and non-invasive groups. Each *emm* type included several subtypes.

Three types, *stG485*, *stG6792* and *stG2078*, predominated among the 42 invasive strains, but the predominance of a specific type was not recognized. In contrast, *stG10*, *stG6* and *stC36* were predominant relative to the other non-invasive strains. The distribution of *emm* types was significantly different between the invasive and non-invasive groups ($P<0.001$).

The PFGE profiles of invasive strains digested with the restriction enzyme *Sma*I are shown in Fig. 3. Strains with the same *emm* type showed uniform DNA profiles and were classified into five clones in accordance with the criteria for interpreting PFGE patterns (Tenover *et al.*, 1995): strains ($n=14$) identified as *stG6792*, *stG2078*, *stG653*, *stC36* and *stG4974* belonged to clone A; strains ($n=11$) identified as *stG6*, *stG652*, *stG5420*, *stG245* and *stC1400* belonged to clone B; strains ($n=10$) identified as *stG485*, *stG643*, and *stC6979* belonged to clone C; strains ($n=3$) identified as *stG10* belonged to clone D; and strains ($n=4$) identified as *stG480* and *stC74a* belonged to clone E. The *emm* types of four strains isolated from patients with STSS were *stG2078*, *stG485*, *stG653* and *stG6792*, respectively. Two patients later died shortly after hospitalization. No bias was observed in the *emm* type of isolates from invasive infections.

Our results of the *emm* type of invasive strains differed from the surveillance results recently reported by Broyles *et al.* (2009). In their results, strains identified as types *stG6*, *stG245*, *stG2078* and *stG643* predominated, and types *stG6792* and *stG485* were heavily outnumbered. At present, although the epidemiology is unknown, it seems that a new *emm* type organism may have entered Japan and may be spreading rapidly among increasing numbers of elderly people with underlying diseases living in densely populated cities.

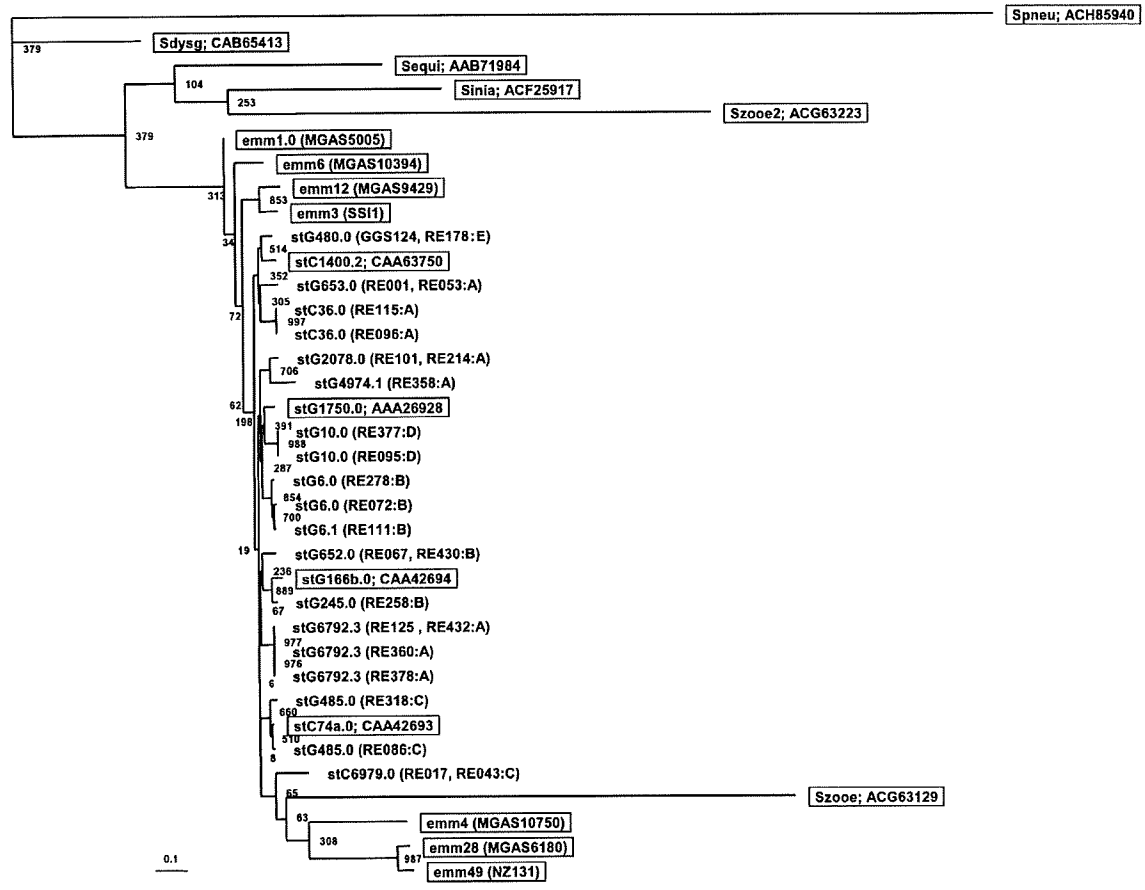

Fig. 4. Phylogenetic tree of the complete M protein in *Streptococcus dysgalactiae* subsp. *equisimilis* ($n=25$) isolated from patients with invasive infections. A phylogenetic tree based on deduced amino acid sequences was constructed by the neighbour-joining method. Bootstrap analyses of 1000 replications were carried out using CLUSTAL W. Each strain number analysed and the clone type is indicated in parentheses. The sequences of the other streptococcal strains were derived from the GenBank/EMBL/DDBJ database. Species have been given a five-letter code: Spneu, *S. pneumoniae*; Sdysg, *S. dysgalactiae* subsp. *dysgalatiae*; Sequi, *S. equi*; Szooe, *S. equi* subsp. *zooepidemicus*; Sinia, *S. iniae*.

Phylogenetic tree of *emm* genes

Fig. 4 shows the phylogeny of the 12 types of M protein in *S. dysgalactiae* subsp. *equisimilis* (n=25) isolated from the patients with invasive infections. A neighbour-joining tree was constructed for the M protein pattern using amino acid sequences corresponding to the complete M protein together with several M-like proteins in other previously analysed streptococcal species: seven strains of GAS, four strains of *S. dysgalactiae* subsp. *equisimilis* and one each of *S. dysgalactiae* subsp. *dysgalactiae*, *S. equi*, *S. iniae* and *S. equi* subsp. *zooepidemicus*.

Although M proteins have a hypervariable region at the N-terminal end, *S. dysgalactiae* subsp. *equisimilis* and GAS harbour extremely homologous M proteins compared with those of other *Streptococcus* species. The phylogenetic tree suggested that the M protein of *S. dysgalactiae* subsp. *equisimilis* was an orthologue of that of GAS.

We recently determined the complete genomic sequence of *S. dysgalactiae* subsp. *equisimilis* GGS_124 (*stG480.0*) isolated from patients with STSS (GenBank accession no. AP010935). The genome size was 2.1 Mbp, and sequence coverage with GAS genomes (Ferretti *et al.*, 2001) was 61–63 % identity. Interestingly, many genes encoding virulence factors in GAS were identified in *S. dysgalactiae* subsp. *equisimilis*. The occurrence of serious infections caused by *S. dysgalactiae* subsp. *equisimilis* in elderly persons with underlying diseases is likely to involve both compromised host defences and GAS-like virulence factors. However, it is unknown how this micro-organism invades deep tissues and vessels. Further investigation is needed to clarify this issue.

ACKNOWLEDGEMENTS

The authors are grateful to Akiko Ono and Hiromi Abrahashi for their assistance. We also thank local laboratory personnel for collection of bacterial isolates: Toshio Takahashi (Shinko Hospital), Tomomi Kohuku (Hygo Prefectural Amagasaki Hospital), Go Yamamoto (Nisikobe Medical Center), Tomoko Kobayashi (Kyurin Laboratory), Hiromi Ikari (Koshigaya Municipal Hospital), Kyoko Sawada (Chiba Children's Hospital), Reiko Hukazawa (Toshiba Hospital) and Iku Kurokawa (Tohoku Rosai Hospital).

REFERENCES

Baracco, G. J. & Bisno, A. L. (2006). Group C and group G streptococcal infections: epidemiologic and clinical aspects. In *Gram-Positive Pathogens*, 2nd edn, pp. 222–229. Edited by V. A. Fischetti, R. P. Novick, J. J. Ferretti, D. A. Portnoy & J. I. Rood. Washington, DC: American Society for Microbiology.

Barnham, M. R. D., Weightman, N. C., Anderson, A. W. & Tanna, A. (2002). Streptococcal toxic shock syndrome: a description of 14 cases from North Yorkshire, UK. *Clin Microbiol Infect* **8**, 174–181.

Beall, B., Facklam, R. & Thompson, T. (1996). Sequencing *emm*-specific PCR products for routine and accurate typing of group A streptococci. *J Clin Microbiol* **34**, 953–958.

Bert, F. & Lambert-Zechovsky, N. (1997). Analysis of a case of recurrent bacteraemia due to group A *Streptococcus dysgalactiae* subsp. *equisimilis* by pulsed-field gel electrophoresis. *Infection* **25**, 250–251.

Brandt, C. M. & Spellerberg, B. (2009). Human infections due to *Streptococcus dysgalactiae* subspecies *equisimilis*. *Clin Infect Dis* **49**, 766–772.

Brandt, C. M., Haase, G., Schnitzler, N., Zbinden, R. & Lütticken, R. (1999). Characterization of blood culture isolates of *Streptococcus dysgalactiae* subsp. *equisimilis* possessing Lancefield's group A antigen. *J Clin Microbiol* **37**, 4194–4197.

Broyles, L. N., Van Beneden, C., Beal, B., Facklam, R., Shewmaker, P. L., Malpiedi, P., Daily, P., Reingold, A. & Fareley, M. M. (2009). Population-based study of invasive disease due to β-hemolytic streptococci of groups other than A and B. *Clin Infect Dis* **48**, 706–712.

Clancy, J., Petipas, J., Dib-Haji, F., Yuan, W., Cronan, M., Kamath, A. V., Bergeron, J. & Retsema, J. A. (1996). Molecular cloning and functional analysis of a novel macrolide-resistance determinant, *mefA*, from *Streptococcus pyogenes*. *Mol Microbiol* **22**, 867–879.

CLSI (2007). *Performance Standards for Antimicrobial Susceptibility Testing*; 17th Informational Supplement, M100-S17. Wayne, PA: Clinical and Laboratory Standards Institute.

Cohen-Poradosu, R., Jaffe, J., Lavi, D., Grisariu-Greenzaid, S., Nir-Paz, R., Valinsky, L., Dan-Goor, M., Block, C., Beall, B. & Moses, A. E. (2004). Group G streptococcal bacteremia in Jerusalem. *Emerg Infect Dis* **10**, 1455–1460.

Facklam, R. (2002). What happened to the streptococci: overview of taxonomic and nomenclature changes. *Clin Microbiol Rev* **15**, 613–630.

Ferretti, J. J., McShan, W. M., Adjic, D., Savic, D. J., Savic, G., Lyon, K., Primeaux, C., Sezate, S., Suvorov, A. N. & other authors (2001). Complete genome sequence of an M1 strain of *Streptococcus pyogenes*. *Proc Natl Acad Sci U S A* **98**, 4658–4663.

Fischetti, V. A. (1989). Streptococcal M protein: molecular design and biological behavior. *Clin Microbiol Rev* **2**, 285–314.

Gerlach, D., Köhler, W., Günther, E. & Mann, K. (1993). Purification and characterization of streptolysin O secreted by *Streptococcus equisimilis* (group C). *Infect Immun* **61**, 2727–2731.

Hashikawa, S., Iinuma, Y., Furushita, M., Ohkura, T., Nada, T., Torii, K., Hasegawa, T. & Ohta, M. (2004). Characterization of group C and G streptococcal strains that cause streptococcal toxic shock syndrome. *J Clin Microbiol* **42**, 186–192.

Hirose, Y., Yagi, K., Honda, H., Shibuya, H. & Okazaki, E. (1997). Toxic shock-like syndrome caused by non-group A β-hemolytic streptococci. *Arch Intern Med* **157**, 1891–1894.

Humar, D., Dattam, V., Bast, D. J., Beall, B., De Azavedo, J. C. S. & Nizetk, V. (2002). Streptolysin S and necrotizing infections produced by group G streptococcus. *Lancet* **359**, 124–129.

Ikebe, T., Murayama, S., Saitoh, K., Yamai, S., Suzuki, R., Isobe, J., Tanaka, D., Katsukawa, C., Tamura, A. & other authors (2004). Surveillance of severe invasive group-G streptococcal infections and molecular typing of the isolates in Japan. *Epidemiol Infect* **132**, 145–149.

Kalia, A., Enright, M. C., Spratt, B. G. & Bessen, D. E. (2001). Directional gene movement from human-pathogenic to commensal-like streptococci. *Infect Immun* **69**, 4858–4869.

Katsukawa, C., Tamaru, A. & Morikawa, Y. (2002). *Streptococcus dysgalactiae* subsp. *equisimilis* possessing Lancefield's group A antigen. *Kansenshogaku Zasshi* **76**, 155–160.

Kugi, M., Tojo, H., Haraga, I., Takata, T., Handa, K. & Tanaka, K. (1998). Toxic shock-like syndrome caused by group G streptococcus. *J Infect* **37**, 308–309.

Murayama, S. Y., Seki, T., Sakata, H., Sunaoshi, K., Nakayama, E., Iwata, S., Sunakawa, K. & Ubukata, K.The Invasive Streptococcal Disease Working Group (2009). Capsular type and antibiotic resistance in *Streptococcus agalactiae* isolates from patients, ranging from newborns to the elderly, with invasive infections. *Antimicrob Agents Chemother* **53**, 2650–2653.

Natoli, S., Fimianim, C., Faglieri, N., Laurenzi, L., Calamaron, A., Frasca, A. M. & Arcuri, E. (1996). Toxic shock syndrome due to group C streptococci. A case report. *Intensive Care Med* **22**, 985–989.

Okumura, K., Hara, A., Tanaka, T., Nishiguchi, I., Minamide, W., Igarashi, H. & Yutsudo, T. (1994). Cloning and sequencing the streptolysin O genes of group C and group G streptococci. *DNA Seq* **4**, 325–328.

Ruoff, K. L., Whiley, R. A. & Beighton, D. (2003). *Streptococcus.* In *Manual of Clinical Microbiology*, pp. 405–421. Edited by P. R. Murray, E. J. Baron, J. H. Jorgensen, M. A. Pfaller & R. H. Yolken. Washington, DC: American Society for Microbiology.

Schnitzler, N., Podbielski, A., Baumgarten, G., Mignon, M. & Kaufhold, A. (1995). M or M-like protein gene polymorphisms in human group G streptococci. *J Clin Microbiol* **33**, 356–363.

Seppälä, H., Skurnik, M., Soini, H., Roberts, M. C. & Huovinen, P. (1998). A novel erythromycin resistance methylase gene (*ermTR*) in *Streptococcus pyogenes. Antimicrob Agents Chemother* **42**, 257–262.

Tait-Kamradt, A., Clancy, J., Cronan, M., Dib-Hajj, F., Wondrack, L. & Yuan, W. (1997). *mefE* is necessary for the erythromycin-resistant M phenotype in *Streptococcus pneumoniae. Antimicrob Agents Chemother* **41**, 2251–2255.

Tenover, F. C., Arbeit, R. D., Goering, R. V., Micelsen, P. A., Murray, B. E., Persing, D. H. & Swaminathan, B. (1995). Interpreting chromosomal DNA restriction patterns produced by pulsed-field gel electrophoresis: criteria for bacterial strain typing. *J Clin Microbiol* **33**, 2233–2239.

Trieu-Cuot, P., Poyart-Salmeron, C., Carlier, C. & Courvalin, P. (1990). Nucleotide sequence of the erythromycin resistance gene of the conjugative transposon Tn*1545. Nucleic Acids Res* **18**, 3660.

Ubukata, K., Sunaoshi, K., Kobayashi, R. & Okuzumi, K. (2006). Large-scale questionnaire surveillance concerning invasive infections with group C and G streptococci. *Kansenshogaku Zasshi* **80**, 480–487.

Vandamme, P., Pot, B., Falsen, E., Kersters, K. & Devriese, L. A. (1996). Taxonomic study of Lancefield streptococcal groups C, G, and L (*Streptococcus dysgalactiae*) and proposal of *S. dysgalactiae* subsp. *equisimilis* subsp. nov. *Int J Syst Bacteriol* **46**, 774–781.

Wagner, J. G., Schlievert, P. M., Assimacopoulos, A. P., Stoehr, J. A., Carson, P. J. & Komadina, K. (1996). Acute group G streptococcal myositis associated with streptococcal toxic shock syndrome: case report and review. *Clin Infect Dis* **23**, 1159–1161.

Wajima, T., Murayama, S. Y., Sunaoshi, K., Nakayama, E., Sunakawa, K. & Ubukata, K. (2008). Distribution of *emm* type and antibiotic susceptibility of group A streptococci causing invasive and non-invasive disease. *J Med Microbiol* **57**, 1383–1388.

Walter, F., Siegel, M. & Malke, H. (1989). Nucleotide sequence of the streptokinase gene from a group-G streptococcus. *Nucleic Acids Res* **17**, 1262.

Whatmore, A. M. & Kehoe, M. A. (1994). Horizontal gene transfer in the evolution of group A streptococcal *emm*-like genes: gene mosaics and variation in Vir regulons. *Mol Microbiol* **11**, 363–374.

Woo, P. C. Y., Fung, A. M. Y., Lau, S. K. P., Wong, S. S. Y. & Yuen, K.-Y (2001). Group G β-hemolytic streptococcal bacteremia characterized by 16S ribosomal RNA gene sequencing. *J Clin Microbiol* **39**, 3147–3155.

JOURNAL OF CLINICAL MICROBIOLOGY, Feb. 2010, p. 635–638 Vol. 48, No. 2
0095-1137/10/$12.00 doi:10.1128/JCM.01362-09

Nonhemolytic *Streptococcus pyogenes* Isolates That Lack Large Regions of the *sag* Operon Mediating Streptolysin S Production[▽]

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Received 13 July 2009/Returned for modification 19 August 2009/Accepted 7 December 2009

Among nonhemolytic *Streptococcus pyogenes* (group A streptococcus) strains (n = 9) isolated from patients with pharyngitis or acute otitis media, we identified three deletions in the region from the *epf* gene, encoding the extracellular matrix binding protein, to the *sag* operon, mediating streptolysin S production.

In clinical laboratories, the property of beta-hemolysis on a blood agar plate is a characteristic used to preliminarily detect pyogenic streptococci such as *Streptococcus pyogenes* (group A streptococcus [GAS]), *Streptococcus agalactiae* (group B streptococcus), and *Streptococcus dysgalactiae* subsp. *equisimilis* (12). GAS produces two hemolysins: oxygen-dependent, labile streptolysin O, encoded by the *slo* gene (8), and oxygen-stable streptolysin S (SLS), encoded by the *sag* operon extending from the *sagA* gene to *sagI* (5). SLS, a potent cytolytic toxin produced by nearly all strains of GAS, is responsible for the zone of hemolysis surrounding GAS colonies grown under routine CO_2 culture conditions.

The *sagA* gene, which is positioned upstream in the *sag* operon, encodes a prepropeptide consisting of 53 amino acid (aa) residues, including a Gly-Gly proteolytic cleavage site that has been predicted to release a propeptide of 30 aa from a 23-aa leader sequence. The propeptide is considered to be the structural element of SLS. The remaining genes in the operon have features consistent with export functions, posttranslational modification of the SLS peptide, and a possible immunity protein (3).

Rarely, nonhemolytic variants of GAS have been isolated from patients with pharyngitis (6, 10), pneumonia (13), sepsis (2, 14), and cellulitis (11). These isolates were probably not producers of SLS, but the molecular cause had previously not been explained. Recently, based on mutational analysis, it was reported that all genetic components of the *sag* operon are required for the expression of functional SLS as an important virulence factor in the pathogenesis of invasive infection (3). In this study, we aimed to determine the reason for nonhemolysis by GAS clinical isolates at the molecular level.

A total of 1,690 samples, including throat swabs (n = 1,513) from patients with pharyngitis/tonsillitis and middle ear fluid (n = 177) from patients with acute otitis media (AOM), were sent to our laboratory by clinical physicians. Real-time PCR was immediately carried out routinely, in parallel with culturing, for all clinical samples on the day they were received. The real-time PCR used in this study was an application of the methods using molecular beacon probes and primers that we had constructed to detect six pathogens, including GAS, in samples from patients with respiratory tract infection (9). The set of primers and the probe for 16S rRNA genes used for the identification of GAS are as follows: sense primer, 5′-GAGA GACTAACGCATGTTAGTA-3′; reverse primer, 5′-TAGTT ACCGTCACTTGGTGG-3′; and probe, 6-carboxyfluorescein–CGCGATCGCGACGATACATAGCCGACCTGGAT CGCG–Black Hole Quencher 1. DNA extraction with the Extragen II kit (TOSOH, Tokyo, Japan) and subsequent DNA amplification with the Mx3000P system (Stratagene, La Jolla, CA) were performed by our protocol (9). On the following day, when no colonies with hemolysis were observed on the blood agar plate, Gram staining and reexamination by real-time PCR were carried out for some nonhemolytic colonies having different shapes, regardless of the positive PCR results for GAS on the preceding day. Next, colonies were confirmed to have characteristics of GAS by (i) an agglutination test for Lancefield group A antigen (Streptex; Mitsubishi Chemical Medience, Tokyo, Japan), (ii) use of the API Strep system (bioMérieux, Tokyo, Japan), and (iii) evaluation for the pyrrolidonyl arylamidase reaction (Oxoid, Hampshire, United Kingdom) in accordance with the *Manual of Clinical Microbiology* (12).

We finally identified nine nonhemolytic GAS strains from among 818 clinical isolates (1.1%) obtained from patients with pharyngitis/tonsillitis or AOM between November 2006 and March 2009. Colonies of these GAS isolates remained nonbeta-hemolytic under aerobic, 5% CO_2, and anaerobic conditions. Representative examples are shown in Fig. 1. The clinical and epidemiologic features of these isolates, including the *emm* type, the sequence type determined by multilocus sequence typing (MLST), and the type of deletion in the *sag* region, are listed in Table 1.

The *emm* types of these strains were determined based on DNA sequence homology by comparison of sequences with entries in the CDC database using the Streptococci Group A Subtyping Request Form Blast 2.0 Server (http://www.cdc.gov/ncidod/biotech/strep/strepblast.htm). DNA sequences approx-

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▽ Published ahead of print on 16 December 2009.

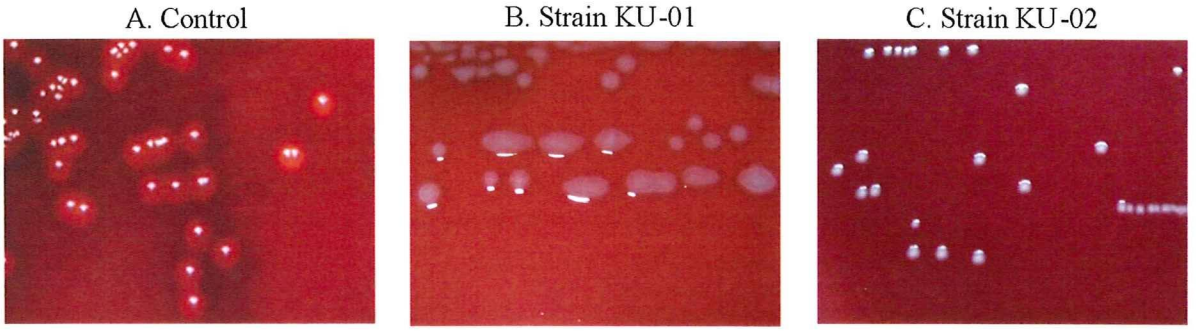

FIG. 1. Nonhemolytic *S. pyogenes* colonies grown on 5% sheep blood agar plates for 18 h at 37°C. (A) Control strain; (B and C) nonhemolytic strains.

imately 14,000 bp in length, extending from the *epf* gene, encoding an extracellular matrix binding protein, to the *sag* operon, encoding SLS, were determined for all nonhemolytic GAS strains. Primers used initially for long-DNA-fragment amplification included a sense primer, 5′-TGTGGATGCCGT TTAGAACA-3′, and a reverse primer, 5′-GAATAGCGACA CGCCTTAGC-3′.

For MLST of the nine GAS strains, DNA sequences from seven housekeeping loci were determined by the methods described by Enright et al. (4), and the sequence results were compared with the data in the *S. pyogenes* database (http://spyogenes.mlst.net/misc/info.asp).

Figure 2 depicts three types of DNA deletions in the region from the *epf* gene to the *sag* operon that were identified among the strains. Deletion type 1 was exhibited by one strain (strain KU-01; *emm1.0*) that lacked a 1,358-bp segment extending from the 3′-terminal region of the *epf* gene to the 5′-terminal region of the *sagB* gene. Six other strains (strains KU-02 to strain KU-07; *emm12.0*) with deletion type 2 lacked a 7,503-bp segment extending from the middle region of the *epf* gene to the *sagD* gene. The remaining two strains (KU-08 and KU-09; *emm1.0*) represented deletion type 3 and had discontinuous deletions in two regions: a 1,709-bp segment in the *epf* gene and another segment of 6,615 bp extending from the middle region of the *epf* gene to the *sagD* gene. All these deletions encompassed the region of the promoter and the *sagA* gene encoding the precursor of SLS.

Two types of *sag* regions have been identified using a DNA database for GAS genomes. In one, the ordinary type, the genes are aligned beginning with the *eno* gene, encoding enolase, and extending through *sagA* to *sagI*. The other type possesses both a *tnp* gene, encoding transposase, and an *epf* gene between the *eno* and the *sagA* genes. GAS strains identified as having *emm2*, *emm3*, and *emm5* represented the former type, while the *emm1*, *emm4*, *emm12*, and *emm28* strains carried the latter type. All nine strains analyzed in this study contained the latter type. Although these unique deletions suggest some associations with a transposon or insertion sequence, such details remain to be clarified.

Transcripts of the *nga* and the *slo* genes are known to be produced by read-through from the *nga* promoter (7). Although the data are not shown here, nucleotide sequences of 4,754 bp in length from the *nga* gene, including the promoter region, to the end of open reading frame of the *slo* gene from the nine strains were identified. No mutations or nucleotide deletions were detected in this region; therefore, the *slo* gene was intact in all strains.

DNA profiles of the nine nonhemolytic strains after pulsed-field gel electrophoresis (PFGE) are shown in Fig. 3. PFGE was performed with the ApaI restriction enzyme (Takara Bio, Kyoto, Japan). The DNA fragments were separated on a 1% agarose gel by using a contour-clamped homogeneous electric field mapper system (Bio-Rad, Tokyo, Japan) for 18 h at 14°C in 0.5× TBE buffer (0.05 M Tris, 0.05 M boric acid, and 1 mM EDTA [pH 8.0]) at 5.7 V/cm with pulse times of 3 to 20 s at an angle of 120° (1). Six strains with *emm12.0* isolated from patients in different regions, i.e., the Chiba and Niigata prefectures, Japan, showed similar DNA restriction patterns. Furthermore, three strains from Gunma prefecture and the Sendai

TABLE 1. Clinical and epidemiologic features of nonhemolytic *S. pyogenes* isolates

Strain no.	Date of isolation	District	Patient			*emm* type	Sequence type	Deletion type involving *sag* operon
			Age (yr)	Gender	Disease			
KU-01	Nov. 2006	Gunma	30	M	AOM	*emm1.0*	28	1
KU-02	Feb. 2008	Chiba	2	M	Pharyngitis	*emm12.0*	36	2
KU-03	Apr. 2008	Chiba	4	F	Pharyngitis	*emm12.0*	36	2
KU-04	May 2008	Chiba	5	F	Pharyngitis	*emm12.0*	36	2
KU-05	May 2008	Chiba	6	M	Pharyngitis	*emm12.0*	36	2
KU-06	May 2008	Chiba	2	F	Pharyngitis	*emm12.0*	36	2
KU-07	Nov. 2008	Niigata	4	M	Pharyngitis	*emm12.0*	36	2
KU-08	Jan. 2009	Sendai	5	F	Pharyngitis	*emm1.0*	28	3
KU-09	Jan. 2009	Sendai	5	M	Pharyngitis	*emm1.0*	28	3

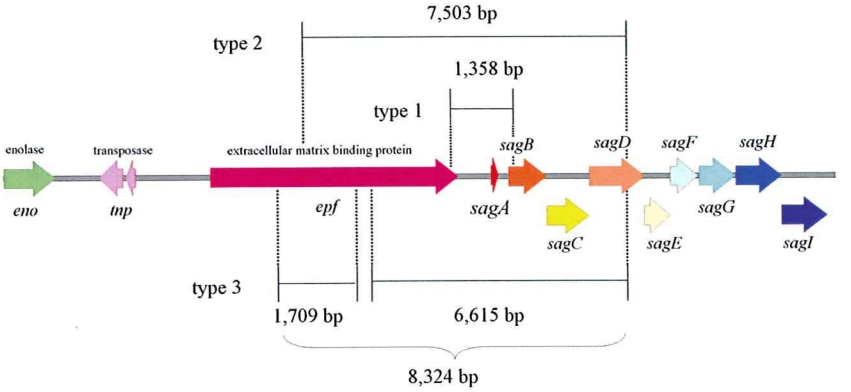

FIG. 2. Three deletion types identified near and/or in the *sag* operon encoding SLS. Large deletions encompassed the regions of the promoter and *sagA*, encoding the precursor of SLS. Deletion type 1, accession number AB518308; deletion type 2, accession number AB518309; deletion type 3, accession number AB518310.

City area identified as *emm1.0* strains showed very similar DNA restriction patterns.

Previously described nonhemolytic GAS strains have included various T antigen types and *emm* types (2, 6, 10, 11, 13, 14). In this study, we analyzed nonhemolytic phenotypes of the *emm1* and *emm12* strains. Evidence suggests that nonhemolytic *emm12* variants spread horizontally among children, considering that several cases occurred in the same area (Chiba prefecture). We also isolated three *emm1* GAS strains, one mucoid type and two nonmucoid types, from samples obtained from different areas. These strains displayed different deletion types in the *sag* operon but showed highly similar PFGE profiles, suggestive of a common origin.

Emergence of the GAS strains described herein suggests that the routine bioassay poses a risk of missing nonhemolytic GAS colonies on blood agar plates, although nonhemolytic GAS variants are considered to be rare.

Nucleotide sequence accession numbers. The deletion type sequences determined in this study have been deposited in GenBank under the following accession numbers: deletion type 1, AB518308; deletion type 2, AB518309; and deletion type 3, AB518310.

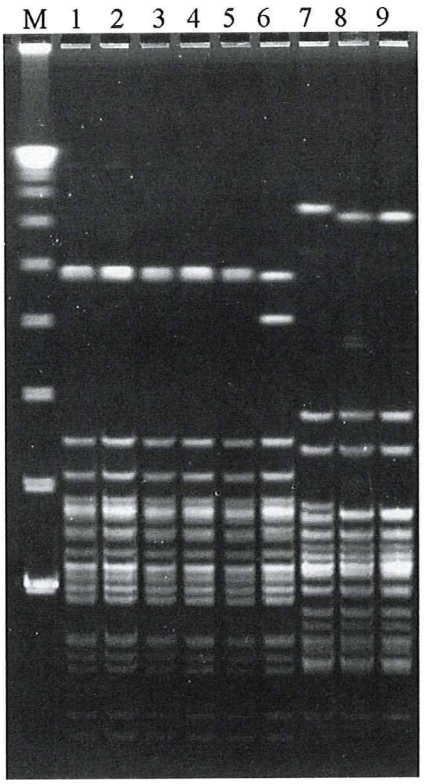

FIG. 3. PFGE patterns for nine nonhemolytic strains. Lanes: M, lambda ladder; 1, KU-02 (*emm12.0*); 2, KU-03 (*emm12.0*); 3, KU-04 (*emm12.0*); 4, KU-05 (*emm12.0*); 5, KU-06 (*emm12.0*); 6, KU-07 (*emm12.0*); 7, KU-01 (*emm1.0*); 8, KU-08 (*emm1.0*); and 9, KU-09 (*emm1.0*).

This work was supported by a grant for a Research Project for Emerging and Re-emerging Infectious Diseases (no. H-20-002) from the Japanese Ministry of Health, Labor and Welfare.

REFERENCES

1. **Chiba, N., M. Morozumi, K. Sunaoshi, S. Takahashi, M. Takano, T. Komori, K. Sunakawa, and K. Ubukata.** 2009. Serotype and antibiotic resistance of isolates from patients with invasive pneumococcal disease in Japan. Epidemiol. Infect. **138:**61–68.
2. **Cimolai, N., C. Trombley, and N. M. Bhanju.** 2002. Nonhemolytic *Streptococcus pyogenes* causing invasive infection. Clin. Pediatr. (Philadelphia) **41:** 453.
3. **Datta, V., S. M. Myskowski, L. A. Kwinn, D. N. Chiem, N. Varki, R. G. Kansal, M. Kotb, and V. Nizet.** 2005. Mutational analysis of the group A streptococcal operon encoding streptolysin S and its virulence role in invasive infection. Mol. Microbiol. **56:**681–695.
4. **Enright, M. C., B. G. Spratt, A. Kalia, J. H. Cross, and D. E. Bessen.** 2001. Multilocus sequence typing of *Streptococcus pyogenes* and the relationships between *emm* type and clone. Infect. Immun. **69:**2416–2427.
5. **Ferretti, J. J., W. M. McShan, D. Ajdic, D. J. Savic, G. Savic, K. Lyon, C. Primeaux, S. Sezate, A. N. Suvorov, S. Kenton, H. S. Lai, S. P. Lin, Y. Qian, H. G. Jia, F. Z. Najar, Q. Ren, H. Zhu, L. Song, J. White, X. Yuan, S. W. Clifton, B. A. Roe, and R. McLaughlin.** 2001. Complete genome sequence of an M1 strain of *Streptococcus pyogenes*. Proc. Natl. Acad. Sci. U. S. A. **98:**4658–4663.
6. **James, L., and R. B. McFarland.** 1971. An epidemic of pharyngitis due to a nonhemolytic group A streptococcus at Lowry air force base. N. Engl. J. Med. **284:**750–752.
7. **Kimoto, H., Y. Fujii, Y. Yokota, and A. Taketo.** 2005. Molecular characterization of NADase-streptolysin O operon of hemolytic streptococci. Biochim. Biophys. Acta **1681:**134–149.
8. **McCormick, J. K., M. L. Peterson, and P. M. Schlievert.** 2006. Toxins and superantigens of group A streptococci, p. 47–58. *In* V. A. Fischetti, R. P. Novick, J. J. Ferretti, D. A. Portnoy, and J. I. Rood (ed.), Gram-positive pathogens, 2nd ed. ASM Press, Washington, DC.
9. **Morozumi, M., E. Nakayama, S. Iwata, Y. Aoki, K. Hasegawa, R. Kobayashi, N. Chiba, T. Tajima, and K. Ubukata.** 2006. Simultaneous detection of pathogens in clinical samples from patients with community-acquired pneu-

monia by real-time PCR with pathogen-specific molecular beacon probes. J. Clin. Microbiol. **44:**1440–1446.
10. **Rubin, L. G., and G. S. Mirkin.** 2000. Apparent false positive detection of group A Streptococcus antigen resulting from pharyngeal infection with a nonhemolytic *Streptococcus pyogenes*. Pediatr. Infect. Dis. J. **19:**672–674.
11. **Sönksen, U. W., K. Ekelund, and B. G. Bruun.** 2007. Case of bacteraemic cellulitis by a non-haemolytic strain of *Streptococcus pyogenes*. Scand. J. Infect. Dis. **39:**262–264.
12. **Spellerberg, B., and C. Brandt.** 2007. Streptococcus, p. 412–429. *In* P. A. Murray, E. J. Baron, J. H. Jorgensen, M. L. Landry, and M. A. Pfaller (ed.), Manual of clinical microbiology, 9th ed. ASM Press, Washington, DC.
13. **Taylor, M. B., and T. Barkham.** 2002. Fatal case of pneumonia caused by a nonhemolytic strain of *Streptococcus pyogenes*. J. Clin. Microbiol. **40:**2311–2312.
14. **Turner, D. P., and S. L. Gunn.** 2007. Fatal case of sepsis caused by a non-haemolytic strain of *Streptococcus pyogenes*. J. Clin. Pathol. **60:**1057.

ORIGINAL ARTICLE 10.1111/j.1469-0691.2009.03047.x

Clinical aspects of invasive infections with *Streptococcus dysgalactiae* ssp. *equisimilis* in Japan: differences with respect to *Streptococcus pyogenes* and *Streptococcus agalactiae* infections

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Abstract

Streptococcus dysgalactiae ssp. *equisimilis* (SDSE) is increasingly being identified as a pathogen responsible for invasive and non-invasive infections. We compared the clinical features of invasive SDSE infections with those of invasive infections caused by *Streptococcus pyogenes* (group A streptococcus (GAS)) and *Streptococcus agalactiae* (group B streptococcus (GBS)). Active surveillance for invasive SDSE, GAS and GBS was maintained over 1 year at 142 medical institutions throughout Japan. Clinical information was collected together with isolates, which were characterized microbiologically. Two hundred and thirty-one invasive SDSE infections were identified, 97 other patients had infections with GAS, and 151 had infections with GBS. The median age of the SDSE patients was 75 years; 51% were male and 79% had underlying diseases. Forty-two SDSE patients (19%) presented to the emergency department. Among the 150 patients (65%) for whom follow-up was completed, 19 (12%) died and eight (5%) had post-infective sequelae (poor outcome). Insufficient white blood cell responses (<5000 cells/μL) and thrombocytopenia on admission each suggested significantly higher risk of poor outcome (ORs 3.6 and 4.5, respectively). Of 229 isolates, 55 (24%) showed an stG6792 *emm* type, which was significantly associated with poor outcome (OR 2.4). Clinical manifestations of invasive SDSE infections were distinct from those of invasive GBS infections. Primary-care doctors should consider invasive SDSE infections when treating elderly patients.

Keywords: Invasive infections, non-invasive infections, *Streptococcus agalactiae*, *Streptococcus pyogenes*, *Streptococcus dysgalactiae* ssp. *equisimilis*

Original Submission: 7 June 2009; **Revised Submission:** 23 August 2009; **Accepted:** 24 August 2009

Editor F. Allerberger

Clin Microbiol Infect

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Introduction

Invasive infections caused by β-haemolytic streptococci apart from Lancefield groups A and B, as well as by *Streptococcus pyogenes* (group A streptococcus (GAS) and *Streptococcus agalactiae* (group B streptococcus (GBS)), are reported increasingly worldwide [1,2]. The other streptococci include groups C, G, F and L; group G is notable because these streptococci can cause bacteraemia [3,4]. According to previous investigations [5], this group includes *Streptococcus dysgalactiae* ssp. *equisimilis* (SDSE), the *Streptococcus anginosus* group, and *Streptococcus canis*.

Recently, SDSE isolates possessing group G antigen have been recovered increasingly from severe invasive streptococcal infections [6]. Brandt *et al.* [7] characterized blood culture isolates of SDSE possessing Lancefield group A antigen. Infection with this pathogen (11 strains) was also sometimes found to lead to streptococcal toxic shock syndrome [8]. We have just completed whole genome analyses of two original isolates (GGS_124 (GenBank accession number

AP010935) and RE378) of SDSE, demonstrating a rate of genome overlap between this subspecies and GAS of 61–63%, whereas the overlap between the subspecies and GBS genomes was 15% (T. Akiyama, K. Ubukata & T. Kirikae, unpublished data).

However, active nationwide surveillance with a collection of large numbers of strains remains to be instituted, as for many years SDSE was considered to be non-pathogenic. We therefore collected isolates of this microorganism as well as of GAS and GBS, with accompanying detailed clinical information, from 142 medical institutions. Our aim was to compare clinical aspects of invasive diseases caused by SDSE with those caused by GAS or GBS in Japan.

Materials and Methods

Surveillance

Active laboratory-based surveillance for invasive SDSE, GAS or GBS infections was organized by the Laboratory of Molecular Epidemiology for Infectious Agents at the Graduate School of Infection Control Sciences, Kitasoto, Japan. SDSE included some isolates of the Lancefield C or A groups. We excluded *S. anginosus* group isolates that showed group C, G or F antigen in the process of isolate identification.

Surveillance was conducted for 1 year (1 August 2006 to 31 July 2007) in 142 medical institutions participating in the Invasive Streptococcal Disease Working Group established at the 19th Annual Meeting of the Japanese Society for Clinical Microbiology. Invasive streptococcal diseases were defined as conditions with isolation of organisms from a normally sterile site (i.e. blood, cerebrospinal fluid, joint fluid, ascites, or pleural effusion) [1,9,10]. Isolates were first identified as streptococci at local hospital laboratories, using standard commercially available latex agglutination assays. Detailed standardized categories of information regarding clinical features (e.g. hospital departments of initial presentation and underlying conditions) and laboratory findings (e.g. white blood cell (WBC) count, platelet count and C-reactive protein (CRP) serum concentration) were obtained from medical charts for all subjects enrolled. Clinical syndromes were assigned on the basis of physicians' diagnoses recorded in the medical charts. Poor outcomes were defined as either death from invasive infections within 3 weeks of onset or invasive disease-associated sequelae following the streptococcal infection (e.g. worsened paralysis or bedridden status). All isolates were sent to the Laboratory of Molecular Epidemiology for Infectious Agents to determine additional characteristics, including Lancefield group, species, M protein gene (*emm*) or capsular type, and antibiotic susceptibility.

Laboratory methods

Isolates were characterized with standard biochemical and enzymatic tests, and were identified as previously described [11]. The *emm* types of SDSE or GAS isolates [12] and the capsular types of GBS [13] isolates were determined as previously reported. All *emm* typing was based on the CDC database (ftp://ftp.cdc.gov/pub/infectious_diseases/biotech/tsemm/). In addition, we quantified the susceptibility of streptococci to 14 antibiotics, including oral and parenteral antibiotics, by agar plate dilution methods using blood agar, as previously described [10]. Depending on the MICs, the presence of streptococcal genes associated with resistance to antimicrobials (e.g. *mef*(A), *erm*(A), or *erm*(B)) was determined. To assess the similarity of isolates, profiling using pulsed-field gel electrophoresis (PFGE) following DNA treatment with the restriction enzyme *Sma*I was also performed [10].

Statistical analysis

Microsoft Excel was used for data analysis. Patient or pathogen characteristics, clinical features and outcomes were compared between paired groups of isolates (SDSE and GAS, SDSE and GBS, or GAS and GBS), using the chi-squared test. To identify clinical laboratory findings associated with fatal outcome, ORs with 95% CIs and p-values according to the chi-squared test were calculated.

Results

We identified 231 patients with invasive infection caused by SDSE in the records from 142 medical institutions during the 1-year study period, during which time 97 GAS and 151 GBS cases were also collected. All isolates of SDSE, GAS or GBS were referred by the hospital laboratories for further microbiological characterization.

As shown in Fig. 1, the age distribution differed significantly between patients with invasive SDSE and those with GBS infection. All patients (n = 231) with invasive SDSE infection were adults, whereas GBS infected some patients 4 months old or younger in addition to adults, especially the elderly. We therefore chose to compare clinical aspects of invasive SDSE diseases with those caused by GAS (n = 82) or GBS (n = 123) in the adult population (≥15 years old).

Isolates (n = 12) of the *S. anginosus* group were excluded from the current surveillance. Lancefield groups G, C and A, respectively, accounted for 216, 12 and three SDSE isolates.

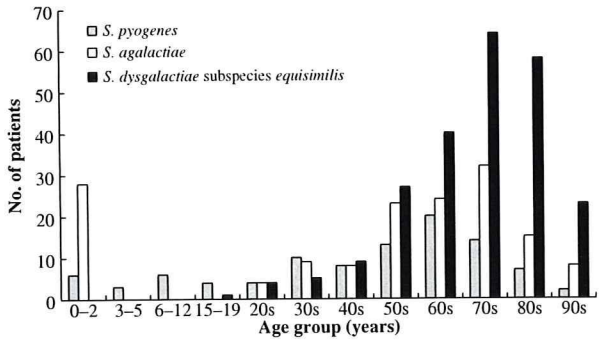

FIG. 1. Number of patients with invasive infections caused by *Streptococcus dysgalactiae* ssp. *equisimilis* (*n* = 231), *Streptococcus pyogenes* (*n* = 97), and *Streptococcus agalactiae* (*n* = 151), shown by age group.

All GAS or GBS isolates were *S. pyogenes* or *S. agalactiae*, respectively.

Patient characteristics and clinical presentations according to the streptococcal group (SDSE, GAS, or GBS)

Patient characteristics, clinical presentations and disease outcomes for all invasive SDSE, GAS and GBS infections are shown in Table 1. The median age of patients with SDSE diseases was 75 years (range, 19–103 years), with the subjects with SDSE infections being significantly older than those with GAS or GBS infections ($p < 0.01$ for each). In our surveillance, all cases of SDSE, GAS and GBS diseases were community-acquired. Forty-two patients (19%) with SDSE infections presented to the hospital emergency department,

TABLE 1. Demographic characteristics, underlying conditions and clinical syndromes in patients with invasive infection caused by *Streptococcus dysgalactiae* ssp. *equisimilis* (*n* = 231), *Streptococcus pyogenes* (*n* = 97), or *Streptococcus agalactiae* (*n* = 151)

Characteristic	No. (%) of patients			p-Value		
	S. dysgalactiae ssp. *equisimilis* (A)	*S. pyogenes* (B)	*S. agalactia* (C)	A vs. B	A vs. C	B vs. C
Demographic characteristic						
Age (years)						
<15	0 (0.0)	15 (15.5)	28 (18.5)	<0.01	<0.01	0.53
15–39	10 (4.3)	18 (22.0)	13 (10.6)	<0.01	0.02	0.03
40–64	50 (21.6)	32 (39.0)	39 (31.7)	<0.01	0.04	0.28
≥65	171 (74.0)	32 (39.0)	71 (57.7)	<0.01	<0.01	<0.01
Gender[a,b]						
Male	117 (51.3)	40 (49.4)	55 (44.7)	0.77	0.24	0.51
Department where presented[a]						
Emergency	42 (19.3)	22 (26.8)	22 (18.0)	0.15	0.78	0.13
Internal medicine	112 (51.4)	36 (43.9)	73 (59.8)	0.25	0.13	0.03
Surgery	64 (29.4)	24 (29.3)	27 (22.1)	0.99	0.15	0.25
Poor outcome[a]						
Death	19 (12.7)	12 (16.7)	8 (10.8)	0.42	0.69	0.30
Death or sequelae	27 (18.0)	19 (26.4)	11 (14.9)	0.15	0.56	0.08
Underlying disease[a]						
None	48 (21.2)	31 (39.7)	16 (11.8)	<0.01	0.07	<0.01
Diabetes mellitus	36 (15.9)	12 (15.4)	22 (16.2)	0.64	0.92	0.72
Malignant disease	35 (15.5)	13 (16.7)	33 (24.3)	0.39	0.02	0.41
Stroke	28 (12.4)	0 (0.0)	5 (3.7)	<0.01	<0.01	0.27
Heart disease	18 (8.0)	4 (5.1)	10 (7.4)	0.80	0.85	0.95
Pulmonary disease	2 (0.9)	0 (0.0)	0 (0.0)	0.93	0.72	–
Liver dysfunction	11 (4.9)	2 (2.6)	17 (12.5)	0.77	<0.01	0.03
Renal dysfunction	13 (5.8)	4 (5.1)	9 (6.6)	0.84	0.72	0.89
Artherosclerotic cardiovascular disease	8 (3.5)	1 (1.3)	11 (8.1)	0.66	0.05	0.12
Autoimmune disease	4 (1.8)	1 (1.3)	5 (3.7)	0.69	0.42	0.68
Other	23 (10.2)	10 (12.8)	8 (5.9)			
Clinical syndrome						
Sepsis without focus	98 (42.4)	27 (32.9)	77 (62.6)	0.06	<0.01	<.01
Cellulitis	52 (22.5)	23 (28.0)	12 (9.8)	0.43	<0.01	<.01
Septic arthritis	23 (10.0)	3 (3.7)	4 (3.3)	0.06	0.02	0.78
Pneumonia	12 (5.2)	6 (7.3)	8 (6.5)	0.74	0.64	0.87
Necrotizing fasciitis	9 (3.9)	5 (6.1)	1 (0.8)	0.67	0.18	0.08
Meningitis	5 (2.2)	3 (3.7)	3 (2.4)	0.79	0.82	0.97
Infectious endocarditis	4 (1.7)	0 (0.0)	4 (3.3)	0.51	0.60	0.24
Streptococcal toxic shock syndorome	2 (0.9)	3 (3.7)	0 (0.0)	0.25	0.76	0.13
Abscess (excluding skin)	2 (0.9)	8 (9.8)	3 (2.4)	<0.01	0.48	0.06
Osteomyelitis	2 (0.9)	0 (0.0)	1 (0.8)	0.94	0.59	0.85
Other	22 (9.5)[c]	4 (4.9)	10 (8.1)			

[a]Patients with unknown data were excluded.
[b]Gender, department where presented, poor outcome, underlying disease and clinical syndrome in the adult population were compared between paired groups (*S. dysgalactiae* ssp. *equisimilis* (SDSE) and group A streptococcus (GAS), SDSE and group B streptococcus (GBS), or GAS and GBS).
[c]Others included urosepsis (*n* = 13), septic spondylitis (*n* = 4), endophthalmitis (*n* = 2), peritonitis (*n* = 2), and biliary tract infection (*n* = 1).

a fraction similar to those of the subjects presenting to the emergency department with infections involving the other two groups.

Underlying diseases were present in 79% of patients with invasive SDSE illnesses; underlying medical conditions in subjects with GAS infections were significantly less frequent than in patients with SDSE or GBS infections ($p < 0.01$ for each). Among patients with SDSE infection, stroke was significantly more frequent than in patients with GAS or GBS infection ($p < 0.01$ for each), whereas in patients with GBS infection, liver dysfunction was significantly more prevalent than in those with SDSE or GAS infection ($p < 0.01$ and $p = 0.03$, respectively). With regard to clinical syndromes, sepsis without focus was significantly more frequent among GBS-infected than among SDSE-infected or GAS-infected patients ($p < 0.01$ for each), whereas cellulitis was significantly less frequent among GBS-infected patients than among SDSE-infected or GAS-infected patients ($p < 0.01$ for each). Subjects infected with SDSE presented more often with septic arthritis than those infected with GBS ($p = 0.02$), whereas patients with GAS infections more often had abscesses involving sites deeper than the skin than did patients with SDSE infection ($p < 0.01$).

Disease outcomes according to the streptococcal group (SDSE, GAS, or GBS)

Among 150 patients (65%) with invasive SDSE diseases for whom follow-up investigation was complete, 19 (13%) died, and eight (5%) had post-infection sequelae. No significant difference in frequency of poor outcome was evident among the three groups. The median time from admission to death was 3 days in subjects who died of SDSE infections, as compared with 1 and 7 days, respectively, for fatalities caused by GAS and GBS. Clinical laboratory data obtained at admission (WBC and platelet counts and CRP serum concentrations) for the three groups are shown in Table 2. In subjects with SDSE infection, a poor WBC response (<5000 cells/μL) and thrombocytopenia were associated with a significantly higher risk of poor outcome, with respective ORs of 3.6 (95% CI 1.2–11.5; $p = 0.04$) and 4.5 (95% CI 1.6–12.2; $p < 0.01$). These two findings were also significantly related to poor outcomes in patients with GAS infections, but not in adults with GBS infections. There was no association of CRP level with poor outcome in the three groups.

***emm* typing for the isolates from adults**

emm typing was carried out for 231 SDSE isolates and for 82 of the 97 *S. pyogenes* isolates; capsular typing was performed for 123 of the 151 *S. agalactiae* isolates. The incidences of *emm* types among SDSE isolates are shown in Table 3. Interestingly, *emm* type stG6792 in SDSE isolates, which was confirmed most frequently ($n = 55$, 24%), was significantly associated with poor outcome of invasive SDSE diseases in comparison with other SDSE *emm* types, with an OR of 2.4 (95% CI 1.0–5.9; p 0.04). Within the stG6792 type, most isolates showed *emm* type stG6792.3 ($n = 54$), whereas PFGE of the isolates of the stG6792.3 type revealed similar profiles. On the other hand, among GAS isolates, *emm* type 1

TABLE 2. Clinical laboratory findings associated with fatal outcome of invasive infections caused by *Streptococcus dysgalactiae* ssp. *equisimilis*, *Streptococcus pyogenes*, or *Streptococcus agalactiae*

	Median or percentage (25/75th percentiles) and (no./total)			
	Poor outcome[a]	Without poor outcome[a]	Analytical data (OR (95% CI))	p-Value
S. dysgalactiae ssp. *equisimilis*				
WBC (/μL)	11 400 (3908–15 200) (22/27)	12 600 (7850–16 050) (107/123)	3.6 (1.2–11.5)	0.04
<5000/μL	27.3% (6/22)	9.3% (10/107)		
PLT (10^4/μL)	11.1 (7.3–15.2) (21/27)	18.8 (12.3–24.5) (100/123)	4.5 (1.6–12.2)	<0.01
<13.0 × 10^4/μL	66.7% (14/21)	13.0% (31/100)		
C-reactive protein (mg/dL)	18.7 (12.3–26.8) (22/27)	4.9 (1.6–21.1) (103/123)	0.2 (0.03–1.8)	0.23
<1 mg/dL	4.5% (1/22)	17.5% (18/103)		
S. pyogenes				
WBC (/μL)	7200 (3800–19 075) (18/19)	10 300 (8000–15 015) (46/53)	4.2 (1.2–15.6)	0.04
<5000/μL	38.9% (7/18)	13.0% (6/46)		
PLT (10^4/μL)	10.1 (7.4–16.5) (18/19)	19.4 (15.6–28.0) (46/53)	7.5 (2.2–25.8)	<0.01
<13.0 × 10^4/μL	61.1% (11/18)	17.4% (8/46)		
C-reactive protein (mg/dL)	22.1 (12.7–28.0) (17/19)	15.6 (5.4–24.6) (45/53)	0.6 (0.06–6.5)	0.89
<1 mg/dL	5.9% (1/17)	8.9% (4/45)		
S. agalactiae				
WBC (/μL)	14 350 (9200–17 225) (10/11)	11 600 (7800–15 150) (47/63)	1.2 (0.1–12.6)	0.64
<5000/μL	10.0% (1/10)	8.5% (4/47)		
PLT (10^4/μL)	10.0 (7.7–18.9) (9/11)	17.9 (12.2–24.3) (46/63)	3.2 (0.7–14.1)	0.23
<13.0 × 10^4/μL	55.6% (5/9)	28.3% (13/46)		
C-reactive protein (mg/dL)	6.7 (1.6–10.0) (9/11)	8.3 (1.1–19.0) (46/63)	1.2 (0.2–6.9)	0.78
<1 mg/dL	22.2% (2/9)	19.6% (9/46)		

WBC, white blood cell count; PLT, platelet count.
[a]Patients with unknown data were excluded.

TABLE 3. Types of *emm* for 231 cases of invasive infection caused by *Streptococcus dysgalactiae* ssp. *equisimilis*

emm type	No. of isolates (*n* = 231)	Poor outcome (*n* = 27)
stG6792[a]	55 (23.8)	11
stG485	37 (16.0)	1
stG6	21 (9.1)	4
stG10	21 (9.1)	2
stG652	17 (7.4)	1
stG2078	16 (6.9)	2
stC36	15 (6.5)	1
stG245	13 (5.6)	2
stG5420	6 (2.6)	1
stG480	5 (2.2)	1
stC6979	5 (2.2)	0
stG4974	4 (1.7)	1
stG653	3 (1.3)	0
Other	11 (4.8)	0
Non-typeable	2 (0.9)	0

[a]This *emm* type includes stG6792.3 (*n* = 54) and stG6792.0 (*n* = 1).

was found most frequently (*n* = 27, 33%). This type was also significantly related to poor outcome of invasive infections, with an OR of 3.4 (95% CI 1.1–10.5; p 0.02). Other *emm* types frequently found in GAS infections were 49 (*n* = 12), 89 (*n* = 5), 11 (*n* = 4), 12 (*n* = 4), and 75 (*n* = 4), whereas *emm* types 12 and 6 predominated in our study of non-invasive GAS infections (T. Wajima, S. Y. Murayama & K. Ubukata, unpublished data).

Capsular type Ib in GBS isolates were not associated with poor outcome of invasive diseases. This type (*n* = 39) was observed most frequently in adults. Other GBS capsular types, i.e. V (*n* = 23), II (*n* = 15), III (*n* = 14), and Ia (*n* = 11), were predominantly observed, showing distribution patterns different from those found in the study of non-invasive GBS infections.

Antibiotic susceptibility

Antibiotic susceptibility testing was performed for all isolates of SDSE, GAS and GBS. Of 231 SDSE isolates, four had the *mef*(A) gene, and 13 and six isolates had *erm*(A) and *erm*(B) genes, respectively; the presence of the latter suggested a high level of resistance to clarithromycin (MIC ≥64 mg/L). In addition, two SDSE isolates showed resistance to fluoroquinolones, such as levofloxacin (MIC ≥32 mg/L), based on substitutions in GyrA (Ser81 to Phe or Tyr) and ParC (Ser79 to Tyr). No penicillin or cephalosporin resistance was observed.

Discussion

To the best of our knowledge, this is the first nationwide surveillance regarding invasive infections caused by SDSE in Japan. The study demonstrates significant differences in clinical aspects, including prognosis, between disease entities caused by SDSE, GAS and GBS. The mortality rate of invasive SDSE diseases in our study (13%) was similar to those previously described in other countries (8–18%) [1,5,14,15].

Host-protective factors, including WBCs, platelets, and CRP, affect the severity and prognosis of infectious diseases, especially those involving invasive pathogens. Disturbed haemostasis is a central finding in severe *S. pyogenes* infection that is associated with M protein-induced platelet activation and thrombus formation [16]. In particular, microthrombi are found both at the local site of infection and at distant sites. Platelets are responsible for maintaining vascular function and haemostasis. With regard to WBCs and CRP, streptococcal erythrogenic toxin B induces apoptosis and proliferation in human leukocytes [17]. Moreover, serial CRP monitoring was found to alert physicians to complications and predict outcome earlier than clinical signs or roentgenograms in 63 children with acute haematogenous osteomyelitis [18]. In our investigations, a poor WBC response (<5000 cells/μL) and thrombocytopenia at admission each showed a significantly higher risk of poor outcome (death or invasive disease-associated sequelae) in patients with invasive SDSE or GAS infections. However, CRP could not predict poor outcomes. We need to continue to examine associations between prognosis and host defence factors.

The M protein of *S. pyogenes* is a major bacterial virulence factor that confers resistance to phagocytosis [19]. Recently, analysis of the *emm* gene, which codes for the amino acid sequence (variable region) at the N-terminal end of the M protein, has often been used for molecular epidemiological studies regarding outbreaks of invasive or non-invasive streptococcal infections [20,21]. Data suggesting horizontal gene transfer and recombination between the *emm* genes of SDSE and GAS strains have been observed in clinical isolates from seven subjects [21]. These genetic transfer and recombination events might have a role in pathogenesis. In our study, *emm* type stG6792 in SDSE isolates (*n* = 55), the type most frequently confirmed in SDSE infection, was significantly more often associated with poor outcome of invasive diseases than other SDSE *emm* types. In contrast to the high frequency in our study, only three strains of stG6792 were observed in a recent article from the USA [1]. Surveillance periods for strains differed between Japan (2006–2007) and the USA (2002–2004). On the basis of the CDC database concerning *emm* type sequences, the stG6792.3 reference strain appeared to be derived from a streptococcal isolate from India, suggesting that this strain might have spread from India to Japan. Similarly, the *emm1* type in GAS isolates (*n* = 27), the type that we found most frequently, was significantly related to poor outcome of invasive GAS infections.

Relationships between *emm* types and prognosis of the SDSE and GAS infections in Japan should be investigated in an ongoing manner. In addition, we need to determine person-to-person transmission routes of SDSE, as the stG6792.3 strain (n = 54) was observed most frequently within the stG6792 type, and similar *Sma*I digestion patterns were found for the isolates using PFGE analysis.

Antibiotic susceptibility testing and resistance gene identification for SDSE isolates in our study revealed clarithromycin and levofloxacin resistance, as described by others [1,2,5]. On the other hand, susceptibility to β-lactam antibiotics, including penicillin and cephalosporins, was high, suggesting that they should be the usual drugs of choice.

Some limitations of our investigation should be noted. Broyles *et al.* [1] recently reported an annual incidence of invasive β-haemolytic streptococcal infections involving groups other than A and B in the USA of 3.17 cases per 100 000 persons. Our surveillance, on the other hand, was not a population-based study of the burden of infections caused by SDSE, GAS and GBS, as official, systemic surveillance has not yet been established in Japan. To our knowledge, however, our study is the largest record of cases of invasive illness caused by SDSE, GAS and GBS in Japan to date.

In our investigation, clinical aspects of invasive SDSE infections appear to differ from those caused by GBS, and to be somewhat more similar to those caused by GAS. These observations might be accounted for by our findings that genes encoding virulence factors (e.g. M protein) in SDSE could be partially shared with those in GAS, on the basis of results of whole genome analyses of original SDSE isolates. Moreover, clinical isolates of SDSE possessing group A antigen have been reported [7]. SDSE has been established as a possible component of the normal flora of the skin, oropharynx, and gastrointestinal and genitourinary tracts. We identified SDSE in respiratory tract specimens from patients with non-invasive SDSE diseases in another study (K. Sunaoshi, S. Y. Murayama & K. Ubukata, unpublished data). However, questions persist as to how SDSE invades deep tissues and vessels. Further investigations to clarify this issue are needed.

In conclusion, primary-care doctors, particularly those treating patients in emergency departments, should consider invasive diseases caused by SDSE, especially when treating elderly subjects with underlying medical conditions.

Acknowledgements

This study, aiming to clarify clinical aspects in invasive streptococcal infections and to determine the molecular epidemiology, was presented in part at a workshop at the 19th Annual Meeting of the Japanese Society for Clinical Microbiology in January 2008 (Edogawa-ku, Tokyo, Japan). We thank A. Ono, N. Chiba and K. Okada for assistance with manuscript preparation. We also express our thanks to staff members at all participating institutions.

Transparency Declaration

This work was supported in part by a grant under the category, 'Research Project for Emerging and Re-emerging Infectious Diseases' (H-19-002), from the Ministry of Health, Labour and Welfare of Japan (to K. Ubukata). None of the authors is aware of any relationship or any degree of conflicting or dual interest, financial or of any other nature, that may affect professional judgement in relation to the article.

References

1. Broyles LN, Van Beneden C, Beall B *et al.* Population-based study of invasive disease due to β-hemolytic streptococci of groups other than A and B. *Clin Infect Dis* 2009; 48: 706–712.
2. Liao CH, Liu LC, Huang YT, Teng LJ, Hsueh PR. Bacteremia caused by group G streptococci, Taiwan. *Emerg Infect Dis* 2008; 14: 837–840.
3. Cohen-Poradosu R, Jaffe J, Lavi D *et al.* Group G streptococcal bacteremia in Jerusalem. *Emerg Infect Dis* 2004; 10: 1455–1460.
4. Sylvetsky N, Raveh D, Schlesinger Y, Rudensky B, Yinnon AM. Bacteremia due to beta-hemolytic Streptococcus group G: increasing incidence and clinical characteristics of patients. *Am J Med* 2002; 112: 622–626.
5. Woo PC, Fung AM, Lau SK, Wong SS, Yuen KY. Group G beta-hemolytic streptococcal bacteremia characterized by 16S ribosomal RNA gene sequencing. *J Clin Microbiol* 2001; 39: 3147–3155.
6. Brandt CM, Spellerberg B. Human infections due to *Streptococcus dysgalactiae* subspecies *equisimilis*. *Clin Infect Dis* 2009; 49: 766–772.
7. Brandt CM, Haase G, Schnitzler N, Zbinden R, Lütticken R. Characterization of blood culture isolates of *Streptococcus dysgalactiae* subsp. *equisimilis* possessing Lancefield's group A antigen. *J Clin Microbiol* 1999; 37: 4194–4197.
8. Hashikawa S, Iinuma Y, Furushita M *et al.* Characterization of group C and G streptococcal strains that cause streptococcal toxic shock syndrome. *J Clin Microbiol* 2004; 42: 186–192.
9. Phares CR, Lynfield R, Farley MM *et al.* Epidemiology of invasive group B streptococcal disease in the United States, 1999–2005. *JAMA* 2008; 299: 2056–2065.
10. Murayama SY, Seki C, Sakata H *et al.* Capsular type and antibiotic resistance in *Streptococcus agalactiae* isolates from patients with invasive infections, ranging from newborns to the elderly. *Antimicrob Agents Chemother* 2009; 53: 2650–2653.
11. Facklam R. What happened to the streptococci: overview of taxonomic and nomenclature changes. *Clin Microbiol Rev* 2002; 15: 613–630.
12. Li Z, Sakota V, Jackson D *et al.* Array of M protein gene subtypes in 1064 recent invasive group A streptococcus isolates recovered from

the active bacterial core surveillance. *J Infect Dis* 2003; 188: 1587–1592.
13. Poyart C, Tazi A, Réglier-Poupet H *et al.* Multiplex PCR assay for rapid and accurate capsular typing of group B streptococci. *J Clin Microbiol* 2007; 45: 1985–1988.
14. Skogberg K, Simonen H, Renkonen OV, Valtonen VV. Beta-haemolytic group A, B, C and G streptococcal septicaemia: a clinical study. *Scand J Infect Dis* 1988; 20: 119–125.
15. Ekelund K, Skinhøj P, Madsen J, Konradsen HB. Invasive group A, B, C and G streptococcal infections in Denmark 1999–2002: epidemiological and clinical aspects. *Clin Microbiol Infect* 2005; 11: 569–576.
16. Shannon O, Hertzén E, Norrby-Teglund A, Mörgelin M, Sjöbring U, Björck L. Severe streptococcal infection is associated with M protein-induced platelet activation and thrombus formation. *Mol Microbiol* 2007; 65: 1147–1157.
17. Viera NT, Romero MJ, Montero MK, Rincon J, Mosquera JA. Streptococcal erythrogenic toxin B induces apoptosis and proliferation in human leukocytes. *Kidney Int* 2001; 59: 950–958.
18. Roine I, Faingezicht I, Arguedas A, Herrera JF, Rodríguez F. Serial serum C-reactive protein to monitor recovery from acute hematogenous osteomyelitis in children. *Pediatr Infect Dis J* 1995; 14: 40–44.
19. Carlsson F, Berggård K, Stålhammar-Carlemalm M, Lindahl G. Evasion of phagocytosis through cooperation between two ligand-binding regions in *Streptococcus pyogenes* M protein. *J Exp Med* 2003; 198: 1057–1068.
20. Kittang BR, Langeland N, Mylvaganam H. Distribution of *emm* types and subtypes among noninvasive group A, C and G streptococcal isolates in western Norway. *APMIS* 2008; 116: 457–464.
21. Mylvaganam H, Bruun T, Vindenes HA, Langeland N, Skrede S. Molecular epidemiological investigation of an outbreak of invasive β-haemolytic streptococcal infection in western Norway. *Clin Microbiol Infect* 2009; 15: 245–252.

J Infect Chemother
DOI 10.1007/s10156-009-0016-1

NOTE

Clinical aspects of invasive infection with *Streptococcus dysgalactiae* subsp. *equisimilis* in elderly patients

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Received: 4 September 2009 / Accepted: 21 October 2009

Abstract The number of patients with severe invasive infections (mainly exhibiting bacteremia) with *Streptococcus dysgalactiae* subsp. *equisimilis* (SDSE) has been increasing worldwide. We herein report the clinical aspects of invasive infections (cellulitis, pneumonia, and urosepsis) occurring with SDSE in 13 elderly patients (mean age 84 years, range 69–99 years) diagnosed at a hospital for elderly individuals during the period January 2005–June 2009. Ten subjects had underlying diseases, including neurologic disorders, diabetes mellitus, and others. Eleven patients presented to the hospital emergency department, and the most common symptom was high fever or respiratory distress. Primary care and emergency department doctors treating elderly patients with high fever should keep in mind invasive SDSE infection as a differential diagnosis, especially when an elderly person has underlying illnesses. To detect SDSE in elderly subjects, blood cultures should be obtained before the administration of antimicrobials because, as we found, the patients' symptoms were limited.

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Published online: 06 January 2010

Keywords *Streptococcus dysgalactiae* subsp. *equisimilis* · Cellulitis · Pneumonia · Urosepsis

Streptococcus dysgalactiae subsp. *equisimilis* (SDSE) was proposed in 1996 as a new taxon involved in human streptococcal infections [1]. This microorganism has Lancefield group C or G antigen, exhibits strong β-hemolysis, and shows streptokinase activity in human plasminogen and proteolytic activity in human fibrin. We have just completed whole-genome analyses of the original two isolates [GGS_124 (GenBank accession no AP010935) and RE378] of SDSE, demonstrating a rate of overlap between this subspecies and group A streptococcal genomes of 61–63%; overlap between the subspecies and group B streptococcal genomes was 15%. Similarly to group A streptococci, SDSE possesses virulence factors such as M protein, streptolysin O, streptolysin S, streptokinase, hyaluronidase, and C5a peptidase. SDSE has been established as possible normal flora of the skin, oropharynx, and gastrointestinal and genitourinary tracts. This pathogen was identified in respiratory tract specimens from patients with noninvasive SDSE diseases [2]. Invasive SDSE infections, mainly exhibiting bacteremia, are now being observed increasingly worldwide [3, 4]. Invasive infections represent the isolation of SDSE from a normally sterile site (i.e., blood, cerebrospinal fluid, joint fluid, ascites, or pleural effusion) [3, 4]. Here we report clinical aspects of invasive infections caused by SDSE in 13 elderly patients diagnosed at a hospital for elderly individuals between January 2005 and June 2009.

Streptococcal isolates with Lancefield group C or G antigen and β-hemolysis were isolated from blood specimens, and detailed information was collected from the hospital clinical laboratory database. SDSE was speciated

based on results of biologic tests, including bacitracin resistance, pyrrolidonyl-arylamidase test (negative), Voges–Proskauer test (negative), and β-D-glucuronidase test (positive). In addition, the *emm* gene, which encodes M protein—a major bacterial virulence factor—was typed, as previously described [4, 5]. To assess the similarity of isolates, DNA profiles after digestion with the restriction enzyme *Sma*I were compared by pulsed-field gel electrophoresis (PFGE) [6]. Patients' clinical features were reviewed using medical records. Clinical data (age, underlying disease, onset situation, main symptom, and vital signs) and laboratory data [white blood cell count (WBC), neutrophil count, C-reactive protein (CRP), platelet count (Plt), hemoglobin (Hb), and others] were obtained at the time blood cultures were performed for each patient. Also, the diagnosis of invasive infection was reviewed by a physician expert in infectious diseases.

Thirteen strains of SDSE and the corresponding elderly patients were studied. The clinical features and outcomes for invasive SDSE infections are shown with microbiologic data in Table 1. Twelve isolates had Lancefield group G antigen. The *emm* genotypes (*stG10.0* and others) and *Sma*I-digested PFGE DNA profiles for 7 isolates (numbers 7–13) varied between strains, suggesting the possible invasion of a different colonized strain in each patient rather than the clonal expansion of a specific subpopulation of isolates. The mean age of the infected patients was 84 years (range 69–99 years); 7 were female; and 10 had underlying diseases including neurologic disorders, diabetes mellitus, and others. Eleven patients with SDSE infections presented to the hospital emergency department, indicating that the infection was mainly community-acquired. The most common symptom was high fever or respiratory distress, without other complaints. Mean body temperature and heart rate were 38.9°C (range 36.4–40.1°C) and 93 beats/min (range 72–120 beats/min). Cellulitis, pneumonia, and urosepsis ($n = 4$ for each) constituted the main invasive infections. In subjects with pneumonia, chest roentgenograms disclosed patchy infiltrates. Mean WBC, neutrophil count, CRP, Plt, and Hb were 12.2×10^9 cells/l (range $5.7–19.2 \times 10^9$ cells/l), 10.9×10^9 cells/l (range $4.6–15.6 \times 10^9$ cells/l), 6.27 mg/dl (range 0.35–28.34 mg/dl), 210×10^9 cells/l (range $59–510 \times 10^9$ cells/l), and 11.2 g/dl (range 7.4–13.4 g/dl), respectively. As treatment, β-lactam antibiotics including penicillins or cephalosporins were administered parenterally in all subjects. No patients died of an invasive infection; any deaths involved other causes. No recurrent SDSE infections were found. The clinical aspects of the invasive SDSE infections in the elderly subjects were considered to be characterized as follows; onset in older-age patients, presence of underlying illnesses, community-acquired onset, and limited patients' symptoms.

In a recent report from Finland considering non-necrotizing bacterial cellulitis, SDSE was observed most often, being isolated in 22% of cultures from either skin lesions or blood [7]. Also, pneumonia and thoracic empyema caused by SDSE were reported from Japan [8, 9]. Similar to the findings of previous reports [8], chest radiographs in our patients with pneumonia indicated patchy infiltrates. The pulmonary lesions may have been induced by the aspiration of SDSE as possible normal flora of the oropharynx, because the functions of both swallowing and coughing are reduced in the elderly. The mortality of invasive group G streptococcal infections was reported previously as 8–18% [1, 10, 11]. Active laboratory-based surveillance for invasive SDSE, *Streptococcus pyogenes* (GAS), and *S. agalactiae* (GBS) infections was conducted for 1 year (August 2006–July 2007) in 142 medical institutions participating in the Invasive Streptococcal Disease Working Group established at the 19th annual meeting of the Japanese Society for Clinical Microbiology, in order to compare the clinical aspects of invasive infections caused by the three species [12]. While 231 invasive SDSE infections were identified, 97 other patients had GAS and 151 had GBS. All patients with invasive SDSE infection were adults (median age 75 years), while GBS infected some patients 4 months old or younger in addition to adults. Underlying diseases were present in 78.8% of the patients with invasive SDSE illnesses; underlying medical conditions were less frequent in subjects with GAS infections than in patients with SDSE or GBS. In addition, all cases of SDSE, GAS, or GBS diseases were community-acquired and 42 patients (18%) with SDSE infections presented to the hospital emergency department, a fraction similar to the proportions of subjects presenting to the emergency department with infections involving the other two bacterial groups.

There are some limitations regarding the clinical features of the SDSE invasive infections reported in the elderly subjects in our study. There may have been a selection bias for the enrolled patients, because our institute is already established as one of the expert hospitals for elderly individuals. In order to clarify risk factors in the subjects susceptible to SDSE, we did not design a setting for control patients, who were frequency matched to the case subjects by age and gender.

In conclusion, based on the detailed information of invasive SDSE infection in our observations, primary care and emergency department doctors treating elderly patients with high fever should keep in mind invasive infections caused by SDSE as a differential diagnosis, especially when the elderly person has underlying illnesses, although we note that no characteristic features giving clues to the diagnosis were found in the present study. To detect this

Table 1 Clinical features and outcomes of invasive infections with *Streptococcus dysgalactiae* subsp. *equisimilis* in 13 elderly patients

Patient no.	Blood culture date	Lancefield group	*emm* Type	Age	Gender	Underlying disease	I or O	Main symptom	Body temperature (°C)
1	2005/5/1	G	*stG652.0*	76	F	Breast cancer	O	Respiratory distress	36.4
2	2005/7/12	G	*stG2078.0*	74	M	Diabetes mellitus	I	Fever	39
3	2006/3/31	C	*stC6979.0*	83	F	Angina pectoris	O	Fever	38.7
4	2006/5/11	G	*stG10.0*	83	M	Stroke	O	Fever	39.2
5	2006/9/18	G	*stG652.1*	69	M	Frontotemporal dementia	O	Fever	39.7
6	2007/7/4	G	ND	87	F	Diabetes mellitus	O	Fever	38.8
7	2007/8/16	G	*stG10.0*	91	M	Healthy	O	Fever	38.5
8	2007/8/21	G	*stG10.0*	99	F	Healthy	O	Fever	38.2
9	2007/10/2	G	*stG6792.4*	90	M	Hypothyroidism	O	Fever	40.1
10	2007/10/9	G	*stG245.0*	81	F	Angina pectoris	O	Fever	40.1
11	2007/10/13	G	*stG652.0*	91	F	Healthy	I	Respiratory distress	39.9
12	2008/11/30	G	*stC46.0*	83	M	Pulmonary emphysema	O	Respiratory distress	37.2
13	2009/3/4	G	*stG2078.0*	85	F	Parkinson disease	O	Fever	39.8

Patient no.	Heart rate (beats/min)	Invasive infection	WBC (neutrophil count $\times 10^9$ cells/l)	CRP (mg/dl)	Plt ($\times 10^9$ cells/l)	Hb (g/dl)	BUN/Cr/CK (mg/dl, mg/dl, IU/l)	Antibiotic	Outcome
1	104	Urosepsis	12.9 (ND)	28.34	510	12.7	71.8/2.6/777	IPM/CS	Died of cancer
2	98	Urosepsis	5.7 (4.6)	8.84	183	10.7	10/0.5/ND	PIPC	Died of aspiration
3	98	Cellulitis	8.1 (8)	0.35[a]	148	12.3	25.3/1/111	CMZ	Recovered
4	105	Septic arthritis	14.3 (13.7)	10.48	209	11.4	15.3/0.9/198	PIPC/TAZ	Died of heart failure
5	77	Urosepsis	6.8 (6.3)	2.05	231	12.6	12/0.5/96	ABPC	Recovered
6	90	Urosepsis	14.9 (13.7)	7.31	289	12.3	17.4/0.6/13	CTM	Recovered
7	72	Cellulitis	13.7 (12.2)	1.59	168	11.4	21.7/0.8/25	CEZ	Recovered
8	96	Pneumonia	13.9 (12.6)	0.49[a]	104	9.6	35.4/0.8/79	PIPC	Died of stroke
9	91	Pneumonia	11.3 (9.8)	1.82	163	7.4	35.1/1.2/133	ABPC/SBT	Recovered
10	88	Cellulitis	17.4 (15.2)	2.33	216	13.4	17.4/0.7/104	PIPC	Recovered
11	120	Pneumonia	10.4 (9.5)	4.42	149	8.8	19.9/0.5/98	CZOP	Recovered
12	88	Pneumonia	19.2 (15.6)	0.37[a]	59	13.4	15.5/0.9/77	PIPC	Recovered
13	77	Cellulitis	10.3 (9.9)	13.09	299	10	32.5/1/667	ABPC/SBT	Recovered

Clinical and laboratory findings were recorded when blood cultures were performed

I or O Inpatient or outpatient, *WBC* white blood cell count, *CRP* C-reactive protein, *Plt* platelet count, *Hb* hemoglobin, *BUN* blood urea nitrogen, *Cr* creatinine, *CK* creatine phosphokinase, *ND* not determined, *IPM/CS* imipenem/cilastatin, *PIPC* piperacillin, *CMZ* cefmetazole, *TAZ* tazobactam, *ABPC* ampicillin, *CTM* cefotiam, *CEZ* cefazolin, *SBT* sulbactam, *CZOP* cefozopran

[a] CRP was increased later, 1 or 2 days after blood cultures were obtained

pathogen in elderly subjects, blood cultures should be obtained before the administration of antimicrobials because, as we found, the patients' symptoms were limited to high fever or respiratory distress.

Acknowledgments This study was funded in part by a grant under the category, ''Research Project for Emerging and Re-emerging Infectious Diseases'' (H-19-002), from the Japanese Ministry of Health, Labour and Welfare (to Dr. K. Ubukata) and a Grant-in-Aid for Scientific Research (B) from the Ministry of Education, Culture, Sports, Science and Technology of Japan (number 21390306, to Dr. T. Takahashi). The authors thank Akiko Ono, Keisuke Okada, and Miho Yoshino for assistance with the manuscript preparation.

References

1. Vandamme P, Pot B, Falsen E, Kersters K, Devriese LA. Taxonomic study of Lancefield streptococcal groups C, G, and L (*Streptococcus dysgalactiae*) and proposal of *S. dysgalactiae subsp. equisimilis* subsp. nov. Int J Syst Bacteriol. 1996; 46:774–81.
2. Sunaoshi K, Murayama SY, Adachi K, Yagoshi M, Okuzumi K, Chiba N, et al. Molecular *emm* genotyping and antibiotic susceptibility of *Streptococcus dysgalactiae* subsp. *equisimilis* isolated from invasive and noninvasive infections. J Med Microbiol. 2010;59:82–8. doi:10.1099/jmm.0.013201-0.
3. Ikebe T, Murayama S, Saitoh K, Yamai S, Suzuki R, Isobe J, et al. Surveillance of severe invasive group-G streptococcal

infections and molecular typing of the isolates in Japan. Epidemiol Infect. 2004;132:145–9.
4. Broyles LN, Van Beneden C, Beall B, Facklam R, Shewmaker PL, Malpiedi P, et al. Population-based study of invasive disease due to β-hemolytic streptococci of groups other than A and B. Clin Infect Dis. 2009;48:706–12.
5. Sunaoshi K, Aburahashi H, Kobayashi R, Yamamoto Y, Okuzumi K, Yoshida A, et al. *Emm* typing by genetic identification of *Streptococcus dysgalactiae* subsp. equisimilis and susceptibility to oral antibiotics. Kansenshogaku Zasshi. 2006;80:488–95.
6. Murayama SY, Seki C, Sakata H, Sunaoshi K, Nakayama E, Iwata S, et al. Capsular type and antibiotic resistance in *Streptococcus agalactiae* isolates from patients with invasive infections, ranging from newborns to the elderly. Antimicrob Agents Chemother. 2009;53:2650–3.
7. Siljander T, Karppelin M, Vähäkuopus S, Syrjänen J, Toropainen M, Kere J, et al. Acute bacterial, nonnecrotizing cellulitis in Finland: microbiological findings. Clin Infect Dis. 2008; 46:855–61.
8. Matsui D, Kitasato Y, Honda S, Ueno K, Tanaka A, Edakuni N, et al. A case of bacterial pneumonia caused by *Streptococcus dysgalactiae* subsp. *equisimilis*, showing patchy consolidations resembling organizing pneumonia. Nihon Kokyuki Gakkai Zasshi. 2007;45:36–42.
9. Ueno K, Kawayama T, Edakuni N, Koga T, Aizawa H. A case of thoracic empyema with gas formation associated with *Streptococcus dysgalactiae* subsp. *equisimilis*. Kansenshogaku Zasshi. 2006;80:527–30.
10. Skogberg K, Simonen H, Renkonen OV, Valtonen VV. Beta-haemolytic group A, B, C and G streptococcal septicaemia: a clinical study. Scand J Infect Dis. 1988;20:119–25.
11. Ekelund K, Skinhøj P, Madsen J, Konradsen HB. Invasive group A, B, C and G streptococcal infections in Denmark 1999–2002: epidemiological and clinical aspects. Clin Microbiol Infect. 2005;11:569–76.
12. Takahashi T, Sunaoshi K, Sunakawa K, Fujishima S, Watanabe H, Ubukata K. Clinical aspects of invasive infections with *Streptococcus dysgalactiae* ssp. *equisimilis* in Japan: differences with respect to *Streptococcus pyogenes* and *Streptococcus agalactiae* infections. Clin Microbiol Infect. doi:10.1111/j.1469-0691.2009.03047.x.

Epidemiol. Infect., Page 1 of 8. © Cambridge University Press 2009
doi:10.1017/S0950268809990239 Printed in the United Kingdom

Serotype and antibiotic resistance of isolates from patients with invasive pneumococcal disease in Japan

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(*Accepted 25 May 2009*)

SUMMARY

Invasive pneumococcal disease (IPD) is of concern in Japan, where the heptavalent pneumococcal conjugate vaccine (PCV7) is unavailable. We determined serotypes, genotypes indicating β-lactam resistance, and antibiotic susceptibilities of 496 isolates from normally sterile sites in patients (193 children, 303 adults) from 186 institutions between August 2006 and July 2007. Disease presentations included sepsis (46·2%), pneumonia (31·5%), and meningitis (17·5%). Mortality was 1·4% in children and 22·1% in adults, many of whom had underlying diseases. In children, serotype 6B (22·5%) was followed by 19F (14·1%), and 14 (13·1%); potential coverages of PCV7 and PCV13 were 75·4% and 93·7%, respectively. In adults, serotype 12F (14·3%) was followed by 3 (11·3%), and 6B (10·3%); 23-valent polysaccharide vaccine (PPV23) coverage was 85·4%. Most serotype 12F strains were gPISP, with *pbp2b* gene alteration; carbapenem had an excellent MIC_{90}. PCV7 is recommended for children and PPV23 for adults to increase prevention against IPD.

Key words: Antibiotic resistance, molecular epidemiology, *Streptococcus pneumoniae* (pneumococcus), surveillance, vaccines.

INTRODUCTION

Streptococcus pneumoniae is a leading cause of invasive infections such as lobar pneumonia, septicaemia, and meningitis, which are major contributors to morbidity and mortality in children and adults. Since the discovery of pneumococcal strains resistant to penicillin G (PEN) [1], these strains have spread rapidly worldwide [2, 3] and have been the subject of several epidemiological surveillance studies of capsule serotype distribution and antibiotic susceptibility in many countries [4–8].

In Japan, the prevalence of PEN-resistant *S. pneumoniae* (PRSP) and PEN-intermediate *S. pneumoniae* (PISP) in clinical isolates has increased rapidly since the late 1990s, especially in younger children [9, 10]. Characteristically, PRSP strains show simultaneous

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This study was presented as one of the workshops at the 19th Annual Meeting of the Japanese Society for Clinical Microbiology, 26 January 2008.

resistance to cephalosporin antibiotics used in ambulatory practice [9]. In PRSP and PISP, genotypic abnormalities in three penicillin-binding protein (PBP) genes, *pbp1a*, *pbp2x*, and *pbp2b*, which encode the PBP1A, PBP2X, and PBP2B enzymes, respectively, have been identified by polymerase chain reaction (PCR) using primers to detect mutations in these genes [9, 11]. The prevalence of PRSP possessing the three abnormal *pbp* genes currently exceeds 50% in Japan [12].

Given this background, therapeutic choices for patients with invasive pneumococcal disease (IPD) in Japan have been gradually eroded. A carbapenem antibiotic such as panipenem (PAM), which has been used only in Japan, Korea, and China, was administered in preference to intravenous third-generation cephalosporins such as cefotaxime (CTX) and ceftriaxone (CRO). Additionally, rapid increases in numbers of adults and elderly persons with various underlying diseases, is thought to increase the threat of IPD.

A heptavalent pneumococcal conjugate vaccine (PCV7) for children has been introduced in many countries [13], beginning with the USA [14]. This vaccine has been reported to contribute to a decrease in IPD when causative strains are covered [15–18]. In contrast, IPD caused by non-PCV7 serotypes of *S. pneumoniae*, such as 19A, continues to increase [19–21]. As a result, a second-generation pneumococcal conjugate vaccine such as PCV13 is now being developed to cover a wider range of serotypes.

We therefore focused on understanding the serotype distribution and antibiotic susceptibility of isolates from IPD in children and adults throughout Japan, where clinical trials of PCV7 for children have been concluded and approval is expected. Here we describe the serotype distribution and antibiotic susceptibility of the isolates according to their *pbp* genotype by PCR. We also extrapolate from the data the expected PCV7 and PCV13 coverage rates for children and those of PPV23 and PCV13 for adults.

MATERIALS AND METHODS

We examined 496 *S. pneumoniae* isolates from patients with IPD [22]. Isolates were cultured from clinical samples processed in the laboratories of 186 medical institutions from August 2006 to July 2007 throughout Japan and then sent to our laboratory with an anonymous application form written by the reporting doctor. All isolates were from normally sterile samples such as cerebrospinal fluid (CSF), blood, or pleural or joint fluid.

Haematological tests in IPD patients

To statistically determine risk factors in adults, we requested an anonymous report including patient's age, disease presentation, underlying disease, white blood cell count (WBC), C-reactive protein (CRP), and platelet count (PLT); and outcome, including presence or absence of neurological sequelae.

Serotype and antimicrobial susceptibility

Serotypes of all *S. pneumoniae* isolates were determined by the capsule swelling reaction using antiserum purchased from the Statens Serum Institute (Denmark) [23]. Minimal inhibitory concentrations (MICs) of penicillin (PEN), ampicillin (AMP), cefotaxime (CTX), meropenem (MEM) and vancomycin (VAN) were determined by agar dilution methods using Muller–Hinton II agar (MH; Becton Dickinson, USA) supplemented with 5% defibrinated sheep blood [24]. *S. pneumoniae* ATCC49619 was used as a quality control strain.

Genotypic identification of resistance by PCR

To confirm that isolates were *S. pneumoniae*, the *lytA* gene encoding the autolysin enzyme specific to *S. pneumoniae* [25] was amplified simultaneously with the three PBP genes. Each primer set used for detection of the three PBP genes was designed to amplify a part of the normal *pbp1a*, *pbp2x*, and *pbp2b* genes detected only in susceptible strains [9]. Portions of each gene corresponding to the primers were positioned in blocks of highly divergent sequences within or near conserved amino-acid motifs. Each reaction tube for PCR contained two primer sets, for detecting *lytA* and *pbp1a* in tube A; *pbp2x* and *pbp2b* in tube B; and *mef*(A) and *erm*(B) in tube C. These tubes contained 30 μl reaction mixture as previously described [9, 22, 26].

One colony was chosen from sheep blood agar and suspended in 30 μl lysis solution [11]. The tube then was placed in a thermal cycler (Gene Amp PCR System 9600R; PerkinElmer Cetus, USA) and heat-treated for 10 min at 60 °C and for 5 min at 94 °C to obtain template DNA. Next, 2 μl template DNA was added to each of the three tubes marked A, B, and C containing 30 μl reaction mixture. PCR cycling