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ORIGINAL ARTICLE

Correlation of virulence genes to clinical manifestations and outcome in patients with *Streptococcus dysgalactiae* subspecies *equisimilis* bacteremia



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Background/Purpose: *Streptococcus dysgalactiae* subsp. *equisimilis* (SDSE) is increasingly recognized as a human pathogen responsible for invasive infection and streptococcal toxic shock syndrome (STSS). The pathogen possesses virulence genes that resemble those found in *Streptococcus pyogenes* (GAS). We analyzed the association between these specific toxic genes, clinical presentations, and outcome in patients with SDSE infections.

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Streptococcus dysgalactiae subsp. *dysgalactiae*;
Streptococcus dysgalactiae subsp. *equisimilis*;
 Superantigen

Methods: Patients (older than 18 years) with community-acquired invasive bacteremia caused by SDSE bacteremia who were undergoing treatment at China Medical University Hospital from June 2007 to December 2010 were included in this study. Multiplex polymerase chain reaction was performed to identify virulence genes of the SDSE isolates. Demographic data, clinical presentations, and outcome in patients with SDSE infections were reviewed and analyzed.

Results: Forty patients with 41 episodes of SDSE bacteremia were reviewed. The median age of the patients with SDSE infection was 69.7 years; 55% were female and 78% had underlying diseases. Malignancy (13, 33%) and diabetes mellitus (13, 33%) were the most common comorbidities. The 30-day mortality rate was 12%. Compared with the survivors, the non-survivors had a higher rate of diabetes mellitus (80% vs. 26%), liver cirrhosis (60% vs. 11%), shock (60% vs. 17%), STSS (60% vs. 8%), and a high Pittsburgh bacteremia score >4 (40% vs. 6%). Most isolates had *scpA*, *ska*, *saga*, and *slo* genes, whereas *speC*, *speG*, *speH*, *speI*, *speK*, *smez*, and *ssa* genes were not detected. *speA* gene was identified only in one patient with STSS (1/6, 17%). All isolates were susceptible to penicillin, cefotaxime, levofloxacin, moxifloxacin, vancomycin, and linezolid.

Conclusion: In invasive SDSE infections, most isolates carry putative virulence genes, such as *scpA*, *ska*, *saga*, and *slo*. Clinical SDSE isolates in Taiwan remain susceptible to penicillin, cefotaxime, and levofloxacin.

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Introduction

Streptococcus dysgalactiae belongs to Lancefield groups C and G, and can be categorized into at least five subgroups historically.^{1,2} In 1998, with the help of multilocus enzyme electrophoresis typing and genomic DNA, an updated classification of *S. dysgalactiae* was proposed by Vieira et al,³ which defined only alpha-hemolytic or nonhemolytic group C streptococcus as *S. dysgalactiae* subsp. *dysgalactiae* (SDSD) and all β -hemolytic groups C and L and human group G streptococci as *S. dysgalactiae* subsp. *equisimilis* (SDSE).

SDSE colonizes the pharynx, skin, gastrointestinal tract, and vagina in humans and is a part of the normal human flora,^{4,5} whereas SDSD was isolated only from animals.⁶ Invasive infections caused by SDSE have increasingly been recognized as a human pathogen since 2003.^{2,7,8} Among these severe infections, streptococcal toxic shock syndrome (STSS) is of grave concern. STSS is usually caused by *Streptococcus pyogenes* (GAS),⁹ and superantigens such as *speA* are implicated in the pathogenesis of STSS. Other virulence factors, such as streptolysin S (*saga*), streptolysin O (*slo*), and streptokinase A (*ska*), have been suggested to have a significant role in the pathogenesis of invasive GAS infections.¹⁰ Similarly, the several virulence factors of SDSE that resemble those of GAS possibly play an important role in the pathogenesis of SDSE infections.¹

Previous studies have been devoted to investigate the pathogenesis of infection caused by SDSD and SDSE; however, few studies have investigated the association between specific toxic genes, clinical presentations, and outcome in patients. This study aims to analyze the association between clinical manifestations, specific toxic genes including superantigens, and outcome in patients with invasive infection caused by SDSE bacteremia.

Materials and methods

Patients, setting, and definitions

This study was conducted retrospectively at China Medical University Hospital, a 2000-bed tertiary hospital in central Taiwan, from June 2007 to December 2010. Inclusion criteria were as follows: (1) age >18 years; (2) presence of at least one positive *S. dysgalactiae* in the blood culture of patients; and (3) consistent symptoms and signs of infection (such as fever and chills) in clinical presentations. Exclusive criteria were the following: (1) positive blood culture without obvious clinical symptoms and signs of infection and (2) hospital-acquired infection. Data on all demographic characteristics, laboratory data, microbiological data, clinical diagnosis, treatment, and outcome in patients were collected from medical records. The primary bacteremia was defined as bacteremia that did not have the obvious infectious sources and secondary bacteremia was defined as bacteremia accompanied with localized infections such as skin–soft tissue infections, bone and joint infection, respiratory tract infection, and genital tract infection.¹¹ Recurrence of bacteremia was defined as recurrent positive blood culture after complete treatment of prior bacteremia.⁵ Fever was defined as body temperature >37.8°C.¹² Shock was defined as a systolic blood pressure of ≤ 90 mmHg or the use of an inotropic agent to maintain blood pressure.¹³ Leukocytosis was defined as a serum white blood cell level of >11,000/ μ L. The severity of bacteremia was evaluated by the Pittsburgh bacteremia score (PBS), and severely ill was defined as a PBS of over 4.^{14,15} STSS was diagnosed according to the criteria proposed by the Working Group on Severe Streptococcal Infections in 1993.¹⁶ The outcome measure in our study was 30-day mortality after hospitalization due to any causes. This study was approved by the Institutional Review Board of China Medical University Hospital (102-REC3-079).

Bacterial identification

During the study period, a total of 41 blood isolates (involving 40 patients) of *S. dysgalactiae* were collected consecutively in our clinical microbiologic laboratory. These isolates were preserved in commercially available trypticase soy broth containing 20% glycerol and stored in a refrigerator at -70°C . The initial bacterial identification of *S. dysgalactiae* isolates and antimicrobial susceptibility testing of minimum inhibitory concentration (MIC) were performed using an Automated Microbiology System BD Phoenix system (Becton Dickinson, Sparks, MD, USA). The breakpoint of MIC was based on the definition of the Clinical and Laboratory Standards Institute.¹⁷

DNA was extracted from 41 isolates using the Genomic DNA minikit (Geneaid, Taipei, Taiwan). The 16S universal 8F GAGAGTTTGATCCTGGCTCAG and 16S universal 1942R TACGGCTACCTTGTTAC-GACT were used for DNA amplification.^{18–20} The size of the polymerase chain reaction (PCR) product was approximately 1.5 kb. Sequencing of the product was performed on a DNA analyzer (ABI 3730XL). Finally, the sequence was compared with the database of the National Center for Biotechnology Information (template source: *S. dysgalactiae* subsp. *dysgalactiae* ATCC 27957; *S. dysgalactiae* subsp. *equisimilis* ATCC 12394; <http://www.ncbi.nlm.nih.gov/>) for the identification of *S. dysgalactiae* subspecies.

Detection of specific virulent genes

Superantigenic genes (*speA*, *speC*, *speG*, *speH*, *speI*, *speJ*, *speK*, *speL*, *speM*, *ssa*, *smez*, *speA* 1–3, 5, and *speA* 1–4) and other specific virulence genes (*scpA*, *ska*, *slo*, and *saga*) were identified using the method of multiplex PCR described in previous studies.^{7,21} The final reaction volume (50 μL) contained 25 μL of master mix (DNA polymerase, dNTP mix, and PCR buffer), 100 ng of template DNA, 200 nM of each superantigenic gene primer pair, and 20 nM of 16S rRNA primers. DNA amplification was performed using a GeneAmp PCR System 9700 (Applied Biosystems, Taipei, Taiwan) with an initial denaturation step at 95°C for 15 minutes, followed by 35 cycles (30 seconds at 94°C , 90 seconds at 57°C , and 90 seconds at 72°C). PCR methods for the identification of other virulence genes were followed by an initial denaturation step at 94°C for 10 minutes, followed by 35 cycles (45 seconds at 94°C , 45 seconds at 50°C , and 60 seconds at 72°C) and the final extension step (7 minutes at 72°C). Samples were analyzed on a 2.0% agarose.

Statistical analysis

Continuous variables were analyzed by median and categorical variables by Chi-square test or Fisher exact test. We use the stepwise logistic regression analysis to assess the influence of independent variables on crude mortality in individuals with SDSE bacteremia. Variables significantly associated with mortality in the univariate analysis were entered into the model. All statistical analyses were performed using SPSS software version 12.0 (SPSS, Chicago, IL,

USA). All tests were two tailed, with $p < 0.05$ being considered statistically significant.

Results

A total of 41 blood isolates of *S. dysgalactiae* were identified in this study. Based on the results of bacterial identification using a BD Phoenix system, 30 isolates (73%) were identified as SDS and the others as SDSE. Finally, all isolates were confirmed as SDSE by means of 16S rRNA sequencing with GeneBank.

The comparison of clinical characteristics of patients with SDSE bacteremia between the survivor and non-survivor groups was summarized in Table 1. Most of the patients (88%) were over 50 years old, and 18 of them (46%) were males. Diabetes mellitus, malignancy, and heart disease were the most common comorbidities observed during this study. Malignancy (11/35, 31%) was the leading underlying disease in the survival group, whereas diabetes mellitus (4/5, 80%) was the most common comorbidity in the non-survival group. The most common clinical diagnoses were primary bacteremia (22/41, 54%) and cellulitis (14/41, 34%). Twelve patients had skin–soft tissue infections involving the lower limbs, and two breast cancer patients had an upper limb infection. All patients were treated with appropriate antibiotics, such as β -lactam antibiotic alone or in combination with glycopeptides, within 2 hours of diagnosis. Patients with septic arthritis or surgical site infection received surgical management. One cervical cancer patient had two episodes of cellulitis at the same site accompanied with bacteremia within a 2-month period. The patient was treated successfully with β -lactam antibiotic alone in both the events. Nine patients developed shock and six patients had STSS. In this study, the mortality rate within 30 days was 12%, patients with diabetes mellitus or liver cirrhosis, presentation of shock, STSS, or PBS > 4 were significant poor prognostic factors in the univariate analysis (Table 1). However, a stepwise logistic regression model identified no variable independently associated with mortality after adjustment by patients' age and sex (Table 2).

Table 3 shows the distribution of the virulence genes in patients with or without STSS. More than 95% of the isolates in the study had invasive, spreading, or nonsuperantigenic toxic genes, including *scpA*, *ska*, *saga*, and *slo* genes. By contrast, superantigenic toxic genes (*speC*, *speG*, *speH*, *speI*, *speK*, *smez*, and *ssa* genes) were not found in any strains. *speA* gene was detected in one patient with STSS (1/6, 17%). The association between these specific genes and clinical presentations (fever, shock, and PBS > 4), clinical diagnosis (cellulitis, bacteremia, and STSS), and 30-day crude mortality rate were not statistically significant.

Table 4 shows antimicrobial susceptibility patterns of 41 SDSE clinical isolates. All isolates were susceptible to penicillin (MIC ≤ 0.0312 $\mu\text{g}/\text{mL}$), cefotaxime (MIC ≤ 0.5 $\mu\text{g}/\text{mL}$), levofloxacin (MIC ≤ 1 $\mu\text{g}/\text{mL}$), moxifloxacin (MIC ≤ 0.25 $\mu\text{g}/\text{mL}$), and linezolid (MIC ≤ 1 $\mu\text{g}/\text{mL}$). However, drug resistance was remarkable for clindamycin (34%, 14; MIC ≥ 1 $\mu\text{g}/\text{mL}$) and erythromycin (49%, 20; MIC ≥ 1 $\mu\text{g}/\text{mL}$).

Table 1 Clinical characteristics of 40 patients with *Streptococcus dysgalactiae* subspecies *equisimilis* infection^{a,b}

Characteristics	Total no. of patients (%)	No. of survivors (%)	No. of nonsurvivors (%)	<i>p</i>
Age (y)				
>50	35/40 (88)	30/35 (86)	5/5 (100)	0.569
Mean (range)	69.7 (32–96)			
Sex				
Male	18/40 (45)	15/35 (43)	3/5 (60)	0.439
Underlying disease				
Diabetes mellitus	13/40 (33)	9/35 (26)	4/5 (80)	0.013 ^c
End-stage renal disease	8/40 (20)	7/35 (20)	1/5 (20)	0.977
Malignancy	13/40 (33)	11/35 (31)	2/5 (40)	0.671
Liver cirrhosis	7/40 (18)	4/35 (11)	3/5 (60)	0.006 ^c
Heart disease	10/40 (25)	8/35 (23)	2/5 (40)	0.386
Hypertension	10/40 (25)	9/35 (26)	1/5 (20)	0.807
Clinical presentation ^a				
Fever	31/41 (76)	29/36 (80)	2/5 (40)	0.048 ^c
Leukocytosis	32/41 (78)	27/36 (75)	5/5 (100)	0.206
Shock	9/41 (22)	6/36 (17)	3/5 (60)	0.028 ^c
PBS > 4	4/41 (10)	2/36 (6)	2/5 (40)	0.015 ^c
Clinical diagnosis ^a				
Primary bacteremia	22/41 (54)	19/36 (53)	3/5 (60)	0.762
Cellulitis	14/41 (34)	13/36 (36)	1/5 (20)	0.762
STSS	6/41 (15)	3/36 (8)	3/5 (60)	0.002 ^c
Septic arthritis	1/41 (2)	1/36 (3)	0/5 (0)	0.589
Pneumonia	3/41 (7)	2/36 (6)	1/5 (20)	0.245
Endometritis	1/41 (2)	1/36 (3)	0/5 (0)	0.706
Surgical site infection ^d	1/41 (2)	1/36 (3)	0/5 (0)	0.706

^a Forty patients with 41 bacteremia episodes as one patient had two episodes of bacteremia.

^b Nonsurvivors were defined as crude mortality within 30 days after hospitalization.

^c Univariate statistical analysis was used to compare between survivor and nonsurvivor groups.

^d One patient had left distal tibia fracture and received open reduction with internal fixation.

PBS = Pittsburgh bacteremia score; STSS = streptococcal toxic shock syndrome.

Discussion

In this study, several important findings were made. First, the Phoenix Automated Microbiology System appears to have limited ability in discriminating between the strains of SDSE and SDS. Hence, we may perform molecular diagnostic tests, such as 16S rRNA sequencing, for the identification of *S. dysgalactiae* subspecies. Second, several putative virulence genes, including *scpA*, *ska*, *saga*, and *slo*, were commonly found in invasive SDSE isolates, but *speA*, a superantigenic gene, may not play a significant role in the pathogenesis of STSS caused by SDSE. Third, penicillin, cefotaxime,

levofloxacin, and moxifloxacin remain *in vitro* activity against SDSE in Taiwan.

Infection caused by SDSE accounts for 5–8% of *streptococcus* diseases in humans²² and usually occurs in aged patients or in those with underlying diseases.^{2,8} The presentation of STSS and mortality rate in invasive SDSE infection in our study were similar to those reported elsewhere.^{2,8,23} The current findings, which were similar to previous studies, showed that diabetes mellitus, malignancy, and heart disease were the common identifiable underlying diseases in patients with SDSE bacteremia;^{5,23} however, our study reported a higher percentage of diagnosis of primary bacteremia.^{5,8,24} In this study, we also found that patients with diabetes mellitus, liver cirrhosis, shock status, PBS > 4, or complications such as STSS had a higher 30-day mortality rate in the univariate analysis. However, none of these variables was statistically significant in the multivariate analysis. Perhaps the small sample size led to insignificant statistical difference. Larger sample size studies may help to clarify the association between mortality and risk factors in the future.

The Phoenix Automated Microbiology System (Becton Dickinson) is a common tool used for bacterial identification in clinical practice. In our research, a total of 41 *S. dysgalactiae* isolates were identified initially, and 30 of them (73%) were classified as SDS. However, SDS is

Table 2 Multivariate risk factors for 30-day crude mortality rate in patients with *Streptococcus dysgalactiae* subspecies *equisimilis* infection

Categories	Odds ratio (95% CI)	<i>p</i>
STSS	9.306 (0.77–113.28)*	0.08
Liver cirrhosis	6.089 (0.53–70.18)*	0.15

CI = confidence interval; STSS = streptococcal toxic shock syndrome.

*Adjusted for age and sex.

Table 3 Distribution of the virulence genes in *Streptococcus dysgalactiae* subspecies *equisimilis* isolates from the patients with or without STSS^a

Virulence genes ^b	STSS, no. of isolates (%)	No STSS, no. of isolates (%)
Evasion of host immune system		
<i>scpA</i>	6/6 (100)	35/35 (100)
Spreading		
<i>ska</i>	6/6 (100)	33/35 (94)
Toxins (nonsuperantigenic)		
<i>slo</i>	6/6 (100)	34/35 (97)
<i>Saga</i>	6/6 (100)	35/35 (100)
Toxins (superantigenic)		
<i>speA</i>	1/6 (17)	4/35 (11)
<i>speC</i>	0/6 (0)	0/35 (0)
<i>speG</i>	0/6 (0)	0/35 (0)
<i>speI</i>	0/6 (0)	0/35 (0)
<i>speJ</i>	1/6 (17)	7/35 (20)
<i>speK</i>	0/6 (0)	0/35 (0)
<i>speL</i>	0/6 (0)	2/35 (6)
<i>speH</i>	0/6 (0)	0/35 (0)
<i>speM</i>	2/6 (33)	5/35 (14)
<i>speA</i> 1–3,5	1/6 (17)	7/35 (20)
<i>speA</i> 1–4	1/6 (17)	3/35 (9)
<i>smeZ</i>	0/6 (0)	0/35 (0)
<i>ssa</i>	0/6 (0)	0/35 (0)

^a No significant differences were observed between two groups.

^b The classification of the virulence genes was based on the method described by Brandt and Spellerberg.¹ STSS = streptococcal toxic shock syndrome.

largely regarded as an animal pathogen⁶ and only two cases of SDDS infection in humans have been reported so far in the English literature.^{25,26} Therefore, we used 16S rRNA sequencing with GeneBank to differentiate *S. dysgalactiae* blood isolates. All the SDDS isolates were confirmed as SDSE by 16S rRNA sequencing. This finding suggests that for the accurate identification of *S. dysgalactiae* subspecies, molecular diagnostic tests, such as 16S rRNA sequencing, appear to be the best choice.

Similar to the findings of Ikebe et al.,⁷ almost all SDSE isolates in the present study carried *scpA*, *ska*, *saga*, and *slo*

genes, and these nonsuperantigenic virulence genes maybe play a role in invasive SDSE infection. Superantigenic virulence gene such as *speA*, similar to those of GAS, have also been found in SDSE, but previous studies showed that the association between these superantigenic genes and invasive SDSE infection or STSS is uncertain. Our analysis did not confirm the association between STSS and *speA*. A comparable observation was also reported by Hashikawa et al.,²⁷ who analyzed 12 strains of group C and G streptococci with STSS and found no *speA* gene. Ikebe et al also reported that the *speA* gene was not detected in 16 patients with STSS due to SDSE, while 12 patients did have *speG*.⁷ Brandt et al analyzed superantigens in 46 invasive SDSE isolates, and *speA*, *speB*, *speC*, *speF*, *speH*, *speI*, *speJ*, *speK*, *speL*, *speM*, *smeZ*, or *ssa*, were not found. Only six isolates in their study had *speG* and two isolates were further identified as having *speG^{dys}*.²⁸ These findings were consistent with the current study that the involvement of *speA* in the pathogenesis of invasive SDSE infections, including STSS, is uncertain. The role of superantigens or associated genes in the pathogenesis of severe SDSE infections remains unclear.

Erythromycin resistance in streptococci, including group G streptococci, has been a major health problem,^{29,30} and certain strains of SDSE have been reported to be resistant to newer macrolides in Taiwan.⁵ In this study, only half of the isolates were susceptible to erythromycin. Both findings may suggest that erythromycin is no longer considered a suitable drug for treating SDSE infections in Taiwan. All the isolates tested were sensitive to cephalosporin and penicillin; the result was similar to Takahashi et al's² report. All the isolates were sensitive to vancomycin, teicoplanin, and linezolid. This is compatible with the Clinical and Laboratory Standards Institute guideline which suggested that nonsusceptible isolates (penicillin MICs > 0.12 and ampicillin MICs > 0.25 µg/mL) are extremely rare in β-hemolytic streptococcus, and routine penicillin susceptibility tests may not be required.¹⁷ Fluoroquinolone-resistant SDSE was first reported in Europe and North America³¹ and was detected in Japan and Portugal as well.^{2,32} However, all our SDSE isolates were sensitive to levofloxacin and moxifloxacin. This was similar to the findings of a previous study conducted in Taiwan.⁵ This finding suggests that fluoroquinolone remains a reliable antimicrobial agent for SDSE infection in Taiwan.

Several limitations were found in this study. First, the study population is small, which may underestimate the association between specific toxic genes and clinical

Table 4 Antimicrobial susceptibility patterns of 41 *Streptococcus dysgalactiae* subspecies *equisimilis* isolates

Drug	MIC50 (µg/mL)	MIC90 (µg/mL)	Range (µg/mL)	No. of susceptible isolates (%)
Penicillin	≤0.0312	≤0.0312	≤0.0312	41/41 (100)
Cefotaxime	≤0.5	≤0.5	≤0.5	41/41 (100)
Erythromycin	0.25	>4	≤0.0625→4	21/41 (51)
Levofloxacin	≤0.5	1	≤0.5–1	41/41 (100)
Moxifloxacin	≤0.25	≤0.25	≤0.25	41/41 (100)
Vancomycin/teicoplanin	≤0.5/≤1	≤0.5/≤1	≤0.5/≤1	41/41 (100)
Linezolid	≤1	≤1	≤1	41/41 (100)
Clindamycin	0.125	>2	0.0625→2	27/41 (66)

MIC = minimal inhibitory concentration.

presentations. Studies with a large sample size may help clarify the relationship between toxic genes and clinical presentations. Second, this study was conducted at a single site; it is unclear if the results can be applied to other sites or populations. Third, no testing of other putative virulence determinants such as M protein, C5a peptidase, and streptokinase was performed; this might have influenced our conclusion. Fourth, we did not use positive and negative control strains in the bacterial identification or detection of genes, and this may affect the accuracy of identification of some virulence genes.

In conclusion, putative virulence genes, such as *scpA*, *ska*, *saga*, and *slo*, were often present in invasive SDSE isolates, but *speA* may play an insignificant role in the pathogenesis of STSS due to SDSE. Penicillin, cefotaxime, and levofloxacin remain to be reliable antimicrobial agents against SDSE in Taiwan. Further prospective molecular studies are warranted to clarify the relationship between toxic genes and clinical manifestations in invasive SDSE infection.

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