Molecular emm genotyping and antibiotic susceptibility of *Streptococcus dysgalactiae* subsp. *equisimilis* isolated from invasive and non-invasive infections

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To analyse the characteristics of infections caused by *Streptococcus dysgalactiae* subsp. *equisimilis*, clinical isolates (n=145) were collected at 11 medical institutions between September 2003 and October 2005. These isolates belonged to Lancefield group A (n=5), group C (n=18) or group G (n=122). Among all isolates, 42 strains were isolated from sterile samples such as blood, synovial fluid and tissue specimens from patients who were mostly over 50 years with invasive infections, and included seven cases of streptococcal toxic shock syndrome and necrotizing fasciitis. In contrast, the remaining 103 were isolated mainly from patients of all age groups with non-invasive infections such as pharyngotonsillitis. These isolates were classified into 25 types based on emm genotyping. A significant difference in emm types was observed between isolates from invasive and non-invasive infections (P<0.001): stG485, stG6792 and stG2078 predominated among isolates from invasive infections. A phylogenetic tree of complete open reading frames of emm genes in this organism showed high homology with those of *Streptococcus pyogenes*, but not with those of other streptococci. The presence of five different clones was estimated based on DNA profiles of isolates from invasive infections obtained by PFGE. Genes for resistance to macrolides [erm(A), three isolates; erm(B), five isolates; mef(A), seven isolates] and levofloxacin (mutations in gyrA and parC, four isolates) were identified in this organism. These results suggest the need for further nationwide surveillance of invasive infections caused by *S. dysgalactiae* subsp. *equisimilis*.

INTRODUCTION

Most β-haemolytic streptococcal pathogens isolated from humans are identified as *Streptococcus pyogenes* (group A streptococci; GAS), *Streptococcus agalactiae* (group B streptococci; GBS), *Streptococcus dysgalactiae* subsp. *equisimilis* and species of the anginosus group that belong to Lancefield groups A, B, C, G or F (Facklam, 2002; Ruoff et al., 2003). In contrast to GAS and GBS, which are known to cause serious and systemic invasive infections, streptococci with Lancefield group C or G antigens were long considered to be commensal organisms that only rarely caused invasive infections as opportunistic pathogens.

In 1996, *S. dysgalactiae* subsp. *equisimilis* was proposed as a new streptococcal taxon (Vandamme et al., 1996). Although rare, *S. dysgalactiae* subsp. *equisimilis* strains having group A antigen rather than group C or G antigen have also been reported (Bert & Lambert-Zechovsky, 1997; Brandt et al., 1999; Katsukawa et al., 2002). Many recent studies have reported that this organism causes invasive and systemic streptococcal infections like GAS (Natoli et al., 1996; Wagner et al., 1996; Hirose et al., 1997; Kugi et al., 1998).
1998; Barnham et al., 2002; Cohen-Poradosu et al., 2004; Hashikawa et al., 2004). The organism has also been reported to cause a wide variety of human infections such as pharyngitis, cellulitis, sepsis, meningitis and endocarditis (Woo et al., 2001).

Our group has reported that most Japanese patients with such invasive infections are older persons with severe underlying diseases (Ubukata et al., 2006). Recently, a population analysis in the USA reported the incidence of invasive diseases caused by \( \beta \)-haemolytic streptococci, mostly \textit{S. dysgalactiae} subsp. \textit{equisimilis} (Broyles et al., 2009).

Notably, \textit{S. dysgalactiae} subsp. \textit{equisimilis} possesses many virulence factors shared with GAS, such as M protein (Fischetti, 1989; Schnitzler et al., 1993), streptolysin \( \Omega \) (Gerlach et al., 1993; Okumura et al., 1994), streptolysin S (Humar et al., 2002) and streptokinase (Walter et al., 1989; Ikebe et al., 2004). It has been suggested that these factors were transmitted from GAS to this species (Kalia et al., 2001).

In the present report, we have described \textit{S. dysgalactiae} subsp. \textit{equisimilis} isolates from patients with invasive and non-invasive infections, and analysed the relationship with patient age and disease, \textit{emm} genotyping and DNA profiles of isolates from invasive infections according to PFGE and antimicrobial susceptibilities.

**METHODS**

**Phenotypic testing of isolates.** From September 2003 to October 2005, a total of 593 \( \beta \)-haemolytic streptococcus isolates identified as causative pathogens were sent to our laboratory from 11 medical institutions throughout Japan. The isolates were accompanied by medical information about the patients using an anonymous questionnaire.

\textit{S. dysgalactiae} subsp. \textit{equisimilis} was identified in accordance with the differentiating characteristics described by Ruoff et al. (2003). These included: (i) agglutination positivity for Lancefield group A, C or G determined using antisera (Streptex; Remel Europe); (ii) strong \( \beta \)-haemolysis; (iii) formation of large, glossy colonies; (iv) bacitracin resistance; (v) negative pyrrolidonylarylamidase test; (vi) negative Voges–Proskauer test; and (vii) positive \( \beta \)-D-glucuronidase test.

Ultimately, 145 isolates were identified as \textit{S. dysgalactiae} subsp. \textit{equisimilis}, and of these, five possessed group A antigen, 18 had group C antigen and 122 had group G antigen.

**Antimicrobial susceptibility.** The susceptibility of all isolates to 12 antimicrobial agents was determined by a microdilution method using cation-adjusted Mueller–Hinton broth (Becton Dickinson) supplemented with 5% lysed horse blood according to the guidelines of the Clinical and Laboratory Standards Institute (CLSI, 2007). Oral antimicrobial agents employed in this study were penicillin G, ampicillin, amoxicillin, cefdinir, cefditoren, faropenem, clarithromycin, azithromycin and levofloxacin. Cefotaxime, panipenem and meropenem were also evaluated as representative parenteral agents.

**Identification of macrolide (ML) and fluoroquinolone resistance genes.** Three ML resistance genes, \textit{erm}(A) (Seppälä et al., 1998), \textit{erm}(B) (Trieu-Cuot et al., 1990) and \textit{mef}(A) (Clancy et al., 1996; Tait-Kamradt et al., 1997), were identified by PCR, as described previously (Wajima et al., 2008). Isolates with the \textit{erm}(A) gene show an inducible ML/lincosamide/streptogramin B resistance phenotype, whilst strains with the \textit{erm}(B) gene show a constitutive ML/lincosamide/streptogramin B resistance phenotype arising from methylation of 23S rRNA. Strains with a \textit{mef}(A) gene show an M phenotype involving an active efflux pump system for 14- and 15-membered MLs.

Four genes related to fluoroquinolone resistance, \textit{gyrA}, \textit{gyrB}, \textit{parC} and \textit{parE}, were analysed using four sets of primers as described previously (Wajima et al., 2008).

**Genotyping of \textit{emm} and bootstrap analysis.** For \textit{emm} genotyping by PCR, a primer set was used as described previously (Beall et al., 1996; Whatmore & Kehoe, 1994). Sequencing reactions for purified PCR products were performed using the primer 5’-TATTGCC-TTAGAAAAATTAAAAACAGG-3’ and an ABI PRISM 3100/3100x1 Genetic Analyser (Applied Biosystems). The first 300 bases of the 5’ end of the \textit{emm} gene were compared with those in the CDC \textit{emm} sequence database (http://www.cdc.gov/ncidod/biotech/strep/streptblast.htm). An \textit{emm} type showing more than 98% identity with a CDC reference strain was identified as that particular \textit{emm} type.

Sequences of open reading frames of \textit{emm} genes in 25 strains were determined using sense primer-1 (3’-ACGGGTACACCTTGGATTGAACTGTTG-5’) and reverse primer-2 (3’-CGTCTTTTGTCCAAAACAGG-5’). The results were compared with those for GAS using clustalW (v.1.83; http://clustalw.ddbj.nig.ac.jp/top-j.html). The Kimura method was used to estimate the number of amino acid substitutions between sequences in each strain. A phylogenetic tree from bootstrap analysis by the neighbour-joining method was obtained using TreeView (v.1.4.0). Sequences of the open reading frames of \textit{emm} genes and deduced amino acids used to depict the phylogenetic tree included seven strains of \textit{Streptococcus pyogenes} (MGAS5005, emm1.0; MGAS9249, emm12; MGAS1680, emm28; SS1, emm3; MGAS10750, emm4; NZ131, emm49; MGAS10394, emm6), four strains of \textit{S. dysgalactiae} subsp. \textit{equisimilis} (CA663750 (protein id), stC4002.2; CAAA42694, stG166b.0; CAA42693, stC74a.0; AAA26928, stG1750.0), \textit{S. dysgalactiae} subsp. \textit{dysgalactiae} (CAB65413, demB encoding M-like protein), \textit{Streptococcus equi} (AA71984, seM encoding M-protein), \textit{Streptococcus iniae} (ACF25917, simA encoding M-like protein) and \textit{S. equi} subsp. \textit{zooepidemicus} (ACG63129 and ACG63223, cspZ.1 and cspZ.2 encoding Emm-like cell surface protein, respectively), with \textit{Streptococcus pneumoniae} (ACH85940, a gene encoding a putative surface protein) used as an outgroup.

**PFGE.** PFGE was carried out by modification of a method described previously (Murayama et al., 2009). Each strain was cultured in 2 ml Todd–Hewitt broth (Becton Dickinson) for 18 h, harvested by centrifugation at 5000 \( g \) at 4 \( ^\circ \)C for 5 min and then washed with saline/EDTA solution. Plugs with embedded bacterial cells were incubated in restriction enzyme buffer containing 30 U SmaI at 30 \( ^\circ \)C for 16 h. Electrophoresis was performed with a CHEF Mapper (Bio-Rad Laboratories). Separation of the fragments was carried out at 6 \( V \ cm^{-1} \) at 14 \( ^\circ \)C for 18 h.

**Statistical analysis.** A \( \chi^2 \) test was used to test for a significant difference between invasive and non-invasive infection groups by age distribution and by \textit{emm} typing.

**RESULTS AND DISCUSSION**

**Age distribution of patients with \textit{S. dysgalactiae} subsp. \textit{equisimilis} infection.** Fig. 1 shows the age distribution of patients with \textit{S. dysgalactiae} subsp. \textit{equisimilis} infection, categorized as either invasive (\( n = 42 \)) or non-invasive (\( n = 103 \)).
The invasive infections comprised sepsis (n=26), purulent arthritis (n=6), cellulitis (n=3), necrotizing fasciitis (n=3) and streptococcal toxic shock syndrome (STSS, n=4). Their causative agents were isolated from normally sterile samples such as blood (n=32), synovial fluid (n=6) and tissue (n=4). The non-invasive infections included pharyngitis, tonsillitis, acute otitis media and local pyogenic infection. These agents were isolated from sputum (n=31), pharynx/tonsils (n=37), pus (n=13), middle ear fluid (n=4) and other sources (n=18).

Invasive infections occurred mostly in patients who were at least 50 years old, especially elderly adults of 60–80 years (P<0.001). Severe underlying conditions such as diabetes mellitus, liver dysfunction, renal dysfunction, medical treatment for malignant disease, immobility and immune deficiency were present in 85.7% of invasive infection cases.

Although group C and G streptococci – the most frequently identified S. dysgalactiae subsp. equisimilis – are usually found as commensal organisms in the throat, skin and occasionally the female genitourinary tract, these organisms are increasingly being recognized as important human pathogens (Brandt & Spellerberg, 2009). Most human infections with S. dysgalactiae subsp. equisimilis are caused by person-to-person transmission and often involve the throat and skin, with patterns similar to those of GAS (Baracco & Bisno, 2006). In patients with severe underlying diseases, the organisms may invade the bloodstream and become widely disseminated to many deep sites where they can cause life-threatening invasive infections.

**Susceptibility to 12 agents**

Table 1 shows the MIC ranges and MIC$_{50}$ and MIC$_{90}$ values of 12 antimicrobial agents for S. dysgalactiae subsp. equisimilis strains. The antimicrobial activities of the oral $\beta$-lactam antibiotics penicillin G, ampicillin, amoxicillin, cefdinir, cefditoren and faropenem were excellent, with MIC$_{90}$ values of $\leq$ 0.031 μg ml$^{-1}$. No strains with reduced $\beta$-lactam susceptibility were recognized. The activities of the parenteral agents cefotaxime, panipenem and meropenem were also excellent.

The activities of clarithromycin, azithromycin and levofloxacin against these strains were less than those of the $\beta$-lactams. Strains possessing ML resistance genes identified by PCR accounted for 10.3% of all strains: three strains (2.1%) possessed an erm(A) gene (stG6979, two strains; stCK401, one strain), five strains (3.4%) had an erm(B) gene (all stG10) and seven strains (4.8%) had a mef(A) gene (stG10, three strains; stC36, two strains; stG2078, one strain; and stG840, one strain).

Four strains isolated from synovial fluid or sputum showed high resistance to levofloxacin, with MIC$_5$ $\geq$ 32 μg ml$^{-1}$. All four strains had amino acid substitutions, changing Ser-81 to Phe or Tyr in GyrA and Ser-79 to Tyr in ParC, together with ML resistance genes erm(B) or mef(A), and they all had emm type stG10.

**Table 1.** MIC range and MIC$_{50}$ and MIC$_{90}$ values of 12 antimicrobial agents for Streptococcus dysgalactiae subsp. equisimilis

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<th>Antibiotic</th>
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<td>Faropenem</td>
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<td>Meropenem</td>
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ML and levofloxacin resistance rates of *S. dysgalactiae* subsp. *equisimilis* were the same as for GAS (Wajima et al., 2008), but different from those reported for GBS in Japan (Murayama et al., 2009).

Typing of *emm* and PFGE profile

Fig. 2 shows the results of *emm* genotyping for 145 *S. dysgalactiae* subsp. *equisimilis* strains classified into invasive
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 Smal 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 stG979 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. 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 Mb, 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 (Hyogo Prefectural Amagasaki Hospital), Go Yamamoto (Nisikyo 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).

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