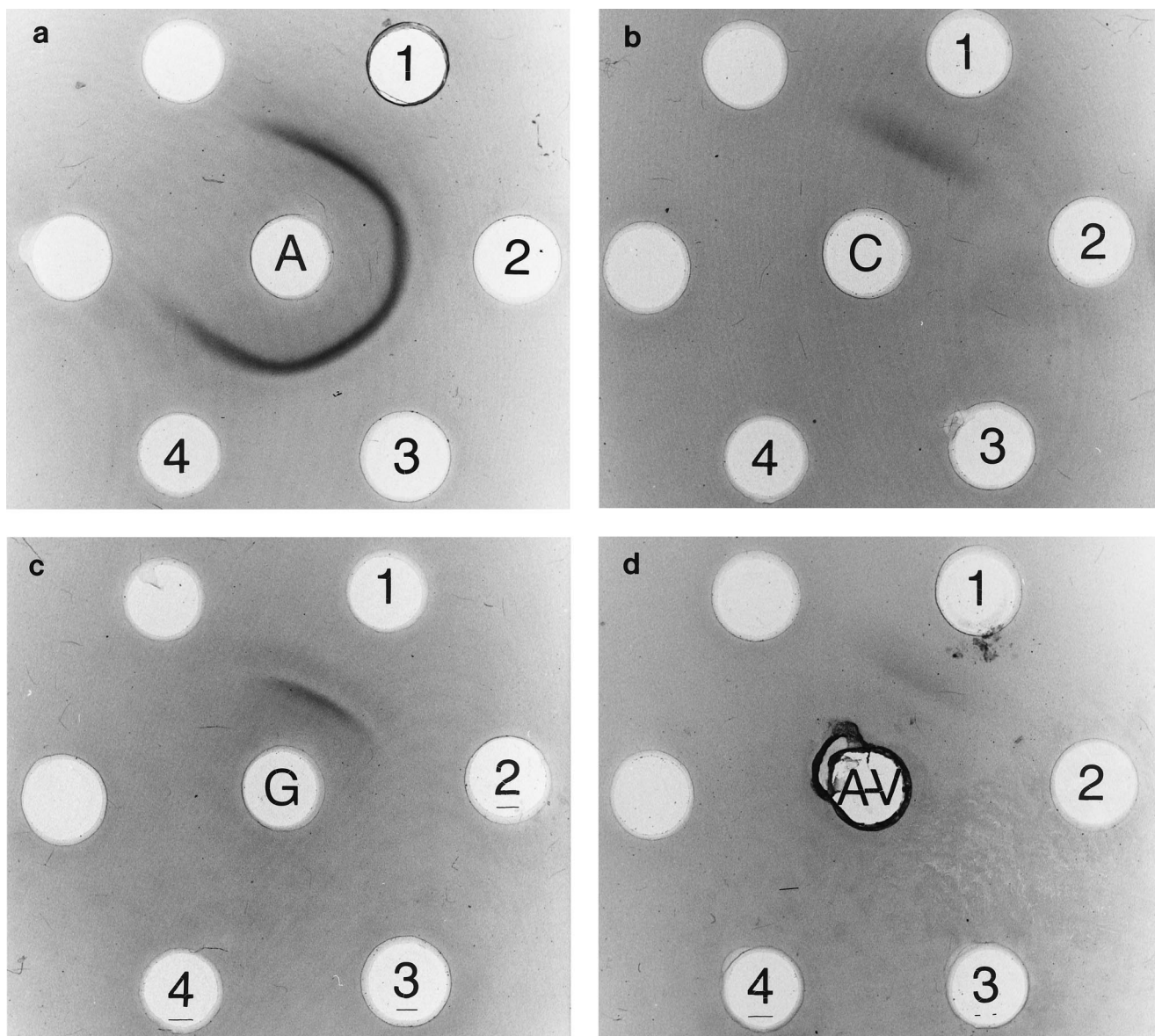


FIG. 1. Ouchterlony immunodiffusion of cell wall carbohydrate extracts (outer wells) tested with rabbit antisera against the antigens A, C, G, and A-variant (central wells). 1, serogroup-specific control antigen; 2, AC-2074; 3, AC-2713; 4, AC-2832.

genes AC-2892; *S. agalactiae* AC-2581; *S. dysgalactiae* subsp. *dysgalactiae* GCS-1338, serogroup C; *S. dysgalactiae* subsp. *equisimilis* ATCC 35666, serogroup C; and *S. dysgalactiae* subsp. *equisimilis* ATCC 12394, serogroup G. Phylogenetic studies were performed by using the neighbor-joining method based on a dissimilarity matrix corrected for multiple mutations per site, using the algorithm of Juke and Cantor. Confidence values for individual branches were determined by bootstrap analysis based on 1,000 bootstrap trees that were generated from resampled characters. All calculations were performed with the software package TREECON, version 1.3b (24).

The presence of genes encoding M- or M-like proteins was determined by PCR with "all M" primers (19). The resulting products were purified and sequenced. The nucleotide sequence encoding the N-terminal hypervariable portion of the M protein was subjected to similarity searches against the National Institutes of Health DNA database (1).

**Remarks and conclusion.** It was shown unequivocally that all three isolates exhibit the serogroup A antigen but not serogroup C, G, or A-variant antigens. Results of the Ouchterlony immunodiffusion are shown in Fig. 1. The *N*-acetylgalactosamine specificity of the group C carbohydrate antigen was not detected by agglutination with the lectin from *D. biflorus* for any of the three strains tested. Isolates AC-2713 and AC-2832 were biochemically identified as *S. dysgalactiae* subsp. *equisimilis* (API code 1512 2041 110; 99.5% identity; T = 1.0). As published previously for isolate AC-2074 (3), our results revealed the following characteristic properties to discriminate group A *S. dysgalactiae* subsp. *equisimilis* from *S. pyogenes*: production of  $\beta$ -glucuronidase, fermentation of ribose, and the absence of pyrrolidonylarylamidase production. A negative Voges-Proskauer reaction provides a means to differentiate between the large-colony-forming *S. dysgalactiae* subsp. *equisimilis* and the minute-colony-forming species of the *S. milleri*

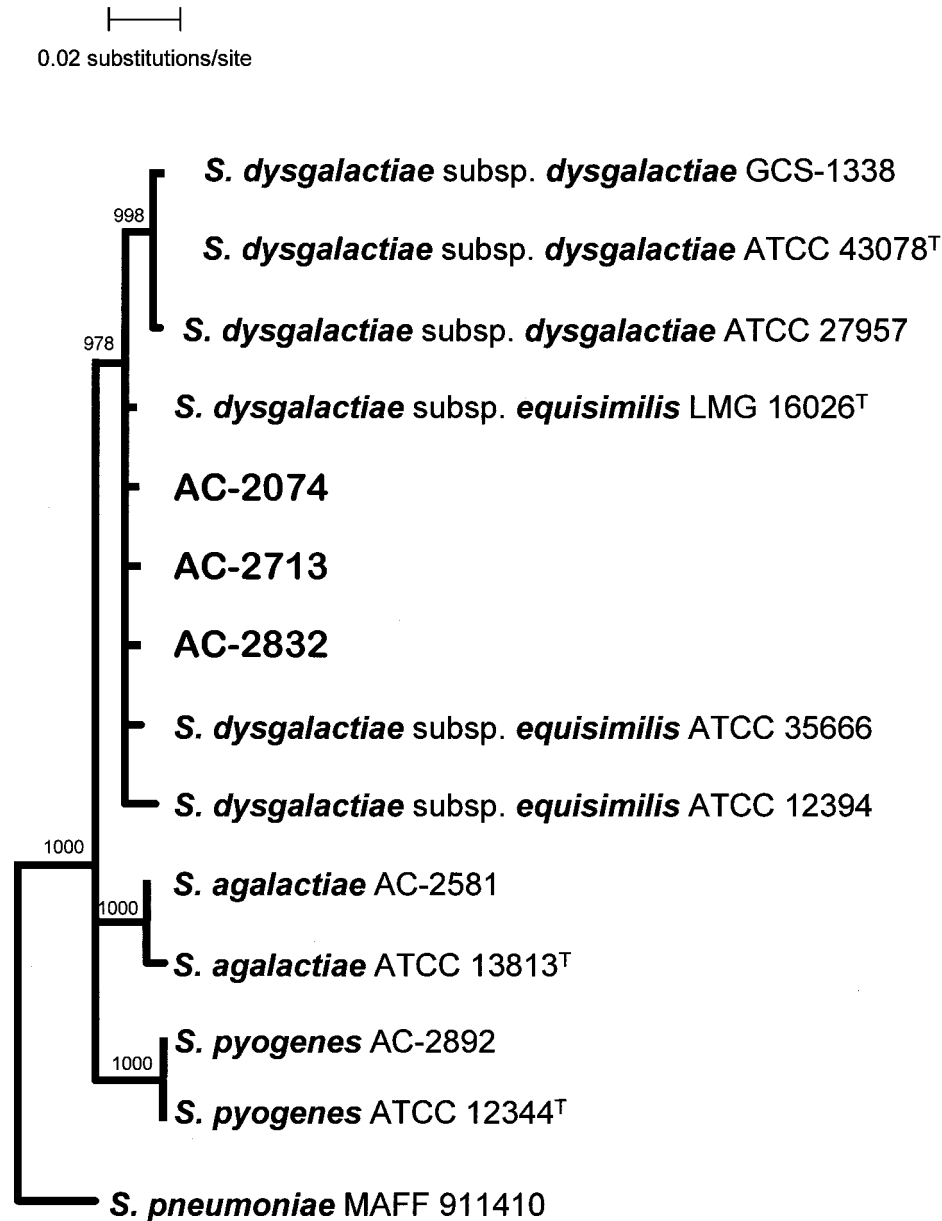


FIG. 2. Rooted phylogenetic tree inferred by analysis of 16S rDNA sequences of the three blood culture isolates AC-2074, AC-2713, and AC-2832 and other members of the pyogenic streptococci by the neighbor-joining method. Bootstrap values of  $\geq 975$  are displayed; nodes with a support of  $< 975$  are drawn unresolved.

group. Hybridization with the Accuprobe oligonucleotide probe specific for *S. pyogenes* was not detected for any of the three strains tested.

A rooted phylogenetic distance tree inferred by analysis of 16S rDNA sequences is shown in Fig. 2. *S. pneumoniae* was arbitrarily chosen as the outgroup taxon. All three blood culture isolates studied clustered with the type strain of *S. dysgalactiae* subsp. *equisimilis*, LMG 16026, as a sister group to *S. dysgalactiae* subsp. *dysgalactiae* in the phylogenetic tree. The phylogenetic analyses revealed a substantial evolutionary distance between *S. pyogenes* and the *S. dysgalactiae* subsp. *equisimilis* blood culture isolates exhibiting the Lancefield serogroup A antigen.

For the three strains studied, genes (*emm* or *emmL*) encoding M- or M-like proteins were detected. The respective nu-

cleotide sequences of strains AC-2713 and AC-2832 revealed a high degree of similarity ( $>80\%$  identity for a portion of at least 200 bp within the 5' region of the respective gene) to the genotype b *emmL* gene described previously for serogroup G *S. dysgalactiae* subsp. *equisimilis* (21); the *emm* or *emmL* gene sequence of strain AC-2074 showed a high degree of similarity to the *emm41* gene of *S. pyogenes*.

Pyogenic streptococci have been divided conventionally on the basis of their Lancefield group antigen, habitat, pathogenicity, and biochemical characteristics (7, 16). With the availability of automated DNA sequencing and a rapidly growing database, the analysis of 16S rRNA sequences has become one of the most powerful tools for phylogenetic analysis and species identification. The finding that the 16S rDNA sequences of all three strains studied are closely related to that of the type

strain of *S. dysgalactiae* subsp. *equisimilis* suggests that biochemical methods, like the API system, are an appropriate diagnostic approach for the identification of this species. The observation of a substantial evolutionary distance between *S. dysgalactiae* subsp. *equisimilis* and *S. pyogenes* is in agreement with results of previous phylogenetic analyses of beta-hemolytic streptococci (13) and supports the application of an oligonucleotide probe specific for *S. pyogenes* in the identification of beta-hemolytic streptococci exhibiting Lancefield's serogroup A antigen. These data clearly show that for species assignment of beta-hemolytic streptococci, the determination of the serogroup is insufficient as the sole diagnostic tool, as outlined previously for group G streptococci (9).

In humans, *S. dysgalactiae* subsp. *equisimilis* may colonize the skin, throat, and genital tract. Recently, this species has been isolated with an increasing frequency as the cause of various human infections, such as cellulitis, pharyngitis, sepsis, meningitis, and endocarditis (6). Just as most patients with infections due to *S. dysgalactiae* subsp. *equisimilis* exhibiting serogroup C or G antigen have underlying diseases, predominantly malignancies (4, 22), the three patients diagnosed with infections due to group A *S. dysgalactiae* subsp. *equisimilis* also suffered from compromising underlying diseases. The finding of two additional patients with bacteremia due to *S. dysgalactiae* subsp. *equisimilis* belonging to Lancefield's serogroup A and the detection of *emm* or *emmL* genes encoding a major virulence factor in all three strains tested provide evidence that strains of *S. dysgalactiae* subsp. *equisimilis* of Lancefield's serogroup A are probably as pathogenic as those exhibiting Lancefield's serogroup C or G antigen. Therefore, it can be concluded that beta-hemolytic streptococci that possess the serogroup A antigen but are negative for the Voges-Proskauer reaction and do not produce pyrrolidonylarylamidase can be found in serious human infections and may be identified as *S. dysgalactiae* subsp. *equisimilis*.

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