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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/strepindex.htm) that 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 (GTATTACCGC GGCTGCTGG), r3L (TTGCGCTCGTTGCGGGACT), r4L (A CGGGGCGGTGTGTACAAG), f1L (GAGTTTGATCCTGGC TCAG), f2L (CCAGCAGCCGCGGTAATAC), and 926f (AA ACTCAAAGGAATTGACGG).

***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.cdc.gov/ncidod/biotech/strep/strepblast.htm]), 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 (*murI*), DNA mismatch repair protein (*mutS*), transketolase (*recP*), xanthine phosphoribosyltransferase (*xpt*), and acetyl coenzyme A acetyltransferase

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TABLE 1. Characteristics of group A *S. dysgalactiae* subsp. *equisimilis* isolated in Japan

Isolate	Yr of isolation	Location	Isolation site and/or clinical disease[a]	*emm* type
2000A-033	2000	Osaka	Lower-limb soft tissue, diabetic gangrene	*stg485.0*
2003A-123	2003	Osaka	Lower-limb joint	*stg485.0*
ST2004-047	2004	Osaka	Sputum, respiratory disease	*stg485.0*
ST2004-224	2004	Osaka	Blood, decubitus ulcer of the lower limb	*stg485.0*
ST2004-241	2004	Osaka	Peritoneal dialysis fluid, shock	*stg485.0*
2000-9	2000	Tokyo	Skin, ulcer	*stg485.0*
TY75	2000	Tokyo	Throat swab, none	*stg485.0*
1572	2001	Tokyo	TSLS	*stg485.0*
1727	2004	Chiba	Blood	*stg485.0*
NIH245	2002	Toyama	TSLS	*stg485.0*
TP-C3663	2003	Toyama	Tinea pedis	*stg485.0*
ST2004-234	2004	Osaka	Blood, lower-limb necrosis	*stg652.5*[b]
NIH285	2004	Hokkaido	Necrotizing fasciitis	*stg652.0*

[a] All isolates except TY75 were isolated from clinical specimens.
[b] New subtype.

(*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′-GGTGAT TATTGGCCCTTCTGG-3′; *gtr*(GAS-Sde)-dn, 5′-CGGTCTG CGACTTCTTTAGCA-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 A_2 (*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*, 434 bp for *slo*, 113 bp for *sagA*, 495 bp for *sla*, 393 bp for *speA*, 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*, *murI105*, *mutS106*, *recP107*, and *xpt104* (GenBank accession numbers AF332633, AF332641, AF332801, AF330237, 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 *skcg* (*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. *equisi-*

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

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

[a] M, male; F, female.
[b] DIC, disseminated intravascular coagulopathy.

milis. 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, stain 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 (*yqiL*) 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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