Genetic Features of Clinical Isolates of *Streptococcus dysgalactiae* subsp. *equisimilis* Possessing Lancefield’s Group A Antigen

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Thirteen *Streptococcus dysgalactiae* subsp. *equisimilis* isolates possessing Lancefield’s group A antigen recovered from people in Japan during 2000 to 2004 were genotyped. The results indicate that a conserved clone has persisted and spread within Japan, and two different *emm* types were observed within members of this clone.

*Streptococcus dysgalactiae* subsp. *equisimilis* belongs to Lancefield’s groups C and G, and it has been recognized as a cause of pharyngitis and skin and soft-tissue infections (6, 10, 12, 27). Further, case reports referring to toxic shock-like syndrome (TSLS) due to group C and G *S. dysgalactiae* subsp. *equisimilis* have been published (2, 13, 16, 19, 20, 22, 28). Recently, some cases of bacteremia or gangrene caused by *S. dysgalactiae* subsp. *equisimilis* belonging to Lancefield’s group A have been reported (5, 7, 18). These group A beta-hemolytic streptococci were identified as *S. dysgalactiae* subsp. *equisimilis* on the basis of the phylogenetic analysis of their 16S rRNA genes and their biochemical characters in spite of the common use of GAS (group A streptococcus) to describe *S. pyogenes*. Thus, these data have demonstrated that *S. pyogenes* is not the only beta-hemolytic streptococcus possessing the group A antigen. However, the genetic characterization of group A *S. dysgalactiae* subsp. *equisimilis* has not been fully studied.

GAS, GCS (group C streptococci), and GGS (group G streptococci) express various M-like proteins on the cell surface. On the basis of the sequence analysis of the 5′ end of the *emm* gene that encodes the M-like protein, *emm* typing has been widely used to characterize these streptococci (3, 4, 13, 16, 17, 26). The Centers for Disease Control and Prevention (CDC) maintains the *emm* sequence database (http://www.cdc.gov/ncidod/biotech/strep/strepblast.htm), which contains >150 *emm* types of GAS and >30 *emm* types of GCS and GGS. Multilocus sequence typing (MLST) data are used to construct a model that analyzes the evolution of GAS, GCS, and GGS (17). In the present study, we collected and characterized group A *S. dysgalactiae* subsp. *equisimilis* isolates from humans, including TSLS patients. We performed epidemiological analysis of these isolates by using a combination of different genotyping methods, *emm* typing, and MLST.

**Bacterial isolates.** The clinical and epidemiological features of the 13 group A *S. dysgalactiae* subsp. *equisimilis* isolates collected in this study are listed in Table 1. All of these isolates were recently recovered from humans, and all of the isolates examined, except one, were associated with disease. Two isolates were recovered from TSLS patients. All of the isolates were confirmed to possess only the group A carbohydrate antigen with a Streptococcus Grouping kit (Oxoid Ltd., Basingstoke, United Kingdom) and Strept LA (Denka Seiken, Japan), and they were identified as *S. dysgalactiae* subsp. *equisimilis* by using the API 20 Strep kit (BioMérieux, Tokyo, Japan).

**16S rRNA gene sequencing.** PCR template DNA was prepared by using InstaGene Matrix (Bio-Rad, Hercules, CA). DNA sequencing of the 16S rRNA genes was performed according to previously described methods (14, 15). The 16S rRNA gene was amplified with primers 27F (AGAGTTTGATCCTGGCTCAG) and 1492r (GGCTACCTTGTTACGACTT). Sequencing was performed with the ABI Prism 3100 genetic analyzer (Applied Biosystems, Foster City, CA) and primers r1L (GTATTACCGCAGGCGCTGTCG), r3L (TTGCGCTCGTGTGGGACT), r4L (A CGGGCCGTTGTGTCAG), f1L (GAGTTTGATCCTGGCTCAG) and 1492r (GGCTACCTTGTTACGACTT). Sequencing was performed with the ABI Prism 3100 genetic analyzer (Applied Biosystems, Foster City, CA) and primers r1L (GTATTACCGCAGGCGCTGTCG), r3L (TTGCGCTCGTGTGGGACT), r4L (A CGGGCCGTTGTGTCAG), f1L (GAGTTTGATCCTGGCTCAG), r2L (CCAGCAGGCCGGTAAATAC), and 926f (AA ACTCAGAGAATGTGCGG).

**emm typing.** *emm* typing was performed as described by Beall et al. (3, 4). The sequence was subjected to a homology search (Streptococci Group A Subtyping Request Form, BLAST 2.0 server [http://www.ncbi.nlm.nih.gov/BLAST] and the *emm* type was determined.

**MLST.** MLST was performed by determining the DNA sequences of the internal portions of seven housekeeping genes that encoded glucose kinase (*gki*), glutamine transport protein (*gtr*), glutamate racemase (*murF*), DNA mismatch repair protein (*mudS*), transketolase (*recP*), xanthine phosphoribosyltransferase (*xpt*), and acetyl coenzyme A acetyltransferase
(yqIL); MLST was performed according to a previously described procedure for GCS and GGS isolates (17). The primer pairs for the gtr loci did not amplify the corresponding MLST target fragment for all of the isolates tested. It was possible that an alteration within primer annealing sites prevented amplification. Therefore, alternative primer pairs for the gtr loci were designed to generate appropriate PCR products on the basis of the sequence information from the S. equi subsp. equi genome sequence (www.sanger.ac.uk) and sequence analysis combined with the inverse PCR method (23). The alternative primers used were as follows: gtr(GAS-Sde)-up, 5′-GTTGAT TATTGGGCCCCCTTCG-3′; gtr(GAS-Sde)-dn, 5′-CGCTCTGT CGACCTCTT TAGCA-3′.

Detection of virulence genes by PCR. PCR with previously described primer pairs was conducted for the detection of the genes coding for C5a peptidase (scpA), streptokinase (ska), streptolysin O (slo), streptolysin S (sagA), extracellular phospholipase A2 (sla), and streptococcal pyrogenic exotoxins (speA, speB, speC, speG, speH, speI, speJ, speL, [M3], speL, [M18], and speM) (16). In all cases, the primers were designed toward a sequence located inside the open reading frame. The expected sizes of PCR products were 759 bp for scpA, 237 bp for ska, 438 bp for slo, 113 bp for sagA, 495 bp for sla, 393 bp for speC, 1,113 bp for speB, 624 bp for speC, 211 bp for speG, 406 bp for speH, 523 bp for speI, 490 bp for speJ, 639 bp for speL [M3], 789 bp for speL [M18], and 672 bp for speM. PCR amplification was carried out by initial denaturation at 94°C for 2 min, followed by 30 cycles at 94°C for 30 s, 52°C for 30 s, and 72°C for 1 min and a final extension at 72°C for 3 min.

The 16S rRNA genes of all of the isolates tested showed completely identical DNA sequences (GenBank accession number AB253330). Two to three base differences existed between the 16S rRNA gene sequences of the isolates tested in this study and that of previously described group A S. dysgalactiae subsp. equisimilis strains (GenBank accession numbers AJ314609, AJ314610, and AJ314611); however, there was no difference between the 16S rRNA gene sequences of the isolates tested and the 5′ region sequences of the 16S rRNA genes of group A S. dysgalactiae subsp. equisimilis strain (GenBank accession number AF239716).

All of the isolates possessed the same set of alleles in six of the seven housekeeping loci, namely, gki108, gtr106, mur1105, mutS106, recP107, and xpt104 (GenBank accession numbers AF332633, AF332641, AF332801, AF332037, AF332816, and AF332829). These Japanese isolates were similar to GCS strain 4288 described by Kalia et al. (17) by MLST. The primer pairs for the yqIL loci did not amplify the corresponding MLST target fragment in any of the isolates tested. It was possible that an alteration within the primer annealing sites prevented amplification. Our attempts to make new primer pairs for the yqIL loci were unsuccessful. In the present study, the results of MLST and 16S rRNA gene sequencing of all of the isolates were completely identical. Our data indicate the dissemination of a single successful group A S. dysgalactiae subsp. equisimilis strain throughout at least four areas of Japan.

The emm types of the isolates tested are shown in Table 1. Of the 13 isolates, 11 were of the stg485 type and the remaining 2 were of the stg652 type. All of the stg485 isolates were of subtype stg485.0, and two stg652 isolates were of subtypes stg652.0 and stg652.5. stg652.5 was a new subtype with only one nucleotide difference from subtype stg652.0. These emm types were found to be associated with GGS isolates (http://www.cdc.gov/ncidod/biotech/strep/strepindex.htm). Both isolates from TSLS patients were of subtype stg485.0. Interestingly, Misawa et al. (21) reported that a group G S. dysgalactiae subsp. equisimilis isolate from a TSLS patient was of emm subtype stg485.0. These data suggest that this emm subtype strain should be kept in mind as a potential pathogen causing TSLS.

The presence of virulence genes was identified by PCR. All of the isolates showed the same virulence gene profile that was PCR positive for speG, scpA, ska, sagA, and slo and PCR negative for sla, speA, speB, speC, speH, speI, speJ, speL [M3], speL [M18], and speM. Previously, we reported that all 16 group G S. dysgalactiae subsp. equisimilis strains isolated from patients with severe invasive infections carried the scpA, ska, slo, and sagA genes (16). In this study, we observed that all group A S. dysgalactiae subsp. equisimilis strains also carried these four virulence genes and the speG gene. Misawa et al. (21) reported that a group G TSLS-causing strain possessed three virulence genes, slo, sagA, and skg (ska). Perhaps some of these virulence genes might relate to pathogenesis of TSLS caused by group A or G S. dysgalactiae subsp. equisimilis.

To the best of our knowledge, this report describes the first case of TSLS caused by group A S. dysgalactiae subsp. equi-

<table>
<thead>
<tr>
<th>Isolate</th>
<th>Yr of isolation</th>
<th>Location</th>
<th>Isolation site and/or clinical diseasea</th>
<th>emm type</th>
</tr>
</thead>
<tbody>
<tr>
<td>2000A-033</td>
<td>2000</td>
<td>Osaka</td>
<td>Lower-limb soft tissue, diabetic gangrene</td>
<td>stg485.0</td>
</tr>
<tr>
<td>2003A-123</td>
<td>2003</td>
<td>Osaka</td>
<td>Lower-limb joint</td>
<td>stg485.0</td>
</tr>
<tr>
<td>ST2004-047</td>
<td>2004</td>
<td>Osaka</td>
<td>Sputum, respiratory disease</td>
<td>stg485.0</td>
</tr>
<tr>
<td>ST2004-224</td>
<td>2004</td>
<td>Osaka</td>
<td>Blood, decubitus ulcer of the lower limb</td>
<td>stg485.0</td>
</tr>
<tr>
<td>ST2004-241</td>
<td>2004</td>
<td>Osaka</td>
<td>Peritoneal dialysis fluid, shock</td>
<td>stg485.0</td>
</tr>
<tr>
<td>2006-9</td>
<td>2000</td>
<td>Tokyo</td>
<td>Skin, ulcer</td>
<td>stg485.0</td>
</tr>
<tr>
<td>TY75</td>
<td>2000</td>
<td>Tokyo</td>
<td>Throat swab, none</td>
<td>stg485.0</td>
</tr>
<tr>
<td>1572</td>
<td>2001</td>
<td>Tokyo</td>
<td>TSLS</td>
<td>stg485.0</td>
</tr>
<tr>
<td>1727</td>
<td>2004</td>
<td>Chiba</td>
<td>Blood</td>
<td>stg485.0</td>
</tr>
<tr>
<td>NIH245</td>
<td>2002</td>
<td>Toyama</td>
<td>TSLS</td>
<td>stg485.0</td>
</tr>
<tr>
<td>TP-C566</td>
<td>2003</td>
<td>Toyama</td>
<td>Tinea pedis</td>
<td>stg485.0</td>
</tr>
<tr>
<td>ST2004-234</td>
<td>2004</td>
<td>Osaka</td>
<td>Blood, lower-limb necrosis</td>
<td>stg652.3b</td>
</tr>
<tr>
<td>NIH285</td>
<td>2004</td>
<td>Hokkaido</td>
<td>Necrotizing fascitis</td>
<td>stg652.0</td>
</tr>
</tbody>
</table>

a All isolates except TY75 were isolated from clinical specimens.

b New subtype.
Two case reports are summarized in Table 2. The ages of the patients were 54 and 70 years. At least one of the underlying conditions was reported in the patients (i.e., alcohol addiction and liver cirrhosis in case 1 and edema in case 2). In previous reports, the underlying conditions have been noted mostly in patients with infections due to *S. dysgalactiae* subsp. *equisimilis* possessing group A, C, or G antigen (5, 7, 13, 16, 18). The clinical manifestations included shock, hepatic insufficiency, renal failure, disseminated intravascular coagulation, erythematous rash, and soft-tissue necrosis.

It has been suggested that the horizontal transfer of the *emm* sequences and the following recombination events occur among beta-hemolytic streptococci such as GAS, GCS, and GGS (1, 24, 25). Such a mechanism could be responsible for the development of gene mosaics and the evolution of the *emm* genes in beta-hemolytic streptococci (29). In the present study, two different *emm* types were observed among the 13 group A *S. dysgalactiae* subsp. *equisimilis* isolates that were recently isolated in Japan, although the results of MLST and 16S rRNA gene sequencing for all of these isolates were completely identical. These two *emm* types included 11 *stg485* isolates obtained during 2000 to 2004 and two *stg652* isolates obtained in 2004. Therefore, it appears that horizontal gene transfer has contributed to variations of the *emm* gene in group A *S. dysgalactiae* subsp. *equisimilis*.

Kalia et al. (17) performed MLST of 34 GCS and GGS strains obtained from humans; they obtained 34 unique combinations of allelic profiles (sequence types). Of these 34 strains, strain 4288 was the most closely related to our isolates. Strain 4288 shares six of the seven housekeeping alleles, carries the Lancefield group C antigen, and belongs to the *stg485* type. It is entirely possible that the Japanese isolates have all seven MLST sequences in common since the corresponding MLST target site (*qglL*) was not amplified and sequenced by an alteration within a primer annealing site(s). Of our 13 group A *S. dysgalactiae* subsp. *equisimilis* isolates, 11 belonged to the same *emm* type. Therefore, these findings indicate that strain 4288 and our isolates may have been derived from the same ancestor or member of the clonal complex, even though they show different group antigens. Further, the basic polysaccharide structure in both groups A and C comprises polymeric L-rhamnose; this indicates a close relationship (8). Moreover, Kalia et al. (17) estimated that interspecies recombinational exchanges from GAS donors to GCS-GGS recipients had occurred recently. Therefore, the lateral transfer of the gene responsible for producing group antigens might cause a change in the group antigen. Recently, the complete genome sequences of several streptococcal species have been reported, and a brief description of each has been presented (11). *S. pneumoniae* and *S. mutans* have highly developed transformation systems, whereas natural transformation is not known to be a common event in *S. pyogenes* or *S. agalactiae*. Although *S. pyogenes* and *S. agalactiae* have many genes that are essential for competence and transformation, they have lost competence probably because phages have assumed a more important role in population diversity. It is possible that *S. dysgalactiae* subsp. *equisimilis* may have its origin in *S. pyogenes* (9). Therefore, phage-mediated genetic transfer is, in fact, the likely mechanism in *S. dysgalactiae* subsp. *equisimilis*, although the complete genome of this species has not been obtained.

In the present study, we investigated the phylogenetic relationship among 13 group A *S. dysgalactiae* subsp. *equisimilis* isolates by using several different genotyping methods. Our data suggest that all of the group A *S. dysgalactiae* subsp. *equisimilis* isolates recovered from Japanese patients could have descended from a common ancestor. Further investigation is required to elucidate the epidemiology of *S. dysgalactiae* subsp. *equisimilis* possessing Lancefield’s group A antigen.

**Nucleotide sequence accession number.** The 16S rRNA gene sequence of strain NIH245 has been deposited in the DDBJ database under accession number AB253330. The new *emm* subtype was deposited in the CDC *emm* sequence database.


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**TABLE 2. Features of TSLS caused by group A *S. dysgalactiae* subsp. *equisimilis* isolated in Japan**

<table>
<thead>
<tr>
<th>Case no. (strain)</th>
<th>Age (yr)</th>
<th>Sex</th>
<th>Isolation site</th>
<th>Underlying condition</th>
<th>Symptoms</th>
<th>Outcome</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 (1572)</td>
<td>54</td>
<td>M</td>
<td>Blood</td>
<td>Alcoholism, liver cirrhosis</td>
<td>Hepatic insufficiency, renal failure, impaired consciousness, fever (40°C), shock, hypotension, DIC, bilateral pleural effusion, jaundice</td>
<td>Death</td>
</tr>
<tr>
<td>2 (NIH245)</td>
<td>70</td>
<td>F</td>
<td>Surgical site</td>
<td>Edema (history of uterine cancer)</td>
<td>Hypotension, shock, erythematous rash, soft-tissue necrosis</td>
<td>Recovery</td>
</tr>
</tbody>
</table>

* a M, male; F, female.

b DIC, disseminated intravascular coagulopathy.
REFERENCES


