

Major Outbreak of Toxic Shock-Like Syndrome Caused by *Streptococcus mitis*

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Severe illness caused by viridans streptococci rarely occurs in immunocompetent hosts. Between December 1990 and May 1991, thousands of patients in the YangZi River Delta area of Jiangsu Province, China, suffered from scarlet fever-like pharyngitis. Fewer cases occurred in subsequent years with the same seasonality. Approximately half of the cases developed complications characteristic of streptococcal toxic shock-like syndrome (TSLs). Throat cultures yielded predominant growth of alpha-hemolytic streptococci. All cases admitted to Haiian People's Hospital were investigated. Clinical specimens were collected, medical records were reviewed, and bacterial isolates were identified phenotypically and analyzed by 16S rRNA gene sequencing and pulsed-field gel electrophoresis (PFGE). Proteins were purified from culture supernatants by extraction, ammonium sulfate precipitation, and fast-protein liquid chromatography. Biological activities of protein components were determined by subcutaneous inoculation into rabbits. A total of 178 cases of non-beta-hemolytic streptococcal scarlet fever-like pharyngitis were studied. In 88 (79.3%) of 111 patients, oropharyngeal swab cultures grew morphologically identical alpha-hemolytic streptococci. A protein in culture supernatants was pyrogenic in rabbits, was mitogenic for splenocytes, and enhanced rabbit susceptibility to endotoxin challenge. The N-terminal amino acid sequence of this 34-kDa protein showed no homology with known *Streptococcus* pyrogenic exotoxins. The organism was identified as *Streptococcus mitis* based on biochemical and 16S rRNA sequence analyses. Representative outbreak isolates from 1990 to 1995 displayed identical PFGE patterns. This TSLs outbreak in southeastern China was caused by a toxigenic clone of *S. mitis*. An apparently novel toxin may explain the unusual virulence of this organism.

Toxic shock-like syndrome (TSLs) caused by *Streptococcus pyogenes* exotoxin was first reported in 1983 (39). Numerous cases of TSLs have been reported since Stevens et al. (34) first described a 1989 outbreak of life-threatening streptococcal TSLs in 20 young individuals in the Rocky Mountains. The Working Group on Severe Streptococcal Infections created criteria in 1993 to define group A streptococcal (GAS) TSLs (3). The streptococcal pyrogenic exotoxins (SPEs) produced by GAS cause fever, erythematous skin rashes, and various immunopathological and cytotoxic effects. The SPEs range in size from 20 to 40 kDa for the cloned toxin gene products (5, 11, 28). The genes for three types of SPEs have been cloned and sequenced (31, 33). All are superantigens that bind to human and mouse major histocompatibility complex (MHC) proteins (7, 15, 24). The SPE type A (SPEA), SPEG, and several variants of the streptococcal mitogenic exotoxin Z are members of a superantigen family. Five additional superantigens (types G, H, J, K, and I) have been identified based on sequence homology (24, 28).

Human cases of TSLs due to non-GAS species have been reported. Schlievert et al. (30) described a 27-year-old woman with a TSLs illness consisting of fever, hypotension, and multiorgan system involvement. Group B beta-hemolytic strepto-

cocci were isolated from urine and vaginal cultures. These isolates produced a substance that behaved like pyrogenic exotoxins in experimental animals. Group C beta-hemolytic streptococci have also been reported to cause TSLs (19).

An outbreak of non-beta-hemolytic streptococcal scarlet fever-like pharyngitis began in the winter of 1990 in the YangZi River Delta area of Jiangsu Province in southeastern China (40). The first case was identified on 15 December 1990 in Haiian County. All initial cases were residents of urban areas of Haiian County, and the outbreak later involved surrounding areas. Cases peaked between February and March 1991, with the last case reported on 6 May 1991 for the 1990 to 1991 outbreak season. Males 15 to 34 years of age had the highest incidence rate. Approximately half of the patients presented with symptoms and/or signs of TSLs characterized by hypotension and multiorgan failure. Most patients responded to antimicrobial agents, glucocorticoids, and intravenous fluids and recovered within weeks. There were very few deaths (40). After numerous reports of apparent scarlet fever in the same area, mostly in primary and middle-school students, thousands of cases were reported during the winter and following spring seasons. Similar smaller outbreaks occurred each subsequent year.

During a 9-year period, all patients hospitalized with scarlet fever-like pharyngitis at one of the local county hospitals were further studied. The present study investigated the clinical manifestations of this scarlet fever-like pharyngitis, isolated and characterized the bacterial strains recovered from these

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patients, and characterized the TSLS-related toxin from these isolates.

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MATERIALS AND METHODS

Participants. Haian People's Hospital, a 300-bed facility in Haian County, serves a population of 950,000. From October 1990 to May 1998, all hospitalized patients who met the case definition of fever, erythematous rash, and scarlet fever-like pharyngitis were included in the study. Patients were excluded if diagnosed with GAS infection, *Staphylococcus aureus* infection, infectious mononucleosis, drug rash, or other viral infections. Oropharyngeal cultures were performed on patients suspected of having GAS infection based on purulent oropharyngeal exudate. Cases enrolled during 1990-1991 season were studied retrospectively. Those enrolled in subsequent years were identified prospectively by one of the authors (B.Z.). During the first outbreak season, physicians and nurses in the hospital were specifically trained to assess children and adults for this infection. Clinical specimens were collected, medical records were reviewed, and a questionnaire was completed that included clinical diagnosis, underlying illnesses, history of sick contacts, and social activities in the prior 2 weeks. Informed consent was obtained from all subjects. The Science and Research Bureau of Fudan University approved the study.

Bacteria isolation and phenotypic identification. Oropharyngeal swab specimens for culture were immediately placed onto 5% sheep blood agar. Plates were incubated for 24 to 48 h in 5% carbon dioxide. Isolates were identified presumptively by colony appearance, pattern of hemolysis, and Gram stain. Isolates were further identified by an API 20 Strep System (bioMérieux, Inc., Hazelwood, Mo.) according to the manufacturer's instructions (14). Blood and urine cultures were routinely performed on patients with temperatures, obtained orally, of $>38^{\circ}\text{C}$. Isolates were tested for susceptibility to penicillin G, ampicillin, ceftriaxone, cefazolin, ceftazidime, gentamicin, ciprofloxacin, norfloxacin and vancomycin. The MICs of antibiotics were determined by E-test according to the manufacturer's instructions. National Committee for Clinical Laboratory Standards breakpoints were used to interpret E-test results (26). Strains were stored at -70°C in Trypticase soy broth for further analysis.

Bacterial genotypic identification. Bacterial genomic DNA was extracted by using a QIAamp DNA Mini kit (Qiagen, Inc., Valencia, Calif.) according to the manufacturer's instructions. A primer set spanning the region of the 16S rRNA gene corresponding to nucleotide positions 5 to 1,540 of *Escherichia coli* (8) was used to amplify the DNA fragment by PCR. The PCR-amplified products were sequenced by using two PCR primers and six additional internal primers as previously described (38). Double orientation sequences of the whole 16S rRNA gene were determined by using the OpenGene sequencing system (Visible Genetics, Inc., Toronto, Ontario, Canada). Sequence sample files were compared to more than 1,100 validated 16S rRNA gene sequences in the MicroSeq database library (Applied Biosystem, Foster City, Calif.) (37).

Toxin isolation and purification. A bacterial isolate recovered during the 1991 spring outbreak season (Sm91) was arbitrarily chosen and cultured in a Bact API 20 culture medium (bioMérieux) at 35.5°C in 5% CO_2 for 18 h. After centrifugation, the supernatant was precipitated with 20, 40, 60, and 80% ammonium sulfate successively at 4°C for 10 h. Precipitates were dissolved in phosphate-buffered saline (5 mM sodium phosphate, 150 mM NaCl; pH 7.2). Dialyzed protein was obtained after sequential anion exchange (DEAE-52), size exclusion on a fast-protein liquid chromatography system, and polymixin B affinity chromatography (29). Active toxin fractions of purified protein were studied by sodium dodecyl sulfate-polyacrylamide gel electrophoresis on a 12% gel, followed by Coomassie brilliant blue staining. A control *S. mitis* strain was provided by the Shanghai Anti-Epidemic and Health Center. The isoelectric point (pI) was determined by agarose thin-layer isoelectric focusing (IEF) (36). The amino acid composition of the purified protein was analyzed with a Beckman 344 HPLC apparatus and then compared to other SPEs (31). The N-terminal amino acid sequencing of the purified protein was achieved by automated Edman degradation in gas-phase sequenator as described previously (18).

Rabbit inoculation. Male New Zealand rabbits (average weight, 2 kg) were monitored daily for fever, diarrhea, and reddening of the ears, mucous membranes, and eyes after subcutaneous injection with 250 μg of purified protein in 0.2 ml of phosphate-buffered saline (20). Control rabbits received the same amount of protein extract from culture supernatants of a nonoutbreak *S. mitis* strain. Rectal temperature was monitored. Spleens were sampled histologically 4 days after inoculation. To assess enhanced susceptibility to endotoxin shock, 4 h

TABLE 1. Clinical features in 178 patients with *S. mitis*-related scarlet fever-like pharyngitis

Clinical features	No. of patients (%)
Temp $\geq 38.9^{\circ}\text{C}$	153 (86.0)
Hypotension ($<90/60$ mm Hg)	87 (49.9)
Clinically diagnosed shock.....	71 (39.9)
Gastrointestinal tract involvement	
Vomiting.....	109 (61.2)
Diarrhea.....	53 (29.8)
Mucous membrane involvement	
Injected pharynx.....	107 (60.1)
Conjunctivitis.....	21 (11.8)
Strawberry tongue	11 (6.2)
Erythematous macular or maculopopular rash	178 (100.0)
Desquamation	178 (100.0)
Complications	
Acute respiratory distress syndrome.....	2 (1.1)
Acute renal failure	17 (9.6)
Laboratory findings	
White blood cell count at $\geq 15.0 \times 10^3$ cells/ mm^3	93 (52.2)
Platelets at $\leq 100 \times 10^3$ cells/ mm^3	30 (16.9)
Creatinine at ≥ 2 times upper limit of normal.....	34 (19.1)
Transaminases at ≥ 2 times upper limit of normal.....	39 (21.9)

after inoculation rabbits were injected intravenously with 10 μg (equivalent to 0.02 to 0.05 50% lethal dose) of *E. coli* endotoxin (O111:B4; Sigma)/kg as described previously (29).

Detection of *spe* gene. Nested PCR was adapted to detect several *spe* genes, including *speA*, *speB*, and *speC*, according to procedures published previously (5).

Bacterial typing. Genomic DNA was extracted from logarithmic-phase bacterial cultures, prepared in low-melting-point agarose plugs (pulsed-field certified agarose; Bio-Rad, Hercules, Calif.), and digested with *Sma*I (New England Biolabs, Beverly, Mass.) (12). The DNA size was determined by using a bacteriophage lambda ladder (Bio-Rad). Electrophoresis was performed with a CHEF DR-III apparatus (Bio-Rad). Run conditions were 240 V while switching from 10 to 50 s for 18.5 h at 14°C . Gels were stained with ethidium bromide, rinsed, and photographed under UV light.

Statistical analysis. Statistical comparisons were performed with Epiinfo software (version 6; Centers for Disease Control and Prevention, Atlanta, Ga.). A *P* value of ≤ 0.05 was considered significant. Phylogenetic analysis by the neighbor-joining method was performed as described previously (1).

RESULTS

During the eight years beginning in December 1990, 178 patients with non-beta-hemolytic streptococcal scarlet fever-like pharyngitis were hospitalized in the Haian People's Hospital. Nearly 90% of the cases investigated occurred during the first two seasons (113 cases [63.5%] in 1990 and 1991 and 45 cases [25.3%] in 1991 and 1992). Sporadic cases occurred during the same season in subsequent years. The average age was 28.5 years (range, 5 to 46 years), and 133 patients (74.7%) were male. Of the 178 patients, 87 (48.9%) presented with characteristic findings of TSLS (3) as summarized in Table 1. All received empirical therapy with broad-spectrum antimicrobials, and there were no deaths.

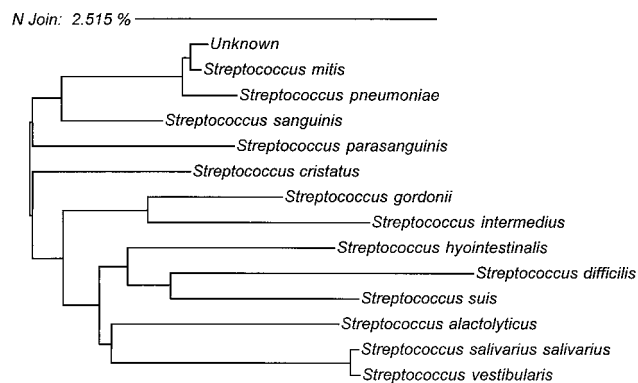


FIG. 1. Neighbor-joining analysis of DNA sequences from specimens found to have homology with the human isolates. Phylogenetic analysis was based on whole 16S rRNA gene sequences. Five clinical isolates arbitrarily selected from each year from 1991 to 1995 show identical 16S rRNA gene sequence information. The scale shows relative phylogenetic distance.

All blood cultures remained negative after 7 days of incubation, and admission urine samples were nondiagnostic. Admission oropharyngeal swab cultures were performed on 111 patients, from which 88 (79.3%) grew either pure culture or predominantly gram-positive alpha-hemolytic streptococci of identical colony morphology. All 88 isolates had identical biochemical reaction profiles and showed 92.2% similarity to *Streptococcus mitis* in the API 20 Strep System. The organisms were susceptible to all antimicrobials tested. Five representative isolates, each from a different season, were further analyzed by 16S rRNA gene sequencing. The sequence of each human oropharyngeal-swab specimen isolate was 100% identical and diverged from the reference *S. mitis* sequence at only 3.5 nucleotide positions (99.8% similarity) (Fig. 1).

A 34-kDa protein precipitated from culture supernatant by 20% ammonium sulfate was pyrogenic in rabbits. The temperature increased steadily over 4 h after injection, with a mean temperature increase of 1.1°C (Fig. 2). Fever decreased over the next 6 to 8 h and returned to normal within several days. All animals injected with the 34-kDa protein also developed diarrhea, as well as redness of the ears and mucous membranes. Gross examination revealed that cardiac tissue was rigid, and histological examination showed punctate hemorrhages. At 4 days the spleens showed increased mitotic features with congestion of red pulp and proliferation of white pulp. Intravenous challenge of animals previously injected with the 34-kDa protein by injecting *E. coli* endotoxin caused death in all animals within 16 to 29 h, confirming increased susceptibility to lethal endotoxic shock. In contrast, culture supernatant from the control *S. mitis* strain displayed none of these effects, and animals survived after *E. coli* endotoxin challenge.

The pI of the 34-kDa protein was 6.2. Amino acid analysis showed a pattern distinct from SPEA, SPEB, and SPEC, with substantially lower molecular ratios in tryptophan, cysteine, methionine, and tyrosine and higher molecular ratios in glycine and alanine (Table 2). The N-terminal amino acid sequence of the 34-kDa protein was VSNLSRGMGA. This showed no homology to other pyrogenic toxins, including SPEA, SPEB, and SPEC. Nested PCR procedures targeting known SPEs

TABLE 2. Comparison of amino acid composition of *S. mitis* 34-kDa protein to those reported for other streptococcal exotoxins

Amino acid	Molecular ratio (%) ^a			
	34 kDa protein	SPEA	SPEB	SPEC
Tryptophan	0.0	0.5	1.6	0.0
Lysine	6.7	10.0	5.5	10.1
Histidine	1.6	2.8	2.8	2.9
Arginine	3.7	1.4	2.8	3.4
Aspartic acid	9.2	14.0	13.0	7.3
Threonine	5.0	6.5	3.6	5.7
Serine	5.0	6.9	8.7	7.7
Glutamic acid	13.6	12.7	9.1	8.1
Proline	5.6	4.2	4.7	1.9
Glycine	11.8	4.2	11.9	5.3
Alanine	10.6	1.9	7.1	2.4
Cysteine	0.3	1.4	0.4	0.5
Valine	8.1	6.4	7.5	3.4
Methionine	0.3	1.4	4.0	1.4
Isoleucine	5.4	5.9	4.3	0.5
Leucine	6.7	9.1	5.5	6.2
Tyrosine	2.1	7.7	5.9	7.7
Phenylalanine	4.3	4.1	1.6	5.3

^a The molecular ratios for SPEA, SPEB, and SPEC were reported elsewhere (31). Boldface type identifies differences in molecular ratios exceeding twofold.

genes, including *speA*, *speB*, and *speC*, were negative for these five representative 34-kDa protein-producing *S. mitis* strains. This suggests that the 34-kDa protein belongs to a novel group of the SPE exotoxins.

The epidemiologic relatedness of the five strains collected during different seasons was further analyzed by PFGE. For comparison, five *S. mitis* isolates from normal human throats collected in the same geographic region and season were included. The PFGE patterns of the outbreak isolates were identical to one another but completely different from the comparison isolates (Fig. 3). These data suggest that the isolates causing the large outbreak of human disease were epidemiologically related and clonal in origin.

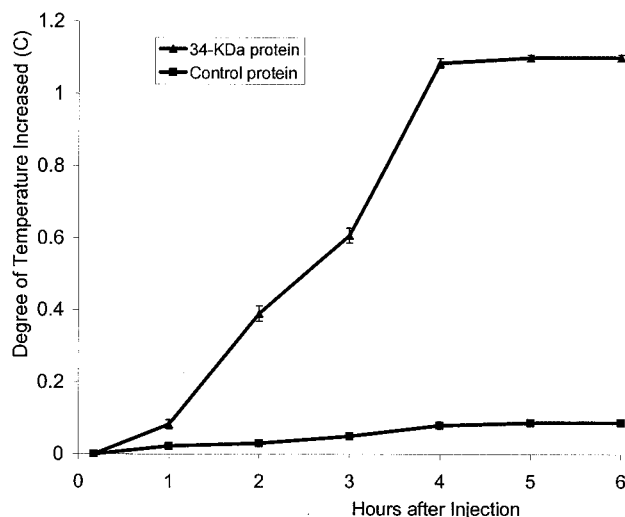


FIG. 2. Fever in rabbits after injection of a secreted 34-kDa *S. mitis* protein. Mean rectal temperature change (in degrees centigrade ± the standard deviation) from four rabbits in each group is shown.

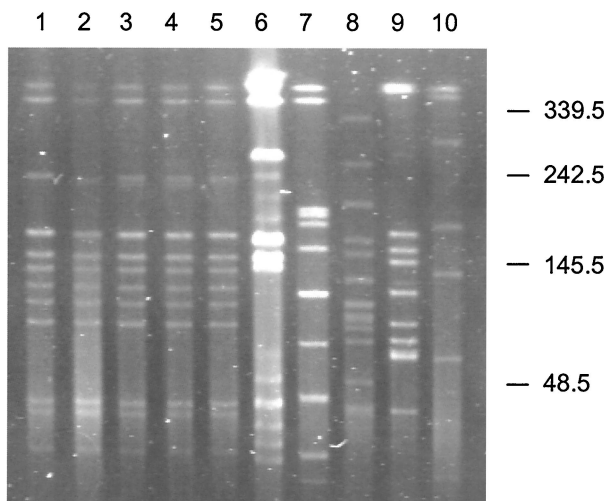


FIG. 3. PFGE patterns of *Sma*I-digested genomic DNA of clinical *S. mitis* isolates. Lanes 1 to 5 are *S. mitis* isolates recovered from 1991 to 1995, respectively. Lanes 6 to 10 were *S. mitis* isolates recovered from the throats of local healthy volunteer during the same period. Molecular sizes are in kilobases.

DISCUSSION

The present study investigated an *S. mitis*-related TSLs outbreak involving thousands of patients in Southeastern China from 1990 to 1998. A total of 178 patients who suffered from non- β -streptococcal scarlet fever-like pharyngitis admitted to a local hospital were investigated. *S. mitis* was recovered from throat swabs of most patients, based on phenotypic and genotypic characteristics, including biochemical profiles and 16S rRNA gene sequences. Representative outbreak strains had identical PFGE patterns, suggesting that a clonal strain of *S. mitis* caused this TSLs outbreak. This report heralds the emergence of a particularly virulent exotoxin-producing strain of viridans streptococci and should promote vigilance for further outbreaks.

S. mitis, one of the more common species of viridans streptococci, has generally been minimally pathogenic and relatively avirulent. Even when it causes endovascular infection such as subacute bacterial endocarditis, the affected patients are often not severely ill (35). When isolated from upper respiratory samples, it is often dismissed as a nonpathogenic commensal. Some aggressive strains have been reported to cause sepsis, acute respiratory distress syndrome, and shock in neutropenic hosts (9, 13, 27). It was reported to cause lower respiratory tract infection in two patients based on culture of transtracheal aspirate and pleural fluid. The first patient presented with empyema and lung abscess, and the second presented with uncomplicated pneumonia complicating lymphoma (27). Extracellular products of *S. mitis* isolates recovered from infants with Kawasaki syndrome behaved as superantigens in a rabbit model (23). Three apparent cases of acute community-acquired viridans streptococcal pneumonia in previously healthy adults have also been reported (9). Viridans streptococci are associated with the development of a rapidly fulminant shock syndrome in neutropenic patients, and these organisms stimulate a panel of cytokines, which may contribute to septic shock

(16, 32). In our study, none of the patients with *S. mitis*-associated TSLs had evidence of systemic infection such as endocarditis or bacteremia.

Since 1980, there has been a resurgence of severe invasive diseases due to group A streptococci (*S. pyogenes*), including necrotizing fasciitis and myonecrosis with or without STLS (25, 33). Both group A streptococci and *S. aureus* produce potent exotoxins that belong to the pyrogenic toxin superantigen family. These simultaneously bind to both the class II MHC molecule and to the V β chain of the T-cell receptor (TCR) outside of the conventional peptide groove (22). Through this interaction, these potent toxins subvert normal immune response by forming a TCR-superantigen-MHC ternary complex that stimulates T cells bearing the appropriate V β region (2). In recent years, many bacterial exotoxins have been shown to act as superantigens and cause toxicity by causing excessive immune activation (7, 24). Whether the *S. mitis* exotoxin described here is truly a superantigen merits further investigation.

STLS is a severe, multisystem illness characterized by the rapid onset of fever and hypotension and is a subset of invasive streptococcal disease. The SPEs (also known as erythrogenic toxins or scarlet fever toxins) include the serologically distinct types A, B, C, D, F, G, and H, as well as streptococcal superantigen and streptococcal mitogenic exotoxin Z (6, 10, 17). The SPEs are responsible for the fever, rash, and severe clinical manifestations of TSLs. Other than some members of the *Streptococcus milleri* group, viridans streptococci, including *S. mitis*, are not considered to possess traditional virulence factors (4). We identified a novel 34-kDa protein from *S. mitis* that was highly pyrogenic and enhanced susceptibility to lethal endotoxin shock.

We previously described an *Enterococcus faecium*-related sepsis outbreak that involved both pigs and humans in 1998 that occurred in the same geographic region and that in some cases also shared clinical features of TSLs (21). We suspect that this region of China may be a reservoir for a novel bacterial exotoxin gene acquired by either *E. faecium* or *S. mitis* and that this may have played a role in both outbreaks. Efforts are being focused on isolating and characterizing a possibly novel *spe* gene.

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