Short Communication

Description of the Pathogenic Features of Streptococcus pyogenes Isolates from Invasive and Non-Invasive Diseases in Aichi, Japan

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SUMMARY: We identified hypervirulent Streptococcus pyogenes in 27 and 420 isolates from patients with invasive and non-invasive diseases, respectively, in Aichi Prefecture, Japan, between 2003 and 2012, in an attempt to understand why the prevalence of streptococcal toxic shock syndrome (STSS) suddenly increased in this location during 2011. Hypervirulent strains belong to the emm1 genotype, with a mutation in the covR/S genes that regulate many other genes, encoding virulence determinants and resulting in the absence of the proteinase streptococcal exotoxin B and the production of virulence factors such as the superantigen streptococcal exotoxin A, the nuclease streptococcal DNase, the cytotoxin NAD-glycohydrolase, and the hemolysin streptolysin O. We found 1 strain from invasive disease and 1 from non-invasive disease with traits similar to those of hypervirulent strains, except that the sda1 gene was absent. We also found 1 non-emm1 strain with phenotypic and genetic traits identical to those of the emm1 hypervirulent strains except that it did not belong to emm1 genotype, from non-invasive diseases cases in 2011. These findings suggested that hypervirulent hypervirulent-like strains from invasive and non-invasive disease cases could have at least partially contributed to the sudden increase in the number of patients with STSS in Aichi during 2011.

Streptococcus pyogenes is an important human pathogen with various clinical manifestations. S. pyogenes commonly causes pharyngitis among schoolchildren, as well as invasive diseases such as sepsis and streptococcal toxic shock syndrome (STSS), which have become more prevalent globally during the last 25 years. STSS is associated with symptoms of hypotension, renal impairment, disseminated intravascular coagulopathy, liver involvement, adult respiratory distress syndrome, generalized erythematous macular rash, soft tissue necrosis, and central nervous disorders, and mortality rates range from 30% to 70% (1,2). Hypervirulent S. pyogenes of the emm1 genotype has recently been associated with more severe invasive diseases (3). According to previous investigations (2–5), emm1 hypervirulent S. pyogenes is defined by the abundant production of numerous virulence factors such as the superantigen, streptococcal exotoxin A (SpeA), the nuclease, streptococcal DNase (Sda1), the cytotoxin nicotinamide adenine dinucleotide (NAD)-glycohydrolase (NADase), and the hemolysin streptolysin O (SLO), but not the proteinase streptococcal exotoxin B (SpeB). The phenotype is mostly explained by mutations in the covR/S genes that comprise a two-component signal transduction system that affects the expression of up to 15% of S. pyogenes genes, including many expressing virulence factors (4). MGAS5005 is a representative globally disseminated clone (5).

Historically, group A streptococcus has caused STSS in 60 to 100 patients annually in Japan. However, the frequency suddenly spiked to 122, 197, and 242 during the years 2010, 2011, and 2012, respectively (6). A similar phenomenon occurred in Aichi Prefecture, Japan, where the annual frequency almost doubled between 2010 (11 cases) and 2011 (18 cases) (<http://www.pref.aichi.jp/eiseiken/mag.html>, in Japanese). Therefore, we investigated the presence of hypervirulent S. pyogenes in 27 and 420 isolates from patients with invasive and non-invasive diseases, respectively, in Aichi between 2003 and 2012.

We identified the hypervirulent S. pyogenes from 27 invasive disease cases as follows. The emm genotype was initially determined using the Centers for Disease Control and Prevention (CDC) protocol to detect SpeB production on BHI agar supplemented with final concentrations of 0.3% yeast extract and 1.5% skim milk. The speA or sda1 genes were detected using conventional PCR (7). Table 1 shows that the biochemical and genetic characteristics of emm1 isolates that did not produce SpeB, as the lack of SpeB production has been used as a marker for hypervirulent strains or strains with CovR/S mutation. One of 4 invasive isolates from an invasive disease case harbored both the speA and sda1 genes, and 2 had only speA. Strains of the emm1 genotype, which has been preferentially isolated from cases of severe invasive disease, possessed speA and/or sda1 genes. Thus, these genes might be associated with the onset of invasive diseases. Two of the 4 SpeB non-producers were positive for NADase and SLO (5,8). We then examined HEp-2 cytotoxicity as well as of the...
might affect the intensity of virulence in gene product and a combination of virulence factors (11), although the mechanism of the latter function bored truncated CovS forms but no (9,10). The two isolates were highly cytotoxic and har- tively regulated SpeB exp ression in these 2 isolates same analytical procedures determined that 33 of 420 cases and designated hypervirulent-like (Table 2). The presence of hypervirulent S. pyogenes might be at least partially associated with the sudden in- crease in STSS in Aichi in the same year. The presence hypervirulent-like isolates that emerged during 2011 (Strain C) (Table 2). This hypervirulent-like strain was found among these isolates from fied among the non-invasive isolates, but 1 hyper- virulent-like strain was found among these isolates from rhabic DNase, which allows the bacterium to escape from neutrophil extracellular traps and enhances progression to a hypervirulent covR/S mutant form (11), although the mechanism of the latter function remains obscure (12). The intensity of each virulence gene product and a combination of virulence factors might affect the intensity of virulence in S. pyogenes. The hypervirulent-like strains (Strains B and C) also harbored a truncated CovS mutatation, which is regarded as a marker of hypervirulence (13). Our find- ings, as well as those from previous studies (3,13) sug- gested that these strains are considerably virulent. Thus, the hypervirulent-like isolates that emerged during 2011 might be at least partially associated with the sudden in- crease in STSS in Aichi in the same year. The presence of hypervirulent S. pyogenes isolates that were phenotypically and genetically indistinguishable from covR/S and ropB (rgg) gene polymorphisms that positively regulated SpeB expression in these 2 isolates (9,10). The two isolates were highly cytotoxic and har- bored truncated CovS forms but no covR and ropB gene mutations. One hypervirulent isolate from 2012 (Strain A) and another from 2011 (Strain B) with traits very similar to those of hypervirulent strains but without the sda1 gene, were identified among the 27 invasive disease cases and designated hypervirulent-like (Table 2). The same analytical procedures determined that 33 of 420 isolates from non-invasive disease cases were of emm1 genotype, and that 13 of these isolates produced little or no SpeB (Table 1). Hypervirulent types were not identified among the non-invasive isolates, but 1 hypervirulent-like strain was found among these isolates from 2011 (Strain C) (Table 2). This hypervirulent-like strain lacked the sda1 gene that encodes Sda1, a potent streptococcal DNase, which allows the bacterium to escape from neutrophil extracellular traps and enhances progression to a hypervirulent covR/S mutant form (11), although the mechanism of the latter function remains obscure (12). The intensity of each virulence gene product and a combination of virulence factors might affect the intensity of virulence in S. pyogenes. The hypervirulent-like strains (Strains B and C) also harbored a truncated CovS mutatation, which is regarded as a marker of hypervirulence (13). Our find- ings, as well as those from previous studies (3,13) sug- gested that these strains are considerably virulent. Thus, the hypervirulent-like isolates that emerged during 2011 might be at least partially associated with the sudden in- crease in STSS in Aichi in the same year. 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Table 1. Biochemical and genetic traits of little or no SpeB producing emm1 S. pyogenes strains isolated from patients with invasive and non-invasive diseases

<table>
<thead>
<tr>
<th>Disease type (No. of emm1 isolates)</th>
<th>Little or no SpeB</th>
<th>speA</th>
<th>sda1</th>
<th>NADase / SLO</th>
<th>NADase</th>
<th>SLO</th>
<th>High HEp-2 Cytotoxicity</th>
<th>CovS mutation</th>
<th>RopB mutation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Invasive (n = 14)</td>
<td>4</td>
<td>1/4</td>
<td>2/4</td>
<td>0/4</td>
<td>2/4</td>
<td>0/4</td>
<td>2/2&lt;sup&gt;i&lt;/sup&gt;</td>
<td>2/4&lt;sup&gt;i&lt;/sup&gt;</td>
<td>0/2&lt;sup&gt;i&lt;/sup&gt;</td>
</tr>
<tr>
<td>Non-invasive (n = 33)</td>
<td>13</td>
<td>9/13</td>
<td>4/13</td>
<td>0/13</td>
<td>1/13</td>
<td>1/13</td>
<td>1/13</td>
<td>13/13&lt;sup&gt;i&lt;/sup&gt;</td>
<td>0/13</td>
</tr>
</tbody>
</table>

<sup>i</sup>: No or very slight transparent zone around cultured colonies. <sup>ii</sup>: 50% of cells were killed at 2<sup>2</sup>-fold diluted culture supernatant. <sup>iii</sup>: Two strains producing both of NADase and SLO were examined. <sup>iv</sup>: Truncation form of 57 amino acids (aa) in length. <sup>v</sup>: One truncated form of 123 aa in length; 12 carrying point mutations of 1332V.

Table 2. Characterization of hypervirulent and hypervirulent-like S. pyogenes strains isolated from patients with invasive and non-invasive diseases

<table>
<thead>
<tr>
<th>Disease type (strain ID)/ Reference strain</th>
<th>emm type</th>
<th>Year of isolation</th>
<th>SpeB&lt;sup&gt;i&lt;/sup&gt;</th>
<th>speA</th>
<th>sda1</th>
<th>NADase/SLO</th>
<th>HEp-2 Cytotoxicity&lt;sup&gt;i&lt;/sup&gt;</th>
<th>CovS mutation</th>
<th>RopB mutation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Invasive (A)</td>
<td>1</td>
<td>2012</td>
<td>–</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>Invasive (B)</td>
<td>1</td>
<td>2011</td>
<td>–</td>
<td>–</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Non-invasive (C)</td>
<td>1</td>
<td>2011</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>+</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>Non-invasive (D)</td>
<td>12</td>
<td>2011</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>–</td>
<td>–</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Non-invasive (E)</td>
<td>3</td>
<td>2006</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Non-invasive (F)</td>
<td>12</td>
<td>2007</td>
<td>–</td>
<td>–</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>MGAS5005</td>
<td>1</td>
<td>1996</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>+</td>
<td>–</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>SF370</td>
<td>1</td>
<td>1985</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>+</td>
<td>–</td>
<td>–</td>
</tr>
</tbody>
</table>

<sup>i</sup>: No (–), slight (+), or obvious (++) transparent zone around cultured cells. <sup>ii</sup>: 50% of cells/well killed at 2<sup>2</sup>-fold diluted culture supernatant. <sup>iii</sup>: Length of aa. <sup>iv</sup>: One aa substitution of RopB protein (+). Position of the mutation is shown. <sup>v</sup>: One aa substitution of CovS protein (+). Position of the mutation is shown.

MGAS5005 implied that this clone was globally preva- lent and may have been the cause of STSS since the 1990s. However, this was not so, because macromolecu- lar analysis of MGAS5005 and the hypervirulent strains using pulsed-field gel electrophoresis indicated that they were different genetically (14). covS mutations were also distinctive between them. MGAS5005 predominantly disseminated throughout the world during the 1990s, and since then it has undergone several genetic events such as a phage conversions, deletions, and insertions despite the lack of major phenotypic changes.

Little is known about the prevalence of non-emm1 hypervirulent S. pyogenes strains that are phenotypically and genetically identical to emm1 hypervirulent strains except for the emm genotype. We therefore ex- amined 400 isolates from patients with invasive and non-invasive diseases. Although 13 isolates were classi- fied as belonging to emm genotypes other than emm1, hypervirulent or hypervirulent-like strains were not identified among the 13 invasive isolates (Table 3). Of the non-emm1 isolates from non-invasive diseases cases, 81 of 387 produced little or no SpeB (Table 3). Among these, one harbored the speA and sda1 genes, 9 had the speA gene, 19 had the sda1 gene, 4 produced NADase, and SLO, 16 produced only NADase, and 3 produced only SLO. Of 44 isolates that harbored at least one of the 2 genes or produced at least one of the 2 toxins, 6 were highly cytotoxic, 12 had the CovS mutations (3 resulted in truncated forms and 9 had one amino acid substitution), and 23 harbored RopB mutations, including 2 truncated form and 21 non-synonymous RopB substitutions (Table 3). Taken together, 1 hypervirulent emm12 strain in 2011 (strain D). 2 hypervirulent-like strains harboring emm3 in 2006 (Strain E) and emm12...
which non-creased STSS prevalence in Japan, the predominant Canada, respectively (6,15). During the period of in-
epidemics of extremely invasive diseases in Japan and 
study found that the emm90 or no SpeB, to examine how strains become hyperviru-
ent strains progressed to hypervirulence is thought to be similar to that of emm1 strains, namely, a covS gene mutation that represses SpeB production and increases the production of several virulence deter-
ants such as slo and hasA (16). Our surveillance 
study found that the emm3 (Strain E) and emm12 (Strains D and F) isolates had essentially the same or most of the characteristics of the emm1 hypervirulent isolates. These isolates might have been sufficient to trigger STSS because of their covS gene mutations, decreased the production of SpeB, and enrichment in important virulence factors such as NADase and SLO. However, a large-scale, nationwide investigation of non-emm1 S. pyogenes isolates is required to clarify the pathogenesis of these strains.

Flores et al. isolated S. pyogenes with an invasive, hypervirulent phenotype in a local ulceration site as in the blood of a patient (17). Walker et al. postulated that S. pyogenes with an invasive phenotype is selected at infection sites rather than after entry into the blood-
stream (11). These 2 articles imply that hypervirulent isolates are simultaneously extant at the initial infection site and in the bloodstream of a patient with STSS. The present study isolated hypervirulent or hypervirulent-like strains with emm1, emm3, and emm12 from the initial infection sites in the vagina, pharynx, and pus, respectively. These findings indicated that hypervirulent or hypervirulent-like isolates at local infection sites could be responsible for triggering STSS. That is, the prevalence of hypervirulent or hypervirulent-like isolates in samples from cases of non-invasive diseases might have contributed to the sudden increase in STSS prevalence in Aichi during 2011.

Since hypervirulent-like strains were identified in patients with both invasive and non-invasive diseases, we tried to induce phage-encoded speA or sda1 genes into 96 strains including 4 hypervirulent-like strains from invasive and non-invasive diseases producing little or no SpeB, to examine how strains become hyperviru-
lent (7,18). We found that the hypervirulent-like isolates did not acquire speA or sda1 genes, but conventional PCR verified that one emm89 genotype strain that ac-
quired the sda1 gene. This was notable because emm89 is the predominant emm genotype among recent isolates from patients with STSS in Japan.

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Conflict of interest None to declare.

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