Genomic Differences in *Streptococcus pyogenes* Serotype M3 between Recent Isolates Associated with Toxic Shock–Like Syndrome and Past Clinical Isolates

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Genomic differences among past *Streptococcus pyogenes* serotype M3 strains isolated in 1973 and before from patients with streptococcal pharyngitis, and recent (1990s) serotype M3 clinical isolates from patients with pharyngitis, and recent M3 isolates from patients with toxic shock–like syndrome were investigated by restriction landmark genomic scanning and by modified random-amplified polymorphic DNA–polymerase chain reaction. Similar polymorphic DNA fragments were identified between the older M3 isolates and the recent isolates; also, the recent M3 clinical isolates from patients with pharyngitis were genetically indistinguishable, by the methods used, from the M3 isolates of patients with toxic shock–like syndrome. Although nucleotide sequences of these regions showed no apparent homology with known virulence factors, the DNA fragments could distinguish the recent M3 strains from the past strains. These results suggested that the recent strains have emerged because of genetic divergence.

Many varieties of symptoms are caused by infections with group A streptococci (*Streptococcus pyogenes; GAS*). The most frequent diseases are acute pharyngitis (strep throat) and impetigo (pyoderma) [1]. Many streptococcal virulence factors involved in these symptoms have been reported, including, among others, pyrogenic exotoxins (SpeA, SpeB, and SpeC) and M protein. Among these virulence factors, the pyrogenic exotoxins are believed to play an important role in infections. The pyrogenic exotoxins are responsible for the rash of scarlet fever and are known as erythrogenic toxins or scarlet fever toxins. The genes *speA* and *speC* are located in the lysogenic bacteriophage [2–4]. The *speB* gene is on the bacterial chromosome and encodes cysteine proteinase [5].

In recent years, a new type of severe infectious disease caused by this pathogen—severe invasive GAS infection, or streptococcal toxic shock–like syndrome (TSLS)—has been spreading [6–9]. Studies by Stevens [9] and Stevens et al. [10] have indicated that the interaction between known virulence factors (SpeA and M protein) of GAS and the host’s defense mechanisms plays an important role in the pathogenesis of TSLS. Host factors must be important for the pathogenesis of the disease. However, although common streptococcal infection is a local disease, streptococcal TSLS is a systemic disease, and TSLS causes such severe symptoms as necrotizing fascitis [9, 10]. Also, some epidemiologic data showed the clonal expansion of a certain serotype strain (type M1 and M3) of severe invasive GAS [7, 11–18]. Such results cause us to speculate that an unknown virulence factor(s) causing TSLS and/or unknown *cis* elements enhancing the original bacterial virulence have been acquired in the strains [19].

In Japan, dominant serotypes of *S. pyogenes* isolates in 1990–1995 were M1, M4, and M12, whereas strains of M3 and M1 serotypes were dominant in causing TSLS during 1992–1995 [7]. The dominance of the M3 serotype in TSLS seemed to be correlated with the rapid increase of M3 serotype strains in streptococcal infections in this period; in other words, the recent M3 clinical isolates that have an ability to cause TSLS seemed to expand clonally.

It has been observed that virulent strains are generally different from avirulent strains at the level of the genome; this difference is caused by acquisition of the cluster of several virulence-associated genes, called the pathogenicity island. For example, 2 genomes of *Salmonella enterica* and *Escherichia coli* K-12 have several large genomic differences, which include the clusters of virulence genes SPI-1–5 [20–25]. Hypothesizing that M3 serotype GAS strains from patients with TSLS must have
some differences from other strains, we tried to find the genetic differences akin to pathogenicity islands on the genomes between older M3 serotype strains and recent M3 clinical isolates, by use of restriction landmark genomic scanning (RLGS) [26, 27] and modified random-amplified polymorphic DNA (RAPD) analysis, also called high-stringency and arbitrary primer (HiSAP) polymerase chain reaction (PCR) [28–32].

Materials and Methods

Strains and isolation of chromosomal DNA. Serotype reference strains used in this study and their sources were as follows: CNCTC 29/58 (Glossy SF130), CNCTC 30/59 (Richard), CNCTC 42/58 (4990), CNCTC 8/49 (S43/100/7), CNCTC 33/58 (SF42), CNCTC 5/64 (T13/51/6), and CNCTC 9/59 (J17C 8320) were gifts from the Czechoslovak National Collection of Type Cultures (CNCTC); T1/119/6, B930/24/3, S43/137/4, and CNCTC 24/59 (J17C) were gifts from R. C. Lancefield in 1958 and were stored in our laboratory; SS95/12 was given by the US Centers for Disease Control in 1956; 5 serotype M3 isolates, K21–K25, were obtained from pharyngitis patients in 1973 at Kanagawa Prefecture; 5 serotype M3 isolates, K31–K35, were recently obtained from pharyngitis patients in 1994 at Kanagawa Prefecture. All clinical isolates designated with the National Institutes of Health (NIH) prefix were isolated from patients with TSLS (table 1), as described elsewhere [7].

Chromosomal DNAs of the strains were prepared after the strains were grown on Todd-Hewitt broth (Difco, Detroit). Streptococcal chromosomal DNA was extracted by the mutanolysin method [4, 33].

RLGS system. RLGS is a powerful technique with enormous potential for biologic and biomedical research and is particularly suited to the identification of genetic differences [26, 27]. Two representative serotype M3 strains, CNCTC 30/59 and NIH1 (see above and table 1), were used for this analysis, as described elsewhere [26, 27]. First, 1.5 µg of bacterial genomic DNA was digested with BglII restriction enzyme as a landmark, after which the landmark site was labeled by [α-32P]deoxynucleotides. The bacterial DNA fragments digested by BglII were separated on 0.8% agarose narrow gels. To resolve these numerous signals, those fragments were subjected to further separation. The agarose narrow gel was treated with HindIII as a secondary restriction enzyme. This cleavage reaction makes the DNA fragments differ in electrophoretic mobility, depending on the distance from the site of restriction enzyme BglII to HindIII. The DNA fragments were subjected to 2-dimensional 5% polyacrylamide gel electrophoresis by connection of the agarose narrow gels to the second gel. The sizes of the DNA fragments range from 2 kb to 70 bp. The final gel samples were dried and autoradiographed (figure 1).

HiSAP-PCR for the detection of polymorphisms and chromosome walking by inverted PCR. HiSAP-PCR was done under the conditions of 40 cycles of 40 s at 93°C, 1 min at 55°C, and 50 s at 72°C (figure 2). The random primers used were a 10-mer kit (Operton Technologies, Alameda, CA) [28, 29]. We could detect the genetic polymorphism among M3 serotype strains by use of the G17 primer (5′-ACGACCGACA-3′) [28]. A 0.4-kb PCR-amplified DNA fragment (probe A) was cloned by use of the pGEM-T vector system I into the vector pGEM-T (Promega, Madison, WI), and the entire nucleotide sequences of probe A (DNA Data Bank of Japan [DDBJ] accession no. AB006751) were determined by the chain-termination method described by Sanger et al. [34]. A self-ligated 8.4-kb EcoRI fragment (including probes B and D-G) that flanks probe A was amplified by the inverted PCR method with an LA-PCR kit (Takara, Kyoto, Japan). The primers that were directed to the outside of probe A were used, and the fragment was detected by Southern hybridization with probe A (figure 3). A 3.8-kb BglII fragment (a part of probe B and probe C) was

![Table 1. Strains of Streptococcus pyogenes used in genetic comparison of recent and past isolates.](image-url)
Figure 1. Results of restriction landmark genomic scanning comparing past and recent serotype M3 isolates of *Streptococcus pyogenes*: CNCTC 30/59 (past) and NIH1 (recent). Nine representative polymorphic spots between strains were detected (arrows).

amplified by use of inverted PCR with primers designed from nucleotide sequences of probe B (DDBJ accession no. AB006752) (figure 3).

**Genomic cloning and physical mapping.** General methods for genomic cloning were those of Sambrook et al. [35]. About 50 μg of bacterial DNA was completely digested with *Bam*HI. A genomic 17.3-kb *Bam*HI fragment (including a part of probe E and probes F–K; figure 3) of M3 serotype strain NIH1 was cloned into the *Bam*HI site of λGEM12 (Promega) and selected with probe G by plaque hybridization. The fragment was subsequently recloned to pBluescript SK– (Stratagene, La Jolla, CA). A physical map of the 26.7-kb region was made from the 17.3-kb *Bam*HI fragment, the PCR-amplified 8.4-kb fragment (probe B and D–G), and the PCR-amplified 3.8-kb fragment (a part of probe B and probe C) (figure 3).

**DNA sequencing and Southern hybridization.** DNA sequencing was done by the chain-termination method described by Sanger et al. [34]. DNA homology was sought by a BLASTP search (basic local alignment search tool designed to support analysis of protein databases) of the National Center for Biotechnology Information.
Conditions were as follows: first, 5 min of washing with a solution of standard saline citrate (SSC) and 0.1% SDS at room temperature; second, 15 min of washing twice with a solution of 2× SSC and 0.1% SDS at room temperature; third, 15 min of washing twice with a solution of 0.1× SSC and 0.5% SDS at 65°C [35]. We used the digoxigenin DNA detection system, following the manufacturer’s instructions (Boehringer Mannheim, Indianapolis) [37].

Southern hybridization with the various probes was done as previously described [35]. About 2 μg of bacterial DNA was digested with BglII and separated on an 0.8% agarose gel. Washing conditions were as follows: first, 5 min of washing with a solution of 2× standard saline citrate (SSC) and 0.5% SDS at room temperature; second, 15 min of washing twice with a solution of 2× SSC and 0.1% SDS at room temperature; third, 15 min of washing twice with a solution of 0.1× SSC and 0.5% SDS at 65°C [35]. We used the digoxigenin DNA detection system, following the manufacturer’s instructions (Boehringer Mannheim, Indianapolis) [37].

Results

We examined whole bacterial genomes by the RLGS method [26, 27] to compare the gross genetic difference between the past and recent M3 strains of GAS. As shown in figure 1, ~500 spots appeared on 2-dimensional gel electrophoresis after cutting genomic DNA of GAS with BglII-HindIII restriction enzymes, and ≥9 polymorphic spots were observed when a past M3-serotyped strain (CNCTC 30/59) and a recent M3-serotyped strain (NIH1) from a patient with TSLS were compared. The strain CNCTC 30/59 we used here lacks the speA gene, which may explain, in part, the polymorphic spots observed here. However, the above result seemed to indicate that there were some more genetic differences between the past M3 isolates and the recent M3 isolates from TSLS patients.

Next, we thought it might be possible, by use of a modified RAPD (HiSAP)-PCR method, to amplify and isolate some of the DNA regions corresponding to the polymorphic DNA fragments [28–32]. We examined 40 kinds of primers (10-mer kit F and G; Operon) to detect genetic differences between the past strains and the recent strains, and we found that only one primer, G17, was available for the detection of genetic differences. The primer G17 detected a difference in amplified fragments among M3 serotype strains; a 0.4-kb fragment was found in all 7 of the M3 isolates from patients with TSLS but not in the serotype M3 reference strains (CNCTC 30/59 and B930/24/3) (figure 2) [28]. In the case of the M3 serotype, this polymorphic DNA fragment was also detected in 1994 in all of 5 M3 isolates (K31–K35) from pharyngitis patients, whereas the polymorphic DNA fragment was not detected in all of 5 M3 isolates (K21–K25) from pharyngitis patients in 1973 (figure 2). The 0.4-kb polymorphic DNA fragment amplified from the TSLS isolate (NIH1) was cloned into pGEM-T vector and named probe A.

We obtained a 26.7-kb region flanking probe A, by chromosomal walking (see Materials and Methods). The physical map is shown in figure 3A, and each fragment cleaved with various restriction enzymes was designated by probes A–K. With each probe, we did Southern hybridization to detect polymorphisms between the M3-serotyped past strains (M3 serotype reference strains CNCTC 30/59 and B930/24/3 and isolates K21, K23, and K25 from pharyngitis patients in 1973) and the M3-serotyped recent strains (isolates K31, K33, and K35 from pharyngitis patients in 1994 and isolates NIH1, NIH12, and NIH16 from TSLS patients) (figure 3A and figure 4).

As shown in figure 4 (lanes 6–11), M3-serotyped recent strains isolated from pharyngitis patients and TSLS patients showed identical patterns of BglII-digested DNA fragment hybridized with DNA probes A–K (all probes used in this study), indicating that the recent M3 strains conserved the DNA regions corresponding to probes A–K. On the other hand, almost all the past strains did not hybridize in the expected fragments with probes A–H, with some exceptions, but hybridized with probes I–K (figure 4, lanes 1–5), indicating that the past strains did not carry all or parts of DNA fragments corresponding to probes A–H (figure 3A). The results are summarized in figure 3B: I, J, and K regions were conserved among all M3 isolates, whereas E, F, and G regions were conserved only among recent M3 isolates. Other regions were conserved among recent M3 isolates but differed in conservation among past M3 isolates. These results clearly show that recently isolated M3 strains are genetically distinct from M3 strains isolated in the past (in 1973 and before).

We further examined whether homologues of the above probes are present in other serotyped strains. A 5.9-kb BglII...
Figure 3. Physical map of 26.7-kb polymorphic region in serotype M3 Streptococcus pyogenes (NIH1) from patient with toxic shock-like syndrome. A, Upper open box indicates 2162-bp sequenced region including probe G. ORF (open-reading frame) and thick horizontal arrow indicate site of 411-bp plausible ORF and direction of a plausible transcriptional unit, respectively. Nucleotide sequences reported here have been deposited to the DNA Data Bank of Japan under accession nos. AB006751 (probe A) and AB006752 (probe G). Lower thick horizontal line indicates total of cloned 26.7-kb DNA fragment. Restriction sites are indicated. Horizontal arrows show 5.9- and 9.4-kb BglII-digested DNA fragments, respectively. Lower open-boxed regions indicate each probe region A–K. Vertical arrow indicates 0.4-kb polymorphic DNA fragment (probe A) isolated by high-stringency and arbitrary primer polymerase chain reaction [28]. B, Box with dark shading indicates region conserved among all M3 isolates; open box, region conserved only among recent M3 isolates; and boxes with light shading, region conserved among recent M3 isolates but different in conservation among past M3 isolates.

fragment hybridized with probe A was detected in the 2 other serotyped isolates from TSLS patients (no. 6 of M4 serotype and no. 32 of T22 serotype; figure 5A, lanes 27 and 31) but not in M1, M4, M6, M12, M13, or M18 strains from patients with pharyngitis and M1, T11, M12, and T28 isolates from patients with TSLS (figure 5A). Probe G hybridized not only with M3-serotyped isolates from patients with TSLS (figure 5B, lanes 19–25) but also with other serotyped isolates from patients with TSLS: M1 (lanes 13–18), M4 (lanes 26 and 27), M12 (lane 29), and T22 (lane 31). Probes E and F showed characteristics similar to those of probe G, except that they hybridized with some past isolates of M1, M6, M12, and M18 serotypes (data not shown). These results showed that parts of the region existing among recently isolated M3 strains have also been conserved among strains of other serotypes, mainly recently isolated, which may suggest the genetic exchange between different serotyped strains.

Because the region of probe G existed among most isolates from patients with TSLS, we sequenced 2162 bp (figure 3), including the probe G region, of serotype M3 NIH1 strain (DDBJ accession no. AB006752) to know whether it encodes some pathogenic factors involved in TSLS. The sequenced region contained a 411-bp plausible open-reading frame that had no homology with known virulence factors but had 80% similarity with the sequences of invasive M1 strain SF370, as determined by searching the University of Oklahoma S. pyogenes database [36].

Discussion

In this study, we identified polymorphic DNA regions between recent (1990s) and past (1973 and before) clinical isolates of the S. pyogenes M3 serotype. The comparison of the total 26.7-kb DNA region clearly showed that recent M3-serotyped strains of S. pyogenes are genetically different from the past strains. In Japan, serotype M3 clinical isolates of S. pyogenes rapidly increased in number during 1992–1995 [7], which suggests an expansion of a certain clone. Our data shown here support the clonal expansion of M3-serotyped S. pyogenes at the genetic level. Most of the past strains did not carry a total of >17 kb homologous DNA regions corresponding to the probes A–H (figure 3A). The recent isolates may have acquired such DNA regions through genetic conversion: for example, bacteriophage or transposon. The HiSAP-PCR method we used here detected only a 0.4-kb DNA fragment (probe A) as a polymorphic DNA fragment between recent and past strains. However, the result of RLGS showed that there were some polymorphic different spots between the past and recent S. pyogenes serotype M3 strains. Two of them were included in the above-mentioned 17-kb region (data not shown). The recent
Figure 4. Southern hybridization of serotype M3 Streptococcus pyogenes strains from past and recent infections by use of probes A–K. Hybridization was done as described elsewhere [35] by use of a digoxigenin DNA detection system (Boehringer Mannheim, Indianapolis) [37]. Strains used: lane 1, CNCTC 30/59; lane 2, B930/24/3; lane 3, K21; lane 4, K23; lane 5, K25; lane 6, K31; lane 7, K33; lane 8, K35; lane 9, NIH1; lane 10, NIH12; lane 11, NIH16. Each probe (probes A–K) was used for hybridization to ~2 μg of chromosomal DNA that was digested with BglII restriction enzyme and further separated on 0.8% agarose gels. Probe D hybridized with an expected 5.9-kb BglII fragment among recently isolated strains but not among past strains.
Figure 5. Southern hybridization of chromosomal DNA from past and recent isolates of group A streptococci (Streptococcus pyogenes; GAS) by use of probes A (A) and G (B). A 5.9-kb BglII-digested band appeared in invasive serotype M3 GAS (lanes 19–25), M4 GAS (lane 27), and T22 GAS (lane 31) by Southern blot with probe A. A 9.4-kb BglII-digested band appeared in invasive serotype M3 GAS (lanes 19–25), M4 GAS (lane 27), and T22 GAS (lane 31) by Southern blot with probe G. Strains used: lane 1, CNCTC 29/58; lane 2, T1/11/96; lane 3, CNCTC 30/59; lane 4, B930/24/3; lane 5, CNCTC 42/58; lane 6, CNCTC 8/49; lane 7, S43/13/94; lane 8, CNCTC 33/58; lane 9, S59/12; lane 10, CNCTC 5/64; lane 11, CNCTC 9/59; lane 12, CNCTC 24/59; lane 13, NIH11; lane 14, NIH17; lane 15, NIH19; lane 16, NIH22; lane 17, NIH27; lane 18, NIH36; lane 19, NIH1; lane 20, NIH12; lane 21, NIH16; lane 22, NIH18; lane 23, NIH21; lane 24, NIH33; lane 25, NIH34; lane 26, NIH6; lane 27, NIH2; lane 28, NIH5; lane 29, NIH30; lane 30, NIH4; lane 31, NIH32; lane 32, NIH35; lane 33, NIH37.
clone of M3 serotype \textit{S. pyogenes} may have obtained additional genetic elements in addition to the 17-kb DNA region.

As shown here, serotype M3 \textit{S. pyogenes} strains isolated from patients with TSLS were indistinguishable, by Southern hybridization, from the strains recently isolated from patients with pharyngitis. These data may show that the M3 clone that recently appeared has the potential to cause severe invasive diseases, particularly necrotizing fasciitis and TSLS, in persons susceptible to invasive infection. Several studies [38, 39] suggested that a large number of cases of pharyngitis caused by potentially virulent clones of \textit{S. pyogenes} increase the exposure of susceptible persons, which may cause a severe invasive GAS infection. This may be the case in Japan; isolates of M3 serotype from pharyngitis patients rapidly increased during 1992–1995, which was correlated with the increase of M3 isolates in patients with TSLS [7].

The region of probe G we sequenced carried an open-reading frame that does not seem to be related to the potential virulence factor. However, it is interesting to note that serotype M1, M4, and T22 GAS isolates from TSLS patients have homology to the probe G that is not detected among old strains isolated from pharyngitis patients. It is very important to learn whether common virulence factors associated with the induction of severe invasive disease exist among isolates of various M serotypes from TSLS patients. For such a purpose, we are now analyzing and sequencing other polymorphic DNA regions we identified.

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References


