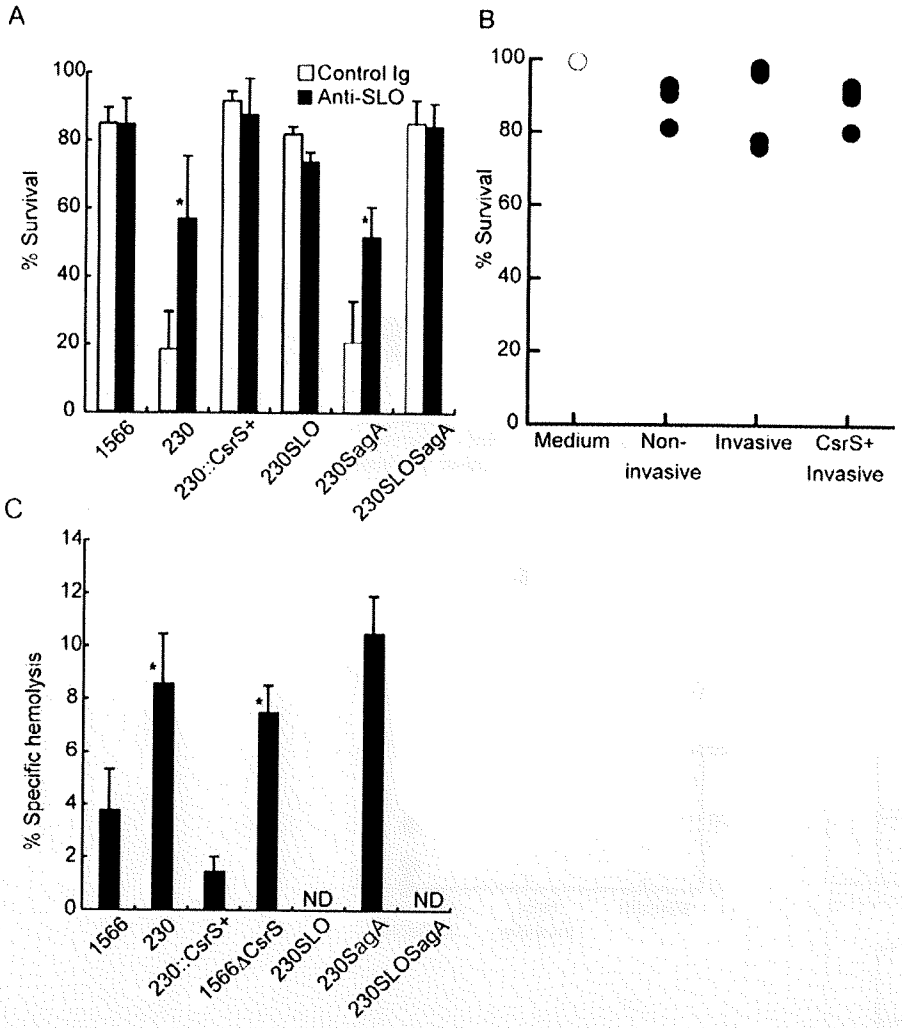


Figure 3. Severe invasive GAS killed PMN by SLO in a contact-dependent manner. (A) The viability of viable PMN that migrated in lower wells of transwell system was estimated as Figure 2. To investigate the role of SLO in PMN survival, lower wells consisted of IL-8 in the presence of either polyclonal rabbit anti-SLO antibodies (25 μg/ml at a final concentration, closed column) or control rabbit IgG (open column), together with either non-invasive GAS (1566), invasive GAS (NIH230), an csrS-transduced NIH230 (NIH230::*csrS+*), an SLO deficient NIH230 mutant (NIH230*slo*), an SLS deficient NIH230 mutant (NIH230*sagA*), or SLO and SLS double mutant (NIH230*slosagA*). Values are mean±SD. *$p<0.05$ estimated by Student's t-test. (B) No substances secreted from GAS reduced PMN viability. PMN was incubated for 2 hours with supernatants from co-culture of IL-8 and invasive or non-invasive GAS and live cell number was examined as described in Figure 2C. (C). SLO-specific hemolytic activity for sheep erythrocytes in supernatants from overnight culture of invasive or non-invasive GAS as listed in (A). Forty-five minutes after incubation, the absorbance of culture supernatants was measured at 540 nm and the SLO-specific hemolytic activity was calculated as described in methods and presented as the mean±SD. The results represent one of two independent experiments. *$p<0.05$ significantly higher than non-invasive strain 1566 estimated by ANOVA. 'ND' represents less than 0.5% hemolysis.
doi:10.1371/journal.pone.0003455.g003

comparable to 1566 strain (Figure 4A), whereas the mutant killed the migrated PMN as well as the parent strain NIH230 (data not shown). These results demonstrate that clinically isolated invasive GAS impaired PMN recruitment and its survival, as a result of productions of ScpC and SLO, respectively.

Enhanced expression of the slo and the scpC genes in severe invasive GAS is attributed to mutation of a transcriptional regulator CsrS

Although sequences of the *slo* gene and the *scpC* gene were identical among clinically isolated non-invasive and severe invasive GAS (data not shown), Figure 5A shows that the *slo* and the *scpC* genes were expressed in the severe invasive GAS greater in extent than those in the non-invasive GAS. The expression of the other virulence-associated genes, such as IgG degrading protease of GAS, Mac-1-like protein (*mac*), nicotine adenine dinucleotide glycohydrolase (*nga*), polysaccharide capsule production (*hasA*), and C5a peptidase (*scpA*), was also upregulted in the severe invasive GAS, greater than that detected in the non-invasive GAS (Figure 5A). Contrarily, the levels of streptococcal pyrogenic endotoxin (*speB*), SLS (*sagA*), and mitogenic factor (*speF*) genes were downregulated in the severe invasive GAS, compared to that found in the non-invasive GAS (Figure 5A and data not shown).

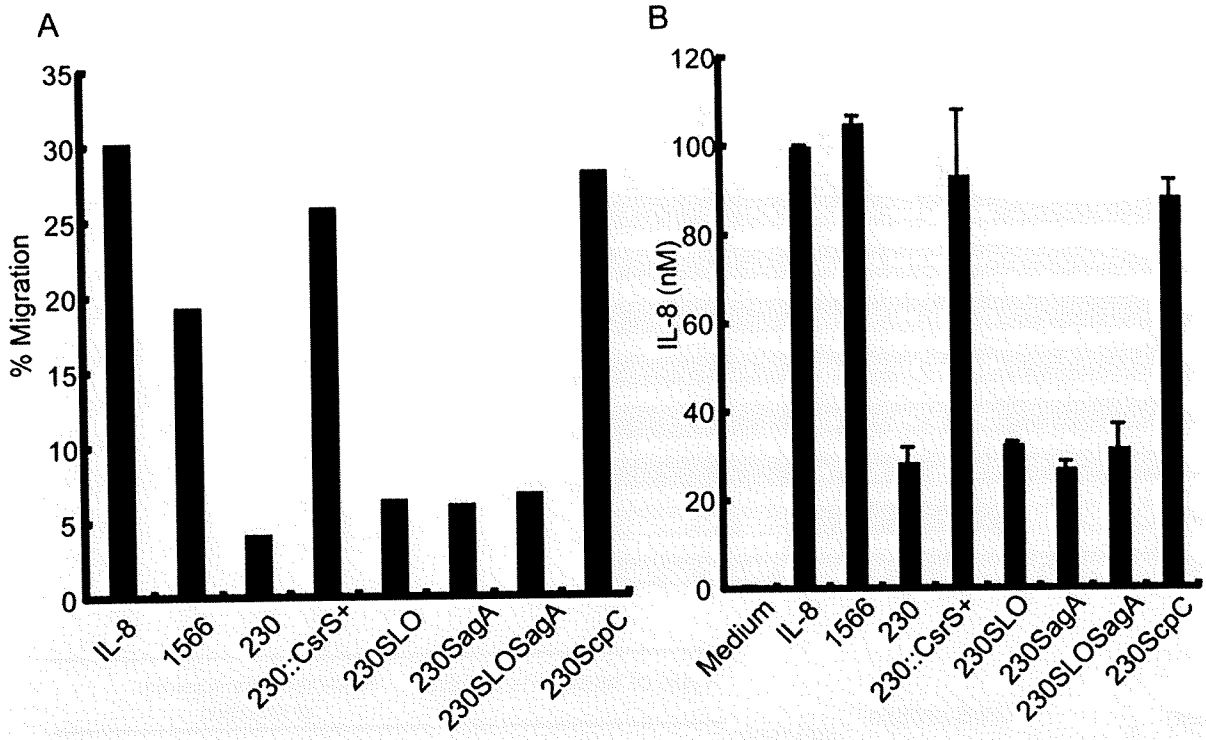


Figure 4. Severe invasive GAS degrade IL-8 by serine protease ScpC, resulting in impaired PMN migration. (A) Migration abilities of PMN in response to IL-8 in the presence of non-invasive and invasive GAS, as listed in Figure 3A plus ScpC deficient NIH230 mutant (NIH230*scpC*). PMNs that migrated into the lower well in a transwell system were estimated by flow cytometry. (B). IL-8 was added into the culture medium (100 nM at a final concentration) and incubated with invasive or non-invasive GAS as listed in (A). Sixty minutes after incubation, the amount of IL-8 in triplicates was measured by sandwich ELISA and presented as the mean±SD. The results represent one of two independent experiments.
doi:10.1371/journal.pone.0003455.g004

These results demonstrate the prominent changes in the transcriptional profile of several virulence-associated genes, including the *slo* and the *scpC*, in the all severe invasive GAS.

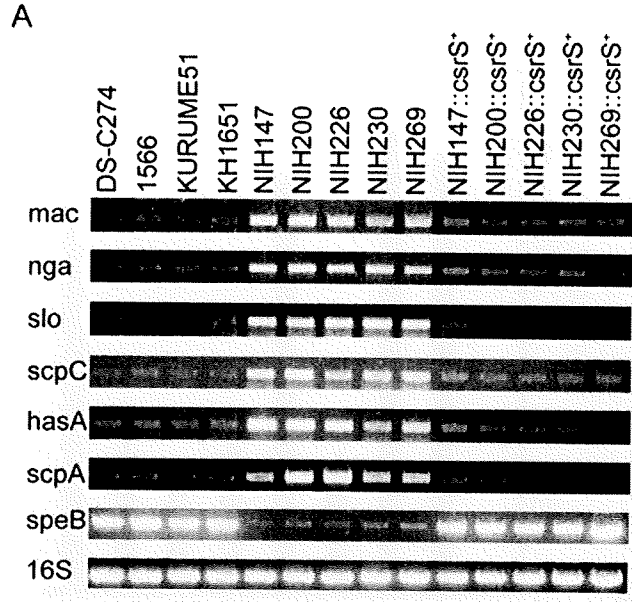
Mutation of *csrR* or *csrS* can cause significant alterations in virulence in mouse models of infection,either increasing lethality or the severity of localized soft tissue lesions [5,6]. GAS isolates from mice with severe invasive disease had mutations in *csrS*, raising the notion that CsrR/S function is important in modulating gene expression during infection. Therefore, we analyzed the linkage between the *csrS* and/or *csrR* genes and the property of invasive GAS infection by sequencing these genes in the *emm49* strains used in this study. The nucleotide sequence of the *csrR* gene was identical in all the isolates, and that of the *csrS* gene was identical among the all non-invasive GAS isolates (data not shown). However, as shown in Figure 5B, the *csrS* genes of all clinically isolated severe invasive GAS had a deletion, a point mutation, or an insertion, thereby, resulting in the creation of translational stop codons (NIH147, NIH226, NIH230, and NIH269) or in a mutation in the presumed kinase domain (NIH200). In order to clarify the role of CsrS regarding expression of the virulence-associated genes and resistance to PMN killing, we introduced the intact *csrS* gene of the 1566 strain into the severe invasive GAS (see Figure 5A). The *csrS*-introduced severe invasive GAS reduced the expression levels of the *slo* and the *scpC* genes, comparable to those detected in the non-invasive GAS. In contrast, the expression of *speB* was upregulated to the level observed in the non-invasive GAS (Figure 5A). In parallel with the expression profile of *slo* and *scpC* in the severe invasive GAS, introduction of the intact *csrS* gene into the severe invasive GAS restored the susceptibility to the killing by PMN ($p = 0.015$ compared with severe invasive isolates +CsrS) (Figure 6A), abrogated the inhibition of PMN migration by degradation of IL-8 ($p = 0.002$ compared with invasive isolates +CsrS) (Figure 4A, 4B, and 6B), and diminished the killing activity for PMN by necrosis ($p = 0.00016$ compared with invasive isolates +CsrS) (Figure 3A, 3C, and 6C), These results strongly suggest that mutations in the *csrS* gene correspond to the immunocompromized activity in the severe invasive isolates, associated with inhibition of PMN recruitment and survival.

csrS mutation is important in the pathogenesis of invasive infections in a mouse model

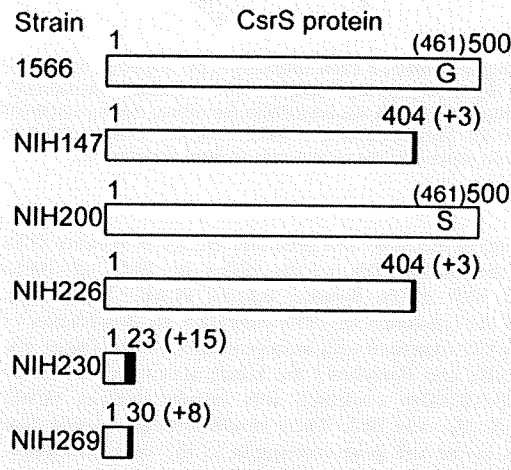
In order to elucidate the role of *csrS*, in infections *in vivo*, we compared the virulence of GAS isolates using a mouse model which infected GAS intraperitoneally. The non-invasive 1566 strain displayed the LD_{50} value approximately 100-fold higher than that of the severe invasive NIH230 strain (Table 1), whereas a *csrS* deletion (1566Δ*csrS*) caused an increase in the LD_{50} value comparable to that of the NIH230 strain. Consistently, an introduction of the intact *csrS* gene into the NIH230 strain (NIH230::*csrS*$^+$) reduced the LD_{50} value to the level observed in the non-invasive strain. These results indicate that *csrS* is an important virulence factor in the mouse model of lethal infections.

As shown in Figure 7A, the NIH230 strain caused bacteremia in mice 24 h after intraperitoneal injection whereas the bacteremia was barely detected in mice infected with NIH230::*csrS*$^+$as well as

A



B



Type of mutations in csrS	Predicted consequence of mutation
Deletion of nucleotides 1215-1219, GAAAA	Creation of stop codon
Change G to A at nucleotide 1381	Reduced phospholylation
Deletion of nucleotides 1215-1219, GAAAA	Creation of stop codon
Change GTTCTTTTTT to TCTGCATTTTC at nuclectide 73-82	Creation of stop codon
Insertion of ATTTTCTCTGC at nucleotide 99	Creation of stop codon

Figure 5. Mutation of the *csrS* gene in the isolates of the patients with severe invasive infections is responsible for increased virulence of GAS. (A) Expression of virulence-associated genes in non-invasive and invasive GAS isolates and mutants transduced with *csrS*, analyzed by RT-PCR. The expression of virulence-associated factors mRNA: IgG degrading protease of GAS, Mac-1-like protein (*mac*), nicotine adenine dinucleotide glycohydrolase (*nga*), *slo*, *scpC*, polysaccharide capsule production (*hasA*), C5a peptidase (*scpA*), and streptococcal pyrogenic endotoxin (*speB*), plus expression of 16S rRNA (*16S*) were shown. (B) The *csrS* mutations in GAS isolates from the patients with severe invasive streptococcal infections. The numbers at the end of the bars indicate the total amino acid residues of CsrS proteins from the start codon in non-invasive GAS (1566) and invasive Gas from the patients (NIH147, NIH200, NIH226, NIH230 and NIH 269). Solid boxes represent the newly created amino acids as a result of frameshift mutations, with length of amino acids ("+ number" within parentheses). In the NIH200 strain, Ser replaced Gly at position 461 of the CsrS protein. Type of mutations are listed at the end of bars.
doi:10.1371/journal.pone.0003455.g005

the 1566 strain ($p = 0.005$ compared with non-invasive isolates, and $p = 0.005$ compared with invasive isolates +CsrS). Histopathologically, in the mice injected with the NIH230 strain, bacteria formed clusters in interstitial tissues in the kidneys and the lungs, accompanied congestion and no inflammatory cells at infectious foci (Figure 7B, 7C and data not shown). Contrarily, no significant pathological alterations were observed in the mice injected with the 1566 and NIH230::*csrS*$^+$ strains (Figure 7B and 7C). Figure 7D shows that subcutaneous infection of NIH230 formed the infected lesions with area significantly larger than those of 1566 and NIH230::*csrS*$^+$. These results suggest that the invasive GAS isolates are more virulent *in vivo* than non-invasive GAS, and impair PMN function *in vivo*, owing, at least in part, to the mutations in the *csrS* gene.

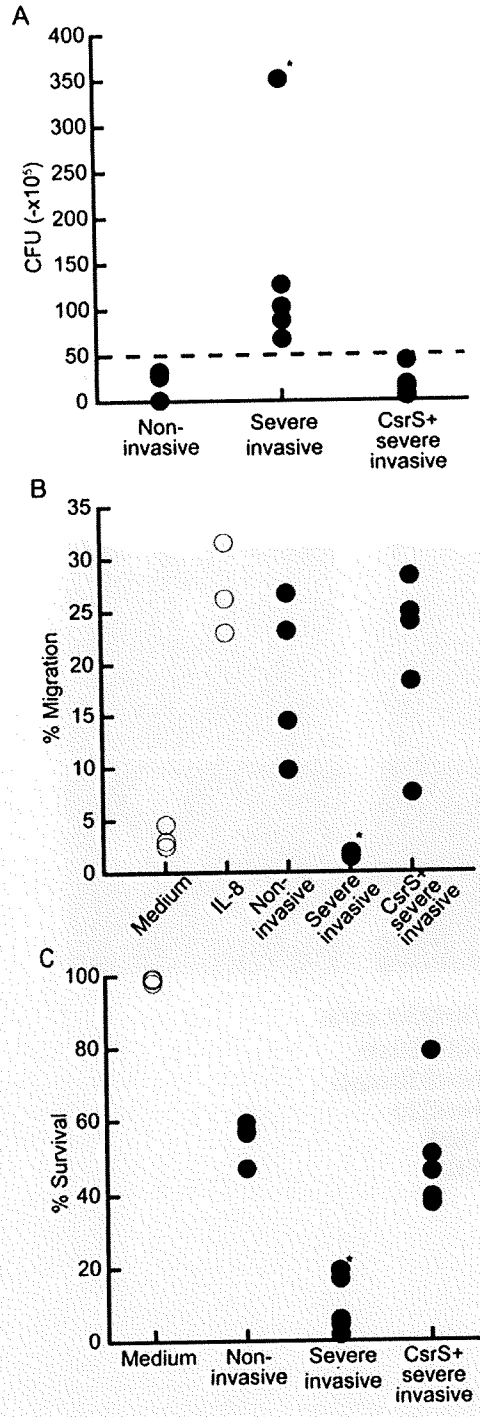


Figure 6. Mutations of CsrS is responsible for increased virulence of GAS to PMN functions. (A) Non-invasive, severe invasive, and invasive strains with overexpression of CsrS strains were incubated with PMN at MOI 10. After 2 hours, the number of live bacteria was counted. The dotted line indicates the number of bacteria applied to the culture. (B)–(C) CsrS transduction into the invasive GAS isolates abrogated the killing activity for PMN as well as the inhibitory effect on PMN migration in a transwell system. (B) The proportion of PMN consisted of both viable and dead cells and (C) The proportion of live PMN that migrated into the lower wells in response to IL-8, in the presence (closed circles) or absence (open circles) of non-invasive GAS, severe invasive GAS isolates or severe invasive GAS isolates transduced with CsrS was analyzed at 60 minutes incubation, as described in Figure. 2B and 2C. *p<0.05 estimated by ANOVA.
doi:10.1371/journal.pone.0003455.g006

scpC and *slo* are insufficient singly for the pathogenesis of invasive infections

Finally, we assessed the influence of enhanced expression of the *scpC* or the *slo* gene on the virulence in a mouse model. As shown in Table 1, NIH230*scpC* and NIH230*slo* exerted the LD_{50} value 3–10 fold lower than that of the non-invasive isolate 1566, but 10–30 fold higher than that of severe invasive isolates. Subcutaneous inoculation of NIH230*scpC* and NIH230*slo* yielded the local infected lesions with area comparable to those of 1566 and NIH230::*csrS*$^+$ during the course of infection (Figure 7D). These results suggest that enhanced expression of ScpC and SLO in invasive GAS plays an important role *in vivo* virulence of GAS infection.

Discussion

It have been demonstrated that CsrS/R is a member of the two-component regulatory systems for regulating the multipe virulence factors of GAS, by using genetically- manipulated GAS mutants [20]. The present study demonstrate that the loss-of-functional mutations in *csrS* gene which were accumulated in clinically isolated GAS from patients with severe invasive infections, but not with *emm*-matched non-invasive strains. The *csrS* mutations enhanced the expression of *scpC* and *slo*, associated with the evasion of PMN functions and *in vivo* virulence. Introduction of the intact *csrS* gene into the severe invasive GAS restored the susceptibility to the killing by PMN and abrogated the activity for inhibition of PMN recruitment and survival, thus, demonstrating an instructional role of the loss-of-functional mutations in *csrS* gene for the evasion of PMN functions, providing unique pathophysiology of invasive GAS infections. Previous studies using animal-passaged GAS have shown that mutations in both *csrS* and *csrR* gene are important for the invasive phenotype [5,6], and mutation frequency of *csrS* and *csrR* seems to be the same [21]. However, the severe invasive isolates analyzed in this study accumulated mutations in the *csrS* gene but not in the *csrR* gene (Figure 5B and data not shown). Then we further examined whether severe invasive isolates of other than emm49 genotype have the mutation of the *csrS* and the *csrR* genes. The frequency of the mutation in the *csrS* gene is higher than that in *csrR* (*csrS* mutation:*csrR* mutation = 59:19) (manuscript in preparation), suggesting the *csrS* mutation is more important in comparison with that of *csrR* in the clinical isolates regardless of *emm* genotypes. Furthermore, the expression of some human invasive disease-

Table 1. LD_{50} values of each strain.

Strain	LD_{50} value
1566	1.03×10^8
NIH230	1.11×10^6
NIH230::*csrS*$^+$	1.52×10^8
1566Δ*csrS*	8.60×10^5
NIH230*slo*	3.33×10^7
NIH230*scpC*	1.04×10^7

doi:10.1371/journal.pone.0003455.t001

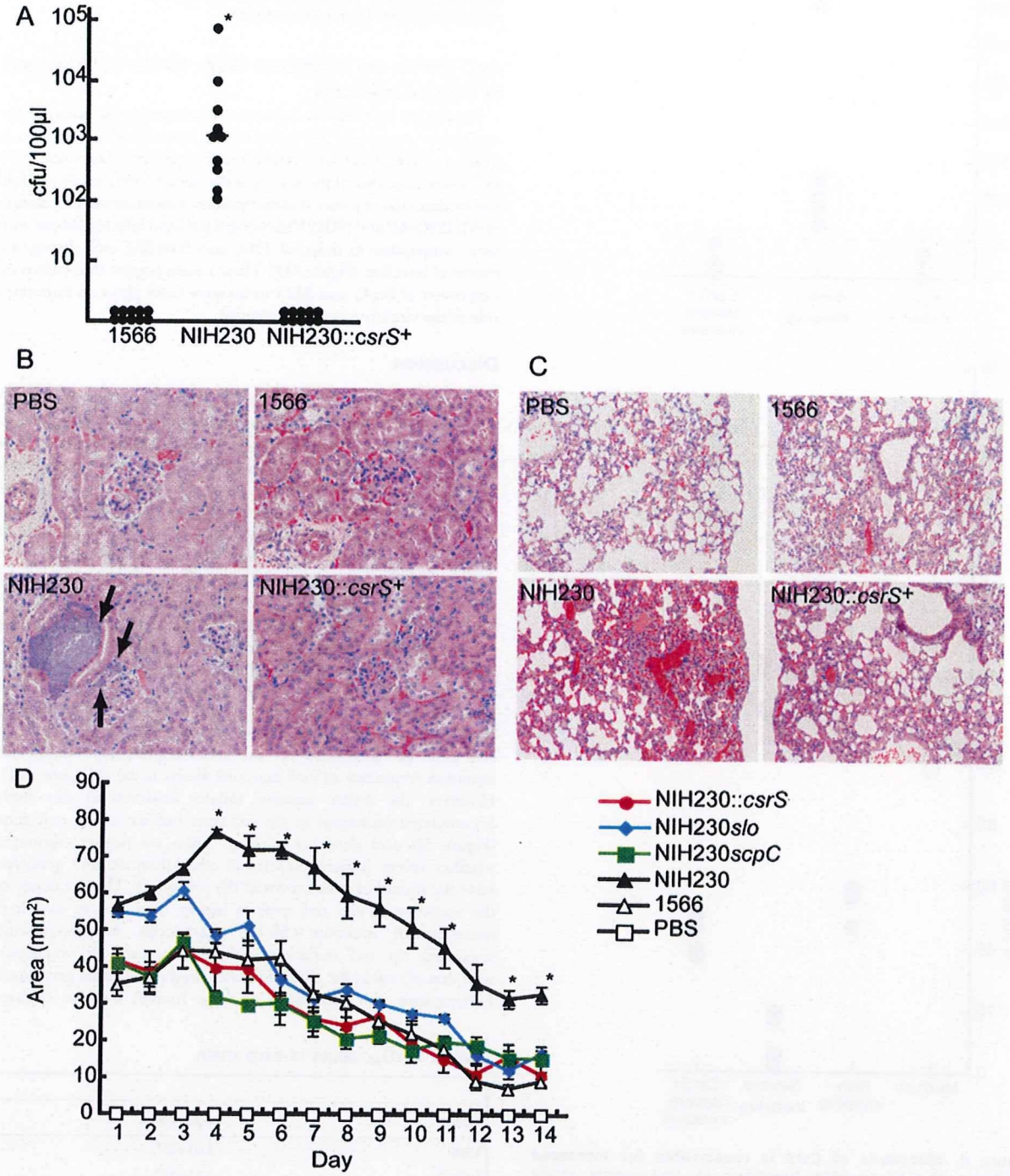


Figure 7. Mutations of the csrS, SLO and ScpC regulate in vivo virulence of GAS in a mouse model. (A) Number of GAS organisms recovered from the blood (100 μL) of each Male ddY mice injected intravenously with 1×10^7 CFU in 100 ml suspension of GAS in PBS. Blood was taken 24 h after injection and the bacterial count was determined after plating on agar. The (-) bar represents median values. *($p<0.05$) estimated by ANOVA. Histopathological changes in the (B) kidney and (C) lungs of mice infected with GAS. Each tissue was extracted 24 h after injecting GAS (1×10^7 CFU). The black arrows indicate clusters of bacteria. (D) The course of subcutaneous infection in hairless mice injected with 1×10^7 CFU in 100 μL suspension of GAS in PBS. Lesion area and body weight were measured daily after infection. Values are mean±SEM ($n=5$). *Area of skin lesion in NIH230 infected mice was significantly higher than all other groups ($p<0.05$) estimated by ANOVA.
doi:10.1371/journal.pone.0003455.g007

associated genes [5] including *slo* was enhanced in the *csrS* mutant (Figure 5B), but not in the *csrR* mutant [20]. On the contrary of a dogma that CsrS/R is a definitive member of the two-component regulatory systems, which involve a coordinate pair of proteins known as the sensor kinase and the response regulator [20], CsrS may transmit a signal not only to CsrR but also to other regulators. This dominant role of CsrS is the first important observation in this study, and its mutation is possibly more important than that of *csrR* in terms of etiopathogenesis of human severe invasive diseases.

Numbers of studies has pointed out virulent factors to evade host defense using genetically-manipulated GAS and animal models [18,22,23], although the significance of each factor to invasive infection is diverse and sometimes controversial, perhaps due to lack of proper non-invasive counterpart. As examples, SpeB [22] and SLS [23] have been proposed as an invasive infection-associated factor by its cytotoxic effect, however, *speB* and *sagA* expression is not enhanced in any *csrS*-mutated severe invasive GAS isolate used in this study and others [5,24]. Furthermore, SLS hemolytic activity of invasive GAS is significantly decreased as compared with non-invasive strains (data not shown) and SLS-deletion in invasive GAS did not affect PMN survival at all, excluding the possibility for the role of SLS in PMN necrosis seen in this study. Extracellular deoxyribonuclease (DNase) is a virulence factor that protects *emm1* type GAS against neutrophil killing by degrading the DNA framework of neutrophil extracelluar traps (NETs) [25,26]. However, we confirmed that addition of DNase in the culture did not alter the level of PI-positive PMN, meaning bright PI staining of PMN is not due to release of NETs from PMN (Figure S1A). DNase activity of the *emm49* severe invasive GAS was lower than that of non-invasive GAS (Figure S1B), possibly due to the difference of *emm* type. The expression of DNase as well as the *slo* and the *scpC* genes in *emm1*-genotype strains was enhanced under the *csrS* mutation [5]. These suggest that DNase may be important but redundant for induction of invasive diseases. Therefore, the second important observation in the present study is that an essential requirement of *csrS* mutation for invasive infection is associated with increased expression of ScpC and SLO and *in vitro* evasion of PMN functions, though we do not exclude the possibility that other CsrS-regulating factors contribute to the escape of invasive GAS from host defense.

SLO and ScpC independently enable GAS to escape from PMN functions; Present data using clinical isolated GAS and a *scpC*-deletion mutant (Figure 4A and 4B) show that enhanced production of serine proteinase ScpC in virulent GAS is essential to impair PMN migration *in vitro* by degradation of IL-8, as others partially have demonstrated [17–19]. The present study also uncovers that increased activity of SLO from invasive GAS isolates induces rapid and extensive necrosis to human PMN. SLO is a cholesterol-binding pore-forming hemolysin as well as cytotoxic for other cells [15]. A study has demonstrated SLO from invasive GAS lyse PMN [27], however this effect is likely due to complement activation by SLO [28] or PMN activation [29] but not due to cytotoxity of SLO itself as judged by their flow cytometry profiles which are distinct from ours (Figure 2A). In the present study, we observed that SLO concentration in a short-time culture with severe invasive GAS did not reach the threshold level to kill PMN by formation of pores (data not shown) and that PMN did not undergo necrosis upon incubation with culture media of severe invasive GAS (Figure 3B), leading to the novel possibility that PMN are probably killed following encounter with invasive GAS in a contact-dependent manner. PMN-binding GAS may make a small interface containing a high concentration of SLO between bacteria and PMN, which resembles to killing mechanism of killer cells to target cells [30]. Collaboration of SLO with other toxins may be critical to induce PMN necrosis as similarly mechanism has been reported [31], although it remains to be examined whether there exist explore interaction-associated molecules on both host and bacterial membrane is needed.

In contrast to the previous view [18], we observed that both of ScpC and SLO together, but not each of them, mediated sufficient *in vivo* virulence (Table 1), thus compatible with the notion that plural virulence-associated factors under the regulation of *csrS* abrogate PMN bactericidal functions and induce invasive diseases in *in vivo* animal model. Consistently, the high mortality and histopathological findings which lacks PMN infiltration in mice tissues infected with *csrS*-mutated GAS (Figure 7) are similar to those seen in clinical invasive GAS infections [32]. Thus, these results suggest that the ability of incompetence for PMN functions by individual GAS strain may determine the induction and clinical outcome of invasive diseases. Several clinical reports seem to support this hypothesis; Leukocytopenia seen in patients with STSS is more severe than that with non-STSS [33], and invasive GAS-infected patients with leukocytopenia show worse prognosis than those without leukocytopenia [33,34]. Furthermore, predisposing factors for severe invasive GAS infection [9], such as diabetes mellitus [35], liver cirrhosis [36], and congestive heart failure [37] are known to impair PMN function. These evidences suggest that the level of PMN function is one of the critical factors to determine the threshold for the onset of invasive GAS infection, which may be the reason for rare outbreaks of invasive GAS infections.

Thus, enhanced expression of virulence factors that could evade PMN function is a key issue at first step to cause invasive bacterial infections. A further study in which collates clinical with bacterial/immunological data may provide with novel clues for early diagnosis and therapeutics of invasive bacterial infections.

Methods

Bacterial strains and culture

The *S. pyogenes* strains used in this study are described in Table S1 [8,38]. *Escherichia coli* DH5α was used as the host for plasmid construction and was grown in liquid Luria-Bertani medium with shaking or on agar plates at 37°C. *S. pyogenes* was cultured in Todd-Hewitt broth supplemented with 0.5% yeast extract (THY medium) without agitation or on tryptic soy agar supplemented with 5% sheep blood. Cultures were grown at 37°C in a 5% CO_2 atmosphere. When required, antibiotics were added to the medium at the following final concentrations: erythromycin, 500 μg/mL for *E. coli* and 1 μg/mL for *S. pyogenes*; spectinomycin (Sp), 25 μg/mL for *E. coli* and *S. pyogenes* both. The growth of *S. pyogenes* was turbidimetrically monitored at 600 nm using Mini-Photo 518R (Taitec, Tokyo, Japan).

Animals

Male 5–6-week-old outbred ddY and hairless mice were purchased from SLC (Shizuoka, Japan) and were maintained in a specific pathogen-free (SPF) condition. All animal experiments were performed according to the guidelines of the Ethics Review Committee of Animal Experiments of the National Institute of Infectious Diseases, Japan.

Isolation of human PMN

PMN were taken from nine healthy volunteers which were composed of 25–52 years old, 7 males and 2 female, and were isolated from venous blood of them using in accordance with a

protocol approved by the Institutional Review Board for Human Subjects, National Institute of Infectious Diseases.

DNA manipulation

DNA amplifications by PCR, DNA restriction-endonuclease digestions, ligations, plasmid preparations, and agarose gel electrophoresis were performed according to standard techniques [42]. PCR reactions were performed using TaKaRa Ex Taq (TaKaRa Bio, Tokyo, Japan). Nucleotide sequence was determined by using the automated sequencer ABI PRISM 3100 Genetic Analyzer (Applied Biosystems, Tokyo, Japan)

Transformation

Calcium chloride ($CaCl_2$) competent *E. coli* cells were prepared and transformed according to a standard protocol [39]. Electrocompetent *S. pyogenes* cells were prepared as described [38].

Construction of deletion or deficient mutants

(i) Construction of the *csrS* mutants. A 1002-bp DNA fragment containing the 5′ terminal of *csrS* and the adjacent upstream chromosomal DNA was amplified from the 1566 chromosomal DNA using the primers for *csrSdel1* and *csrSdel2* (Table S2), and a 1104-bp fragment containing the 3′ terminal of *csrS* and the adjacent downstream chromosomal DNA was amplified from the NIH230 chromosomal DNA using the primers for *csrSdel3* and *csrS4del4* (Table S2); these 2 PCR products were ligated by *Bam*HI and *Eco*RI and by *Eco*RI and *Pst*I, respectively. The digested fragments were cloned into the erythromycin-resistant and temperature-sensitive shuttle vector pJRS233 [40] in order to create the plasmid pJRSΔ*csrS*. This plasmid was then purified from *E. coli* and introduced into the strains NIH230 and 1566 by electroporation. The transformants were selected by observing the growth on erythromycin agar at 30°C. The cells in which pJRSΔ*csrS* had been integrated into the chromosome were selected by the growth of the transformants at 39°C with erythromycin selection. The plasmid-integrated strain was serially passaged in a liquid culture at 30°C without erythromycin selection in order to facilitate the excision of the plasmid and thus, leaving the desired mutation in the chromosome. The replacement of the native *csrS* gene by the *csrS*-deleted mutant allele was verified by PCR, and the resultant strains were named as NIH230Δ*csrS* and 1566Δ*csrS*, respectively.

(ii) Construction of the *slo* mutant. A 1061-bp DNA fragment containing the internal region of *slo* was amplified from the NIH230 chromosomal DNA using the primers for *slo-del3* and *slo-del4* (Table S2). The PCR products were ligated by *Bam*HI and *Eco*RI. This fragment was then cloned into the integration shuttle vector pSF152 [41] to create the plasmid pSF152*slo* that was then used for the chromosomal inactivation of the *slo* gene, as described previously [40]. The inactivated mutant strain NIH230*slo* (*slo*::*aad9* Sp^r) was then selected by using spectinomycin-containing agar plates. Deficiency of the native *slo* gene was verified by PCR. Loss of SL hemoltitic activity of these mutants was confirmed by the standard SLS hemolysis assay (Figure 3C).

(iii) Construction of the *sagA* mutants. A 635-bp DNA fragment containing the 5′ terminal of *sagA* and the adjacent upstream chromosomal DNA was amplified from the NIH230 chromosomal DNA using the primers for sagA0-Xb and sagA2-Bm (Table S2) and a 1037-bp fragment containing the 3′ terminal of sagA and the adjacent downstream chromosomal DNA was amplified from the NIH230 chromosomal DNA using the primers for sagA3-Bm and sagA4-Ps; these 2 PCR products were ligated by *Xba*I and *Bam*HI and by *Bam*HI and *Pst*I, respectively. The digested fragments were then cloned into the temperature-sensitive shuttle vector pJRS233 to create the plasmid pJRSΔsagA that was then used to create NIH230ΔsagA, as described above. Loss of SLS hemoltitic activity of these mutants was confirmed by the standard SLS hemolysis assay.

(iv) Construction of the *scpC* mutant. A 1240-bp DNA fragment containing the internal region of *scpC* was amplified from the NIH230 chromosomal DNA using the primers for *scpC-del5* and *scpC-del6* (Table S2). The PCR products were ligated by *Bam*HI and *Eco*RI. This fragment was then cloned into the integration shuttle vector pSF152 [41] to create the plasmid pSF152*scpC* that was then used to create NIH230*scpC*, as described above.

Construction of the strains integrating the intact *csrS* gene

The *csrS* gene replacement was performed by allelic recombination. Specifically, the chromosomal DNA derived from the GAS strain 1566 was purified and used as a template for the PCR amplification of the *csrS* gene. The primers used were 5′-GGGGATCCTGAGATTCCTCTCACTAAAC-3′ (sense) and 5′-GGGAATTCTCTAATACACTATTTTACC-3′ (antisense). The PCR fragment was ligated into the plasmid pSF152 [41], and the resultant plasmid pSF*csrS* was used for chromosomal integration into the mutated *csrS* gene of isolates from patients of severe invasive infections, as described previously [41]. The integrated strains (Sp^r) were then selected by using spectinomycin (Sp)-containing agar plates. The integration of the *csrS* gene was confirmed by PCR.

RT-PCR

S. pyogenes was grown in THY media at 37°C without aeration, and total RNA was extracted at OD_{600} of 0.75 by using the RNeasy Mini extraction kit (Qiagen). RT-PCR was performed by using a One Step RNA PCR Kit (AMV) (TaKaRa Shuzo Co., Kyoto, Japan) according to the manufacturer's recommendation using the RT-PCR primer pairs shown in Table S3.

GAS infection in a mouse model

To determine LD_{50}, We injected several dilutions of 0.5 mL of GAS isolate suspensions in phosphate-buffered saline (PBS) intraperitoneally into male 5–6-week-old ddY outbred mice (5 mice/dilution, 9 dilutions for each GAS isolate). The exact numbers of the colony-forming units of the injected bacteria were determined by incubating adequate dilutions of each GAS sample on sheep blood agar plates. The data were analyzed for significance according to the Probit method to determine the LD_{50} values for a 7-day period. For a subcutaneous infection model, male hairless mice Hos:Hr-1 were injected 1×10^7 CFU in 100 µl suspension of GAS in PBS. Lesion area and body weight were measured daily, and analyzed

Histopathology

For histological analysis, the tissues from GAS-infected mice were fixed in 10% formalin/PBS. The paraffin-embedded sections were stained with hematoxylin and eosin (Sapporo General Pathology Laboratory Co. Ltd., Hokkaido, Japan).

Phagocytosis and killing assay

Phagocytosis and killing assay by PMN were performed as previously described with some modifications [14]. 5×10^5 human PMN and 5×10^6 bacteria opsonized with human plasma and labeled with alexa 488 (Invitrogen, Carlsbad, CA) in a well of 24 well plates. After incubation for 60 min, PMN were harvested and stained with alexa-594 labeled anti-alexa 488 polyclonal Ab (Invitrogen) in order to distinguish non-phagocytosed but attached bacteria to PMN. The proportion of phagocytosed PMN were analyzed by FACS Calibur (BD Biosciences, San Jose, CA), For killing assay, PMN and opsonized bacteria in the same MOI as phagocytosis assay were incubated for 2 hours at 37°C, adding antibiotics at 60 min to eliminate non-phagocytosed bacteria. Corrected PMN were lysed in 0.1% saponin / PBS for 20 min on ice. Bacteria were washed with PBS and cultured on soy beans agar plate overnight, for counting the number of colonies.

Migration assay

Chemotaxis assay were performed as previously described with modification [42]. Briefly, 5×10^5 PMN in RPMI medium containing 25 mM HEPES and 1% FCS were in Transwell inserts (3 μm pore size, Coaster, Corning, NY) placed in 24-well plates containing 600 μl medium, or 100 nM IL-8 solution (Peprtec, London, UK), which were incubated with or without 5×10^6 bacteria for 1 hour at 37°C in advance of the assay. After 1 hour incubation, cells in the lower wells were collected and 10^4 10 μm microsphere beads (Polysciences Inc., Warrington, MA) were added. Cells were stained with propidium iodine (Sigma, St Louis, MI) for flow cytometry to quantify viable PMN and were analyzed using FACSCalibur. In some experiments, cholesterol (Sigma), 25 μg/mL anti-SLO polyclonal Ab (American Research Product, Inc., Belmont, MA), or rabbit IgG was added in 24 well plates.

ELISA

The amount of IL-8 in supernatant after incubation with bacteria was determined by Ready-to-Go human IL-8 ELISA kit (eBioScience, San, Diego, CA) according to manufacturers' protocol.

SLO-hemolysis assay

The activity of SLO in supernatant is measured as previously described [43]. Briefly, overnight culture supernatants of various strains were subjected to centrifugation, and were filtrated through a 0.45 μm membrane. Dithiothreitol and trypan blue were then added to each sample to a final concentration of 4 mM and 13 μg/mL respectively, and the mixtures were incubated at room temperature for 10 min. A 0.2 ml aliquot of 5% (v/v) sheep erythrocyte in PBS was added to 0.4 ml of each treated sample. After 30 min incubation at 37°C, the mixtures were subjected to centrifugation, and absorbance of the supernatants fluids was measured at 540 nm. To confirm that hemolysis was due to SLO, control reaction including culture supernatants to which water-soluble cholesterol (Sigma), a specific SLO inhibitor, had been added to yield a final concentration of 250 μg/mL.

SLS-hemolytic assay

Overnight culture of various strains were frozen at −80°C, thawed, and centrifuged to obtain the supernatants. Serially diluted culture supernatants (0.1 ml) in PBS containing 250 μg/mL water-soluble cholesterol were incubated at room temperature for 10 min. A 0.1 ml aliquot of 5% (v/v) sheep erythrocyte was added and incubated for 1 h at 37°C. The mixture were subjected to brief centrifugation, and absorbance of the supernatants fluids was measured at 540 nm. To confirm that hemolysis was due to SLS, control reaction including culture supernatants to which trypan blue (Sigma), a specific SLS inhibitor, had been added to yield a final concentration of 13 μg/ml.

Supporting Information

Figure S1 DNase activity is not involved in the virulence of emm49 severe invasive GAS isolates. a) To investigate the role of DNase in PMN survival, the viability of PMN that migrated in lower wells of transwell system was estimated as PMNs were applied into the upper well (5×105 cells) of a transwell system, and lower wells consisted of IL-8 in the presence or absence of DNase I (100 mg/ml at a final concentration), together with either non-invasive GAS (1566), or invasive GAS (NIH230). PMN migrated in lower wells were stained with propidium iodine and were analyzed using flow cytometry. b) Activity of DNase in emm49 GAS. 10 ng of Calf thymus DNA was incubated with or without culture supernatants from non-invasive, severe invasive, and CsrS-transduced severe invasive GAS for 15 min at 37°C. Activity to degrade calf thymus DNA was visualized by 1% agarose gel electrophoresis. Methods in vitro migration assay As shown 5×105 PMN in RPMI medium containing 25 mM HEPES and 1% FCS were in Transwell inserts (3 μm pore size, Coaster) placed in 24-well plates containing 600 μl medium, 100 nM IL-8 solution (Peprtec), 100 μg/mL deoxyribonuclease I (Sigma, St Louis, MI) which were incubated with or without 5×106 bacteria for 1 hour at 37°C in advance of the assay. After 1 hour incubation, cells in the lower wells were collected and 104 10 μm microsphere beads (Polysciences) were added. Cells were stained with propidium iodine (Sigma) for flow cytometry to quantify viable PMN and were analyzed using FACSCalibur (BD BioScience). DNase activity assays Supernatants were collected from overnight cultures of bacterial strains grown in THB. Calf thymus DNA (10 ng) was combined with bacterial supernatant in final volume of 50 ml buffer (300 mM Tris-HCl (pH 7.5), 3 mM CaCl2, 3 mM MgCl2) for 15 min at 37°C. To halt DNase activity, 10 ml of 0.5 M EDTA (pH 8.0) was added to the reaction. Visualization of DNA degrad tion was done in 1% agarose gel electrophoresis.
Found at: doi:10.1371/journal.pone.0003455.s001 (0.60 MB TIF)

Table S1 Strains and plasmids used in this study
Found at: doi:10.1371/journal.pone.0003455.s002 (0.03 MB DOC)

Table S2 Primers used for the construction of deletion mutants
Found at: doi:10.1371/journal.pone.0003455.s003 (0.03 MB DOC)

Table S3 Primers used in RT-PCR
Found at: doi:10.1371/journal.pone.0003455.s004 (0.03 MB DOC)

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Author Contributions

Conceived and designed the experiments: MA TI HW. Performed the experiments: MA TI HK. Analyzed the data: MA TI TT. Contributed reagents/materials/analysis tools: MA TI HK HW. Wrote the paper: MA TI TT HW.

References

1. Bisno AL, Brito MO, Collins CM (2003) Molecular basis of group A streptococcal virulence. Lancet Infect Dis 3: 191–200.
2. Cunningham MW (2000) Pathogenesis of group A streptococcal infections. Clin Microbiol Rev 13: 470–511.
3. Davies HD, McGeer A, Schwartz B, Green K, Cann D, et al. (1996) Invasive group A streptococcal infections in Ontario, Canada. Ontario Group A Streptococcal Study Group. N Engl J Med 335: 547–554.
4. Mitchell TJ (2003) The pathogenesis of streptococcal infections: from tooth decay to meningitis. Nat Rev Microbiol 1: 219–230.
5. Sumby P, Whitney AR, Graviss EA, DeLeo FR, Musser JM (2006) Genome-wide analysis of group a streptococci reveals a mutation that modulates global phenotype and disease specificity. PLoS Pathog 2: e5.
6. Walker MJ, Hollands A, Sanderson-Smith ML, Cole JN, Kirk JK, et al. (2007) DNase Sda1 provides selection pressure for a switch to invasive group A streptococcal infection. Nat Med 13: 981–985.
7. Ikebe T, Murai N, Endo M, Okuno R, Murayama S, et al. (2003) Changing prevalent T serotypes and emm genotypes of Streptococcus pyogenes isolates from streptococcal toxic shock-like syndrome (TSLS) patients in Japan. Epidemiol Infect 130: 569–572.
8. Ikebe T, Endo M, Ueda Y, Okada K, Suzuki R, et al. (2004) The genetic properties of Streptococcus pyogenes emm49 genotype strains recently emerged among severe invasive infections in Japan. Jap J Infect Dis 57: 187–188.
9. ith A, Lamagni TL, Oliver I, Efstratiou A, George RC, et al. (2005) Invasive group A streptococcal disease: should close contacts routinely receive antibiotic prophylaxis? Lancet Infect Dis 5: 494–500.
10. Hidalgo-Grass C, Dan-Goor M, Maly A, Eran Y, Kwinn LA, et al. (2004) Effect of a bacterial pheromone peptide on host chemokine degradation in group A streptococcal necrotising soft-tissue infections. Lancet 363: 696–703.
11. Nathan C (2006) Neutrophils and immunity: challenges and opportunities. Nat Rev Immunol 6: 173–182.
12. Urban CF, Lourido S, Zychlinsky A (2006) How do microbes evade neutrophil killing? Cell Microbiol 8: 1687–1696.
13. Voyich JM, Musser JM, DeLeo FR (2004) Streptococcus pyogenes and human neutrophils: a paradigm for evasion of innate host defense by bacterial pathogens. Microbes Infect 6: 1117–1123.
14. Kobayashi SD, Braughton KR, Whitney AR, Voyich JM, Schwan TG, et al. (2003) Bacterial pathogens modulate an apoptosis differentiation program in human neutrophils. Proc Natl Acad Sci U S A 100: 10948–10953.
15. Bhakdi S, Bayley H, Valeva A, Walev I, Walker B, et al. (1996) Staphylococcal alpha-toxin, streptolysin-O, and Escherichia coli hemolysin: prototypes of pore-forming bacterial cytolysins. Arch Microbiol 165: 73–79.
16. Hirsch JG, Bernheimer AW, Weissmann G (1963) Motion picture study of the toxic action of streptolysins on leucocytes. J Exp Med 118: 223–228.
17. Edwards RJ, Taylor GW, Ferguson M, Murray S, Rendell N, et al. (2005) Specific C-terminal cleavage and inactivation of interleukin-8 by invasive disease isolates of Streptococcus pyogenes. J Infect Dis 192: 783–790.
18. Hidalgo-Grass C, Mishalian I, Dan-Goor M, Belotserkovsky I, Eran Y, et al. (2006) A streptococcal protease that degrades CXC chemokines and impairs bacterial clearance from infected tissues. EMBO J 25: 4628–4637.
19. Sumby P, Zhang S, Whitney AR, Falugi F, Grandi G, et al. (2008) A chemokine-degrading extracellular protease made by group A Streptococcus alters pathogenesis by enhancing evasion of the innate immune response. Infect Immun 76: 978–985.
20. Federle MJ, McIver KS, Scott JR (1999) A response regulator that represses transcription of several virulence operons in the group A streptococcus. J Bacteriol 181: 3649–3657.
21. Engleberg NC, Heath A, Miller A, Rivera C, DiRita VJ (2001) Spontaneous mutations in the CsrRS two-component regulatory system of Streptococcus pyogenes result in enhanced virulence in a murine model of skin and soft tissue infection. J Infect Dis 183: 1043–1054.
22. Terao Y, Mori Y, Yamaguchi M, Shimizu Y, Ooe K, et al. (2008) Group A streptococcal cysteine protease degrades C3 (C3b) and contributes to evasion of innate immunity. J Biol Chem 283: 6253–6260.
23. Miyoshi-Akiyama T, Takamatsu D, Koyanagi M, Zhao J, Imanishi K, et al. (2005) Cytocidal effect of Streptococcus pyogenes on mouse neutrophils in vivo and the critical role of streptolysin S. J Infect Dis 192: 107–116.
24. Kansal RG, McGeer A, Low DE, Norrby-Teglund A, Kotb M (2000) Inverse relation between disease severity and expression of the streptococcal cysteine protease, SpeB, among clonal M1T1 isolates recovered from invasive group A streptococcal infection cases. Infect Immun 68: 6362–6369.
25. Sumby P, Barbian KD, Gardner DJ, Whitney AR, Welty DM, et al. (2005) Extracellular deoxyribonuclease made by group A streptococcus assists pathogenesis by enhancing evasion of the innate immune response. Proc Natl Acad Sci U S A 102: 1679–1684.
26. Buchanan JT, Simpson AJ, Aziz RK, Liu GY, Kristian SA, et al. (2006) DNase expression allows the pathogen group A streptococcus to escape killing in neutrophil extracellular traps. Curr Biol 16: 396–400.
27. Sierig G, Cywes C, Wessels MR, Ashbaugh CD (2003) Cytotoxic effects of streptolysin o and streptolysin s enhance the virulence of poorly encapsulated group a streptococci. Infect Immun 71: 446–455.
28. Bhakdi S, Tranum-Jensen J (1985) Complement activation and attack on autologous cell membranes induced by streptolysin-O. Infect Immun 48: 713–719.
29. Walev I, Hombach M, Bobkiewicz W, Fenske D, Bhakdi S, et al. (2002) Resealing of large transmembrane pores produced by streptolysin O in nucleated cells is accompanied by NF-kappaB activation and downstream events. FASEB J 16: 237–239.
30. Pipkin ME, Lieberman J (2007) Delivering the kiss of death: progress on understanding how perforin works. Curr Opin Immunol 19: 301–308.
31. Madden JC, Ruiz N, Caparon M (2001) Cytolysin-mediated translocation (CMT): a functional equivalent of type III secretion in gram-positive bacteria. Cell 104: 143–152.
32. Bakleh M, Wold LE, Mandrekar JN, Harmsen WS, Dimashkieh HH, et al. (2005) Correlation of histopathologic findings with clinical outcome in necrotizing fasciitis. Clin Infect Dis 40: 410–414.
33. Eriksson BK, Andersson J, Holm SE, Norgren M (1998) Epidemiological and clinical aspects of invasive group A streptococcal infections and the streptococcal toxic shock syndrome. Clin Infect Dis 27: 1428–1436.
34. Hasegawa T, Hashikawa SN, Nakamura T, Torii K, Ohta M (2004) Factors determining prognosis in streptococcal toxic shock-like syndrome: results of a nationwide investigation in Japan. Microbes Infect 6: 1073–1077.
35. Marhoffer W, Stein M, Maeser E, Federlin K (1992) Impairment of polymorphonuclear leukocyte function and metabolic control of diabetes. Diabetes Care 15: 256–260.
36. Propst-Graham KL, Preheim LC, Vander Top EA, Snitily MU, Gentry-Nielsen MJ (2007) Cirrhosis-induced defects in innate pulmonary defenses against Streptococcus pneumoniae. BMC Microbiol 7: 94.
37. Iversen PO, Woldbaek PR, Tonnessen T, Christensen G (2002) Decreased hematopoiesis in bone marrow of mice with congestive heart failure. Am J Physiol Regul Integr Comp Physiol 282: R166–172.
38. Ikebe T, Endoh M, Watanabe H (2005) Increased expression of ska gene in emm49-genotyped Streptococcus pyogenes strains isolated from patients of severe invasive streptococcal infections. Jap J Infect Dis 58: 272–275.
39. Sambrook J, Fritsch EF, Maniatis T (1990) T. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.
40. Perez-Casal J, Price JA, Maguin E, Scott JR (1993) An M protein with a single C repeat prevents phagocytosis of Streptococcus pyogenes: use of a temperature-sensitive shuttle vector to deliver homologous sequences to the chromosome of S. pyogenes. Mol Microbiol 8: 809–819.
41. Tao L, LeBlanc DJ, Ferretti JJ (1992) Novel streptococcal integration shuttle vectors for gene cloning and inactivation. Gene 120: 105–110.
42. Ato M, Stäger S, Engwerda CR, Kaye PM (2001) Defective CCR7 expression on dendritic cells contributes to the development of visceral leishmaniasis. Nat Immunol 3: 1185–1191.
43. Ruiz N, Wang B, Pentland A, Caparon M (1998) Streptolysin O and adherence synergistically modulate proinflammatory responses of keratinocytes to group A streptococci. Mol Microbiol 27: 337–346.
44. Working Group on Severe Streptococcal Infections (1993) Defining the group A streptococcal toxic shock syndrome. JAMA 269: 390–391.

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Genetic Features of Clinical Isolates of *Streptococcus dysgalactiae* subsp. *equisimilis* Possessing Lancefield's Group A Antigen▽

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Thirteen *Streptococcus dysgalactiae* subsp. *equisimilis* isolates possessing Lancefield's group A antigen recovered from people in Japan during 2000 to 2004 were genotyped. The results indicate that a conserved clone has persisted and spread within Japan, and two different *emm* types were observed within members of this clone.

Streptococcus dysgalactiae subsp. *equisimilis* belongs to Lancefield's groups C and G, and it has been recognized as a cause of pharyngitis and skin and soft-tissue infections (6, 10, 12, 27). Further, case reports referring to toxic shock-like syndrome (TSLS) due to group C and G *S. dysgalactiae* subsp. *equisimilis* have been published (2, 13, 16, 19, 20, 22, 28). Recently, some cases of bacteremia or gangrene caused by *S. dysgalactiae* subsp. *equisimilis* belonging to Lancefield's group A have been reported (5, 7, 18). These group A beta-hemolytic streptococci were identified as *S. dysgalactiae* subsp. *equisimilis* on the basis of the phylogenetic analysis of their 16S rRNA genes and their biochemical characters in spite of the common use of GAS (group A streptococcus) to describe *S. pyogenes*. Thus, these data have demonstrated that *S. pyogenes* is not the only beta-hemolytic streptococcus possessing the group A antigen. However, the genetic characterization of group A *S. dysgalactiae* subsp. *equisimilis* has not been fully studied.

GAS, GCS (group C streptococci), and GGS (group G streptococci) express various M-like proteins on the cell surface. On the basis of the sequence analysis of the 5′ end of the *emm* gene that encodes the M-like protein, *emm* typing has been widely used to characterize these streptococci (3, 4, 13, 16, 17, 26). The Centers for Disease Control and Prevention (CDC) maintains the *emm* sequence database (http://www.cdc.gov/ncidod/biotech/strep/strepindex.htm) that contains >150 *emm* types of GAS and >30 *emm* types of GCS and GGS. Multilocus sequence typing (MLST) data are used to construct a model that analyzes the evolution of GAS, GCS, and GGS (17). In the present study, we collected and characterized group A *S. dysgalactiae* subsp. *equisimilis* isolates from humans, including TSLS patients. We performed epidemiological analysis of these isolates by using a combination of different genotyping methods, *emm* typing, and MLST.

Bacterial isolates. The clinical and epidemiological features of the 13 group A *S. dysgalactiae* subsp. *equisimilis* isolates collected in this study are listed in Table 1. All of these isolates were recently recovered from humans, and all of the isolates examined, except one, were associated with disease. Two isolates were recovered from TSLS patients. All of the isolates were confirmed to possess only the group A carbohydrate antigen with a Streptococcus Grouping kit (Oxoid Ltd., Basingstoke, United Kingdom) and Strept LA (Denka Seiken, Japan), and they were identified as *S. dysgalactiae* subsp. *equisimilis* by using the API 20 Strep kit (BioMérieux, Tokyo, Japan).

16S rRNA gene sequencing. PCR template DNA was prepared by using InstaGene Matrix (Bio-Rad, Hercules, CA). DNA sequencing of the 16S rRNA genes was performed according to previously described methods (14, 15). The 16S rRNA gene was amplified with primers 27f (AGAGTTTGATCCTGGCTCAG) and 1492r (GGCTACCTTGTTACGACTT). Sequencing was performed with the ABI Prism 3100 genetic analyzer (Applied Biosystems, Foster City, CA) and primers r1L (GTATTACCGC GGCTGCTGG), r3L (TTGCGCTCGTTGCGGGACT), r4L (A CGGGCGGTGTGTACAAG), f1L (GAGTTTGATCCTGGC TCAG), f2L (CCAGCAGCCGCGGTAATAC), and 926f (AA ACTCAAAGGAATTGACGG).

***emm* typing.** *emm* typing was performed as described by Beall et al. (3, 4). The sequence was subjected to a homology search (Streptococci Group A Subtyping Request Form, BLAST 2.0 server [http://www.cdc.gov/ncidod/biotech/strep/strepblast.htm]), and the *emm* type was determined.

MLST. MLST was performed by determining the DNA sequences of the internal portions of seven housekeeping genes that encoded glucose kinase (*gki*), glutamine transport protein (*gtr*), glutamate racemase (*murI*), DNA mismatch repair protein (*mutS*), transketolase (*recP*), xanthine phosphoribosyltransferase (*xpt*), and acetyl coenzyme A acetyltransferase

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TABLE 1. Characteristics of group A *S. dysgalactiae* subsp. *equisimilis* isolated in Japan

Isolate	Yr of isolation	Location	Isolation site and/or clinical disease[a]	*emm* type
2000A-033	2000	Osaka	Lower-limb soft tissue, diabetic gangrene	*stg485.0*
2003A-123	2003	Osaka	Lower-limb joint	*stg485.0*
ST2004-047	2004	Osaka	Sputum, respiratory disease	*stg485.0*
ST2004-224	2004	Osaka	Blood, decubitus ulcer of the lower limb	*stg485.0*
ST2004-241	2004	Osaka	Peritoneal dialysis fluid, shock	*stg485.0*
2000-9	2000	Tokyo	Skin, ulcer	*stg485.0*
TY75	2000	Tokyo	Throat swab, none	*stg485.0*
1572	2001	Tokyo	TSLS	*stg485.0*
1727	2004	Chiba	Blood	*stg485.0*
NIH245	2002	Toyama	TSLS	*stg485.0*
TP-C3663	2003	Toyama	Tinea pedis	*stg485.0*
ST2004-234	2004	Osaka	Blood, lower-limb necrosis	*stg652.5*[b]
NIH285	2004	Hokkaido	Necrotizing fasciitis	*stg652.0*

[a] All isolates except TY75 were isolated from clinical specimens.
[b] New subtype.

(*yqiL*); MLST was performed according to a previously described procedure for GCS and GGS isolates (17). The primer pairs for the *gtr* loci did not amplify the corresponding MLST target fragment for all of the isolates tested. It was possible that an alteration within primer annealing sites prevented amplification. Therefore, alternative primer pairs for the *gtr* loci were designed to generate appropriate PCR products on the basis of the sequence information from the *S. equi* subsp. *equi* genome sequence (www.sanger.ac.uk) and sequence analysis combined with the inverse PCR method (23). The alternative primers used were as follows: *gtr*(GAS-Sde)-up, 5′-GGTGAT TATTGGCCCTTCTGG-3′; *gtr*(GAS-Sde)-dn, 5′-CGGTCTG CGACTTCTTTAGCA-3′.

Detection of virulence genes by PCR. PCR with previously described primer pairs was conducted for the detection of the genes coding for C5a peptidase (*scpA*), streptokinase (*ska*), streptolysin O (*slo*), streptolysin S (*sagA*), extracellular phospholipase A_2 (*sla*), and streptococcal pyrogenic exotoxins (*speA*, *speB*, *speC*, *speG*, *speH*, *speI*, *speJ*, *speL* [M3], *speL* [M18], and *speM*) (16). In all cases, the primers were designed toward a sequence located inside the open reading frame. The expected sizes of PCR products were 759 bp for *scpA*, 237 bp for *ska*, 434 bp for *slo*, 113 bp for *sagA*, 495 bp for *sla*, 393 bp for *speA*, 1,113 bp for *speB*, 624 bp for *speC*, 211 bp for *speG*, 406 bp for *speH*, 523 bp for *speI*, 490 bp for *speJ*, 639 bp for *speL* [M3], 789 bp for *speL* [M18], and 672 bp for *speM*. PCR amplification was carried out by initial denaturation at 94°C for 2 min, followed by 30 cycles at 94°C for 30 s, 52°C for 30 s, and 72°C for 1 min and a final extension at 72°C for 3 min.

The 16S rRNA genes of all of the isolates tested showed completely identical DNA sequences (GenBank accession number AB253330). Two to three base differences existed between the 16S rRNA gene sequences of the isolates tested in this study and that of previously described group A *S. dysgalactiae* subsp. *equisimilis* strains (GenBank accession numbers AJ314609, AJ314610, and AJ314611); however, there was no difference between the 16S rRNA gene sequences of the isolates tested and the 5′ region sequences of the 16S rRNA genes of group A *S. dysgalactiae* subsp. *equisimilis* strain (GenBank accession number AF239716).

All of the isolates possessed the same set of alleles in six of the seven housekeeping loci, namely, *gki108*, *gtr106*, *mur1105*, *mutS106*, *recP107*, and *xpt104* (GenBank accession numbers AF332633, AF332641, AF332801, AF330237, AF332816, and AF332829). These Japanese isolates were similar to GCS strain 4288 described by Kalia et al. (17) by MLST. The primer pairs for the *yqiL* loci did not amplify the corresponding MLST target fragment in any of the isolates tested. It was possible that an alteration within the primer annealing sites prevented amplification. Our attempts to make new primer pairs for the *yqiL* loci were unsuccessful. In the present study, the results of MLST and 16S rRNA gene sequencing of all of the isolates were completely identical. Our data indicate the dissemination of a single successful group A *S. dysgalactiae* subsp. *equisimilis* strain throughout at least four areas of Japan.

The *emm* types of the isolates tested are shown in Table 1. Of the 13 isolates, 11 were of the *stg485* type and the remaining 2 were of the *stg652* type. All of the *stg485* isolates were of subtype *stg485.0*, and two *stg652* isolates were of subtypes *stg652.0* and *stg652.5*. *stg652.5* was a new subtype with only one nucleotide difference from subtype *stg652.0*. These *emm* types were found to be associated with GGS isolates (http://www.cdc.gov/ncidod/biotech/strep/strepindex.htm). Both isolates from TSLS patients were of subtype *stg485.0*. Interestingly, Misawa et al. (21) reported that a group G *S. dysgalactiae* subsp. *equisimilis* isolate from a TSLS patient was of *emm* subtype *stg485.0*. These data suggest that this *emm* subtype strain should be kept in mind as a potential pathogen causing TSLS.

The presence of virulence genes was identified by PCR. All of the isolates showed the same virulence gene profile that was PCR positive for *speG*, *scpA*, *ska*, *sagA*, and *slo* and PCR negative for *sla*, *speA*, *speB*, *speC*, *speH*, *speI*, *speJ*, *speL* (M3), *speL* (M18), *and speM*. Previously, we reported that all 16 group G *S. dysgalactiae* subsp. *equisimilis* strains isolated from patients with severe invasive infections carried the *scpA*, *ska*, *slo*, and *sagA* genes (16). In this study, we observed that all group A *S. dysgalactiae* subsp. *equisimilis* strains also carried these four virulence genes and the *speG* gene. Misawa et al. (21) reported that a group G TSLS-causing strain possessed three virulence genes, *slo*, *sagA*, and *skcg* (*ska*). Perhaps some of these virulence genes might relate to pathogenesis of TSLS caused by group A or G *S. dysgalactiae* subsp. *equisimilis*.

To the best of our knowledge, this report describes the first case of TSLS caused by group A *S. dysgalactiae* subsp. *equisi-*

TABLE 2. Features of TSLS caused by group A *S. dysgalactiae* subsp. *equisimilis* isolated in Japan

Case no. (strain)	Age (yr)	Sex[a]	Isolation site	Underlying condition	Symptoms[b]	Outcome
1 (1572)	54	M	Blood	Alcoholism, liver cirrhosis	Hepatic insufficiency, renal failure, impaired consciousness, fever (40°C), shock, hypotension, DIC, bilateral pleural effusion, jaundice	Death
2 (NIH245)	70	F	Surgical site	Edema (history of uterine cancer)	Hypotension, shock, erythematous rash, soft-tissue necrosis	Recovery

[a] M, male; F, female.
[b] DIC, disseminated intravascular coagulopathy.

milis. Two case reports are summarized in Table 2. The ages of the patients were 54 and 70 years. At least one of the underlying conditions was reported in the patients (i.e., alcohol addiction and liver cirrhosis in case 1 and edema in case 2). In previous reports, the underlying conditions have been noted mostly in patients with infections due to *S. dysgalactiae* subsp. *equisimilis* possessing group A, C, or G antigen (5, 7, 13, 16, 18). The clinical manifestations included shock, hepatic insufficiency, renal failure, disseminated intravascular coagulation, erythematous rash, and soft-tissue necrosis.

It has been suggested that the horizontal transfer of the *emm* sequences and the following recombination events occur among beta-hemolytic streptococci such as GAS, GCS, and GGS (1, 24, 25). Such a mechanism could be responsible for the development of gene mosaics and the evolution of the *emm* genes in beta-hemolytic streptococci (29). In the present study, two different *emm* types were observed among the 13 group A *S. dysgalactiae* subsp. *equisimilis* isolates that were recently isolated in Japan, although the results of MLST and 16S rRNA gene sequencing for all of these isolates were completely identical. These two *emm* types included 11 *stg485* isolates obtained during 2000 to 2004 and two *stg652* isolates obtained in 2004. Therefore, it appears that horizontal gene transfer has contributed to variations of the *emm* gene in group A *S. dysgalactiae* subsp. *equisimilis*.

Kalia et al. (17) performed MLST of 34 GCS and GGS strains obtained from humans; they obtained 34 unique combinations of allelic profiles (sequence types). Of these 34 strains, stain 4288 was the most closely related to our isolates. Strain 4288 shares six of the seven housekeeping alleles, carries the Lancefield group C antigen, and belongs to the *stg485* type. It is entirely possible that the Japanese isolates have all seven MLST sequences in common since the corresponding MLST target site (*yqiL*) was not amplified and sequenced by an alteration within a primer annealing site(s). Of our 13 group A *S. dysgalactiae* subsp. *equisimilis* isolates, 11 belonged to the same *emm* type. Therefore, these findings indicate that strain 4288 and our isolates may have been derived from the same ancestor or member of the clonal complex, even though they show different group antigens. Further, the basic polysaccharide structure in both groups A and C comprises polymeric L-rhamnose; this indicates a close relationship (8). Moreover, Kalia et al. (17) estimated that interspecies recombinational exchanges from GAS donors to GCS-GGS recipients had occurred recently. Therefore, the lateral transfer of the gene responsible for producing group antigens might cause a change in the group antigen. Recently, the complete genome sequences of several streptococcal species have been reported, and a brief description of each has been presented (11). *S. pneumoniae* and *S. mutans* have highly developed transformation systems, whereas natural transformation is not known to be a common event in *S. pyogenes* or *S. agalactiae*. Although *S. pyogenes* and *S. agalactiae* have many genes that are essential for competence and transformation, they have lost competence probably because phages have assumed a more important role in population diversity. It is possible that *S. dysgalactiae* subsp. *equisimilis* may have its origin in *S. pyogenes* (9). Therefore, phage-mediated genetic transfer is, in fact, the likely mechanism in *S. dysgalactiae* subsp. *equisimilis*, although the complete genome of this species has not been obtained.

In the present study, we investigated the phylogenetic relationship among 13 group A *S. dysgalactiae* subsp. *equisimilis* isolates by using several different genotyping methods. Our data suggest that all of the group A *S. dysgalactiae* subsp. *equisimilis* isolates recovered from Japanese patients could have descended from a common ancestor. Further investigation is required to elucidate the epidemiology of *S. dysgalactiae* subsp. *equisimilis* possessing Lancefield's group A antigen.

Nucleotide sequence accession number. The 16S rRNA gene sequence of strain NIH245 has been deposited in the DDBJ database under accession number AB253330. The new *emm* subtype was deposited in the CDC *emm* sequence database.

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REFERENCES

1. **Albertí, S., C. Garcia-Rey, M. I. Garcia-Laorden, R. Dal-Re, J. Garcia-de-Lomas, and the Spanish Surveillance Group for Respiratory Pathogens.** 2005. Survey of *emm*-like gene sequences from pharyngeal isolates of group C and group G streptococci collected in Spain. J. Clin. Microbiol. **43:**1433–1436.
2. **Barnham, M. R., N. C. Weightman, A. W. Anderson, and A. Tanna.** 2002. Streptococcal toxic shock syndrome: a description of 14 cases from North Yorkshire, UK. Clin. Microbiol. Infect. **8:**174–181.
3. **Beall, B., R. Facklam, T. Hoenes, and B. Schwartz.** 1997. Survey of *emm* gene sequences and T-antigen types from systemic *Streptococcus pyogenes* infection isolates collected in San Francisco, California; Atlanta, Georgia; and Connecticut in 1994 and 1995. J. Clin. Microbiol. **35:**1231–1235.
4. **Beall, B., R. Facklam, and T. Thompson.** 1996. Sequencing *emm*-specific PCR products for routine and accurate typing of group A streptococci. J. Clin. Microbiol. **34:**953–958.
5. **Bert, F., and N. Lambert-Zechovsky.** 1997. Analysis of a case of recurrent bacteraemia due to group A *Streptococcus equisimilis* by pulsed-field gel electrophoresis. Infection **25:**250–251.
6. **Bisno, A. L., C. M. Collins, and J. C. Turner.** 1996. M proteins of group C streptococci isolated from patients with acute pharyngitis. J. Clin. Microbiol. **34:**2511–2515.
7. **Brandt, C. M., G. Haase, N. Schnitzler, R. Zbinden, and R. Lutticken.** 1999. Characterization of blood culture isolates of *Streptococcus dysgalactiae* subsp. *equisimilis* possessing Lancefield's group A antigen. J. Clin. Microbiol. **37:**4194–4197.
8. **Coligan, J. E., T. J. Kindt, and R. M. Krause.** 1978. Structure of the streptococcal groups A, A-variant and C carbohydrates. Immunochemistry **15:**755–760.
9. **Davies, M. R., D. J. McMillan, G. H. Van Domselaar, M. K. Jones, and K. S. Sriprakash.** 2007. Phage 3396 from a *Streptococcus dysgalactiae* subsp. *equisimilis* pathovar may have its origins in *Streptococcus pyogenes*. J. Bacteriol. **189:**2646–2652.
10. **Efstratiou, A., E. L. Teare, D. McGhie, and G. Colman.** 1989. The presence of M proteins in outbreak strains of *Streptococcus equisimilis* T-type 204. J. Infect. **19:**105–111.
11. **Ferretti, J. J., D. Ajdic, and W. M. McShan.** 2004. Comparative genomics of streptococcal species. Indian. J. Med. Res. **119**(Suppl.)**:**1–6.
12. **Fox, K., J. Turner, and A. Fox.** 1993. Role of beta-hemolytic group C streptococci in pharyngitis: incidence and biochemical characteristics of *Streptococcus equisimilis* and *Streptococcus anginosus* in patients and healthy controls. J. Clin. Microbiol. **31:**804–807.
13. **Hashikawa, S., Y. Iinuma, M. Furushita, T. Ohkura, T. Nada, K. Torii, T. Hasegawa, and M. Ohta.** 2004. Characterization of group C and G streptococcal strains that cause streptococcal toxic shock syndrome. J. Clin. Microbiol. **42:**186–192.
14. **Hiraishi, A.** 1992. Direct automated sequencing of 16S rDNA amplified by polymerase chain reaction from bacterial cultures without DNA purification. Lett. Appl. Microbiol. **15:**210–213.
15. **Hiraishi, A., Y. K. Shin, Y. Ueda, and J. Sugiyama.** 1994. Automated sequencing of PCR-amplified 16S rDNA on 'Hydrolink' gels. J. Microbiol. Methods **19:**145–154.
16. **Ikebe, T., S. Murayama, K. Saitoh, S. Yamai, R. Suzuki, J. Isobe, D. Tanaka, C. Katsukawa, A. Tamaru, A. Katayama, Y. Fujinaga, K. Hoashi, H. Watanabe, and the Working Group for Streptococci in Japan.** 2004. Surveillance of severe invasive group-G streptococcal infections and molecular typing of the isolates in Japan. Epidemiol. Infect. **132:**145–149.
17. **Kalia, A., M. C. Enright, B. G. Spratt, and D. E. Bessen.** 2001. Directional gene movement from human-pathogenic to commensal-like streptococci. Infect. Immun. **69:**4858–4869.
18. **Katsukawa, C., A. Tamaru, and Y. Morikawa.** 2002. *Streptococcus dysgalactiae* subsp. *equisimilis* possessing Lancefield's group A antigen. Kansenshogaku Zasshi **76:**155–160.
19. **Keiser, P., and W. Campbell.** 1992. 'Toxic strep syndrome' associated with group C Streptococcus. Arch. Intern. Med. **152:**882–884.
20. **Kugi, M., H. Tojo, I. Haraga, T. Takata, K. Handa, and K. Tanaka.** 1998. Toxic shock-like syndrome caused by group G Streptococcus. J. Infect. **37:**308–309.
21. **Misawa, Y., S. Okugawa, K. Ubukata, K. Okuzumi, M. Okada, K. Moriya, and K. Koike.** 2006. A case of severe necrotizing cellulitis caused by group G *Streptococcus dysgalactiae* subsp. *equisimilis*. Kansenshogaku Zasshi **80:**436–439.
22. **Roth, S., K. Andrassy, K. H. Schmidt, E. Gunther, and E. Ritz.** 1999. Febrile lady with acute renal failure and desquamating erythema. Am. J. Kidney Dis. **34:**150–154.
23. **Sambrook, J., and D. W. Russell.** 2001. Molecular cloning: a laboratory manual, 3rd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
24. **Schnitzler, N., A. Podbielski, G. Baumgarten, M. Mignon, and A. Kaufhold.** 1995. M or M-like protein gene polymorphisms in human group G streptococci. J. Clin. Microbiol. **33:**356–363.
25. **Simpson, W. J., J. M. Musser, and P. P. Cleary.** 1992. Evidence consistent with horizontal transfer of the gene (*emm12*) encoding serotype M12 protein between group A and group G pathogenic streptococci. Infect. Immun. **60:**1890–1893.
26. **Tanaka, D., Y. Gyobu, H. Kodama, J. Isobe, S. Hosorogi, Y. Hiramoto, T. Karasawa, and S. Nakamura.** 2002. *emm* typing of group A streptococcus clinical isolates: identification of dominant types for throat and skin isolates. Microbiol. Immunol. **46:**419–423.
27. **Turner, J. C., A. Fox, K. Fox, C. Addy, C. Z. Garrison, B. Herron, C. Brunson, and G. Betcher.** 1993. Role of group C beta-hemolytic streptococci in pharyngitis: epidemiologic study of clinical features associated with isolation of group C streptococci. J. Clin. Microbiol. **31:**808–811.
28. **Wagner, J. G., P. M. Schlievert, A. P. Assimacopoulos, J. A. Stoehr, P. J. Carson, and K. Komadina.** 1996. Acute group G streptococcal myositis associated with streptococcal toxic shock syndrome: case report and review. Clin. Infect. Dis. **23:**1159–1161.
29. **Whatmore, A. M., and M. A. Kehoe.** 1994. Horizontal gene transfer in the evolution of group A streptococcal *emm*-like genes: gene mosaics and variation in Vir regulons. Mol. Microbiol. **11:**363–374.

TOPICS クリニカルトピックス

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1994年 広島大学生物生産学部卒業。2000年広島大学大学院生物圏科学研究科博士過程修了，同年国立感染症研究所細菌第一部研究員，05年国立感染症研究所細菌第一部主任研究官。研究テーマは劇症型溶血性レンサ球菌感染症の発症機序に関する研究。

Key words：劇症型溶血性レンサ球菌感染症，免疫回避

劇症型溶血性レンサ球菌感染症の発症機序
—菌の特徴と免疫回避機構—

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Abstract
劇症型溶血性レンサ球菌は，病態の進行が劇的で，ショック症状から死に至らしめる。この感染症の病理像の特徴の一つとして，病巣に菌の集積が見えるにもかかわらず，多核白血病の遊走がみらればい。この遊走阻害には少なくとも2つの酵素が関与しており，劇症型感染症起因株において，これらの毒素の発現量が増大していることが判明した。

はじめに

劇症型溶血性レンサ球菌感染症(streptococcal toxic shock syndrome)は1987年に米国で最初に報告され[1, 2]，その後，先進国ばかりでなく発展途上国からも報告されている。日本における最初の典型的な症例は1992年に報告されており[3]，現在までに500人を超える患者が確認されている。そして，このうち約40%が死亡しているというきわめて致死率の高い感染症である。この感染症の主な病原菌は，A群レンサ球菌(*Streptococcus pyogenes*)であり，古くから咽頭炎，扁頭炎，猩紅熱，続発症としてリウマチ熱や急性糸球体腎炎などを引き起こすことが知られている。なぜ，近年になり劇症型溶血性レンサ球菌感染症が出現したか，そのような病原性因子が関与しているか，現在までの知見をまとめた。

1．劇症型溶レン菌感染症患者分離株の病態および疫学

劇症型溶血性レンサ球菌感染症は，初期症状として，四肢の疼痛，腫脹，発熱，血圧低下などがみられ，発病から病態の進行が非常に急激かつ劇的で，いったん発病すると数十時間以内に急性腎不全，成人型呼吸窮迫症候

Mechanism of the onset of streptococcus toxic shock-like syndrome-Characteristics and the immunity evasion mechanism of bacteria- : Tadayoshi Ikebe, Manabu Ato, Kazuo Kobayashi, Haruo Watanabe, 1) Department of bacteriology, National Institute of Infectious Diseases, 2) Department of immunology, National Institute of Infectious Diseases

表　劇症型溶血性レンサ球菌感染症の感染症法に基づく医師及び獣医師の届出基準

(1) 定義

β溶血を示すレンサ球菌を原因とし、突発的に発症して急激に進行する敗血症性ショック病態である。

(2) 臨床的特徴

初発症状は眼頭痛、発熱、消化管症状（食欲不振、吐き気、嘔吐、下痢）、全身倦怠感、低血圧などの敗血症症状、筋痛などであるが、明らかな前駆症状がない場合もある。後発症状としては軟部組織病変、循環不全、呼吸不全、血液凝固異常（DIC）、肝腎症状など多臓器不全を来し、日常生活を営む状態から24時間以内に多臓器不全が完結する程の急激な進行を示す。

(3) 届出基準

ア．患者（確定例）

医師は、(2) の臨床的特徴を有する者を診察した結果、症状や所見から劇症型溶血性レンサ球菌感染症を疑い、かつ、(4) の届出に必要な要件を満たすと診断した場合には、法第12条第1項の規定による届出を７日以内に行わなければならない。

イ．感染症死亡者の死体

医師は、(2) の臨床的特徴を有する死体を検案した結果、症状や所見から、劇症型溶血性レンサ球菌感染症を疑い、かつ、(4) の届出に必要な要件を満たし、劇症型溶血性レンサ球菌感染症により死亡したと判断した場合には、法第12条第1項の規定による届出を7日以内に行わなければならない。

(4) 届出に必要な要件（以下のアの（ア）及び（イ）かつイを満たすもの）

ア．届出のために必要な臨床症状

（ア）ショック症状

（イ）（以下の症状のうち2つ以上）

肝不全、腎不全、急性呼吸窮迫症候群、DIC、軟部組織炎（壊死性筋膜炎を含む）、全身性紅斑性発疹、痙攣・意識消失などの中枢神経症状

イ．病原体診断の方法

検査方法　分離・同定による病原体の検出

検査材料　血液、壊死軟部組織

群(ARDS)，播種性血管内凝固症候群(DIC)，多臓器不全(MOF)，軟部組織壊死を引き起こし，患者をショック症状から死に至らしめる。

Stevensら[4,5]の報告によると，劇症型溶血性レンサ球菌感染症の最も一般的な初期症状は急激に始まる四肢の疼痛であり，その部位の圧痛を示す。疼痛は，通常，四肢で見られる。疼痛の開始前に，約20％の患者では，発熱，悪寒，筋肉痛，下痢のようなインフルエンザ様の症状を示す場合がある。臨床所見として，発熱が，最も一般的な徴候である（ただし，患者の10％では発見時にすでにショックによる低体温を示す例がある）。錯乱状態(confusion)は患者の55％で見られ，患者によっては，昏睡や好戦的な姿勢を示すことがある。局所的な腫脹，圧痛，疼痛，紅斑のような軟部組織感染の徴候は，皮膚の傷口が存在する場合によく見られる。発熱を持つ患者で紫色の水疱が圧痛のある部位にみられると，筋炎や壊死性筋膜炎のような深部の軟部組織感染を起こしている可能性が考えられる[6]。Stevenら[4]の報告によると，劇症型溶血性レンサ球菌感染症の患者の約35％は皮膚(minor trauma, surgical procedures, intravenous drug abuse)，ある

いは約20％は粘膜(pharynx, vagina)からの*S. pyogenes*の感染であり，残りの約45％は，正確な菌の侵入部位が不明である。

1999年4月に施行された「感染症の予防及び感染症の患者に対する医療に関する法律(感染症法)」による集計によると，2000年には45例，2001年には43例，2002年には90例，2003年には52例，2004年には53例，2005年には60例が報告されている。2006年の法の改正で，劇症型溶血性レンサ球菌感染症の届出基準が一部変更され，今まで起因菌をA群レンサ球菌に限定していたが，今回の改正でβ溶血を示すレンサ球菌にまで広げられた。感染症法に基づく医師及び獣医師の届出を表に示す(http://www.mhlw.go.jp/bunya/kenkou/kekkaku-kansenshou11/01-05-06.html)。改正後，年間約100例が報告されている（2006年　107例，2007年　96例)。

2．劇症型／重症溶レン菌感染症患者分離株の分子疫学

A群レンサ球菌には，数多くの表層抗原因子が知られている。このうちM蛋白質は，型特異的であり，100以上の型が知られていることから[7]，菌の疫学マーカーとしてよく用いられている。M蛋白は，抗オプソニン作用[8,9]を有し，細胞への接着にも関与しており，病原因子として知られている。分離株のM型別を行うことは病因との関連を知る上で重要である。近年，M型別を血清学的方法ではなく，M蛋白をコードする遺伝子(*emm*)の塩基配列を決定することで，遺伝子による型別が可能となった。

2008年7月31日までに衛生微生物技術協議会溶血性レンサ球菌レファレンスシステムセンター（図1）に集められた劇症型／重症溶レン菌感染症患者分離株に390株ついて，*emm*遺伝子型を調べたところ，最も多い型は，*emm1*型で，44.4％（173株）を占める。続いて*emm3*型(11.8％)，*emm28*型(6.9％)，*emm12*型(6.7％)である。劇症型溶血性レンサ球菌感染症患者から分離される*S. pyogenes*の*emm*型は，1992-1995年までは，*emm3*型と*emm1*型が主であったが，1995年以降，*emm3型*は減少し，*emm1*型が主流となっている[10,11]。また，近年は*emm1*，*emm3*以外の型の菌も多く分離されてきているのが特徴である。国立感染症研究所に集められた劇症型／重症溶レン菌感染症患者分離株の*emm*型は，31種類にも及んでいる。

3．A群レンサ球菌の病原因子

A群レンサ球菌の病原因子は，他の細菌と比べ非常に多彩であるとともに，A群レンサ菌の中でも保有している病原性因子が菌株により異なる。接着因子として，フィブロネクチン結合タンパク質，ラミニン結合タンパク質，Mタンパク質などが知られおり，これらは，粘膜上皮や細胞外マトリクスなどに接着するときに重要な役割を示すことが知られている。細胞障害に関与するものとして，ストレプトリジンOやストレプトリジンS，NADアーゼなどが知られている。ストレプトキナーゼは，線溶系を活性化し，血液凝固を阻止する因子として知られている。タンパク分解酵素の中には，システインプロテアーゼであるSpeB，補体であるC5aやC3を分解するC5aペプチダーゼ，C3分解プロテアーゼ，IL-8分解酵素であるScpC/SpyCEPなどがある。この他の分解酵素として，抗体を分解するEndoSやMac/IdeSなどが知られている。Sicタンパク質は，補体阻害

A群レンサ球菌のT, M型別試験，および劇症型A群レンサ球菌感染症に関する情報についての窓口は以下の機関になっておりますので，お問い合わせをお願いいたします。

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図1　衛生微生物協議会溶血性レンサ球菌レファレンスシステムセンター窓口

因子として機能する。さらに，T細胞活性化因子として，SpeA, SpeC, SpeG, SpeH, SpeI, SpeJ, SpeK, SpeL, SpeMなどのスーパー抗原も知られている。

4．劇症型溶血性レンサ球菌感染症患者分離株に関する知見

劇症型溶血性レンサ球菌感染症の病理像の特徴の一つとして，病巣に菌の集積が見えるにもかかわらず，溶血性レンサ球菌による感染を最前線で防御する多核白血球の遊走がみられないことが報告されている[12]。このことは，宿主防御因子，特に多核白血球の病巣における欠如が劇症型溶血性レンサ球菌感染症に重要な役割をもっていることが示唆される。我々は，2000年以降分離され始めだした*emm49*型の劇症型/重症溶血性レンサ球菌感染症患者分離株と非劇症型感染症患者分離株について，好中球に対するIL-8添加時の遊走能お

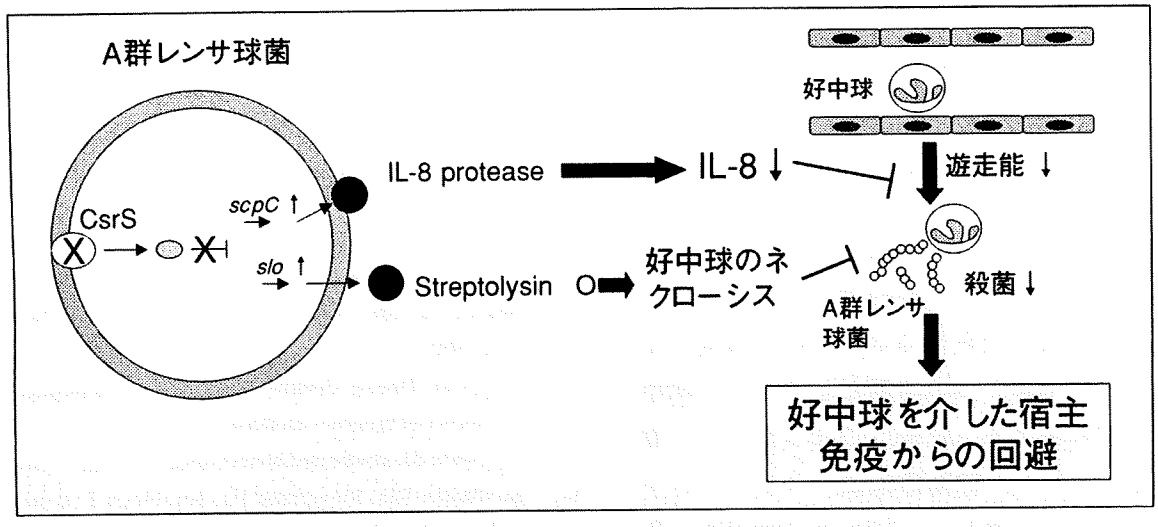

図2　劇症型溶血性レンサ球菌感染症患者分離株の免疫回避
劇症型溶血性レンサ球菌感染症患者由来分離株は，*csrS*遺伝子に変異が生じた結果，csrSにより抑制されていたIL-8プロテアーゼやストレプトリジンO遺伝子の発現量が増大する。その結果，IL-8の減少により，好中球の遊走能の減少を引き起こすとともに，好中球のネクローシスを引き起こし，宿主免疫から回避する。

よび殺菌能について解析した。その結果，劇症型溶血性レンサ球菌感染症患者分離株は，非劇症型患者分離株と比較して，好中球の遊走能が低下し，好中球のほとんどが死んでいることが判明した。この原因として，劇症型溶血性レンサ球菌感染症でみられる多核白血球の病巣における欠如は，2段階の作用によって行われていることが明らかとなった[13]。一つ目は，好中球の遊走性の阻止である。これはA群レンサ球菌がもつセリンプロテアーゼであるScpC/SpyCEPタンパク質が，好中球の遊走因子であるIL-8を分解し，好中球の遊走を阻害することによるものである。二つ目は，好中球のネクローシスである。これは，A群レンサ球菌が分泌する細胞障害因子であるストレプトリジンOタンパク質が好中球をネクローシスさせることによるものである。

非劇症株と劇症型患者分離株とでは，これらの遺伝子の配列に違いが見られないことから，遺伝子の発現量を調べたところ，劇症型溶血性レンサ球菌感染症患者分離株のほうが，非劇症株より，発現量の増大が確認された[13]。このことから，劇症型溶血性レンサ球菌感染症患者分離株は，IL-8プロテアーゼやストレプトリジンO遺伝子の発現量が増大し，好中球の機能障害を起こしていることが判明した[13]。

IL-8プロテアーゼやストレプトリジンOの遺伝子の発現量の増大が何に起因しているのかを調べたところ，劇症型溶血性レンサ球菌感染症患者分離株において，CsrS(CovS)という二成分制御因子のセンサータンパク質に変異があることが判明した[13]。このタンパク質は，環境の変化に応じてシグナルを負の転写制御因子に伝えるセンサータンパク質である。転写制御因子により発現が抑えられていた遺伝子群（IL-8プロテアーゼやストレプトリジンOをコードする遺伝子を含む）は，*csrS*遺伝子に変異が生じることにより，脱抑制され，IL-8プロテアーゼやス

トレプトリジンOをコードする遺伝子の発現量が増大することが示唆された（図2）[13]。

おわりに

劇症型溶血性レンサ球菌感染症は，病態の進行が非常に急激かつ劇的で，患者をショック症状から死に至らしめる。我々の研究の結果から，好中球からの防御として二つの病原因子が明らかとなったが，これらだけで，劇症型溶血性レンサ球菌感染症のメカニズムを説明することは難しい。二つの病原因子の発現量の上昇は，すべて負の制御因子である*csrS*の変異からはじまっていると考えられる。この制御因子は，様々な遺伝子の発現を負に制御していることから，この遺伝子の変異により，様々な病原性遺伝子の発現が増大しているものと考えられる。この制御下にある病原因子をさらに追求することで，劇症型溶血性レンサ球菌感染症の全体像が解明されることが期待される。

文献

1) Weiss KA, Laverdiere M: Group A Streptococcoccus invasive infections; A review. Can J Surg 40: 18-25, 1997
2) Stevens DL: The flesh-eating bacterium: what' next? J Infect Dis 179 (Suppl 2): S366-374, 1999
3) Shimizu Y, Ohyama A, Kasama K, *et al.*: Case report of toxic shock-like syndrome due to group A streptococcal infection. Kansenshogaku Zasshi 67: 236-239, 1993
4) Stevens DL, Tanner MH, Winship, *et al.*: Reappearance of scalet fever toxin A among streptococci in the Rocky Mountain West: severe group A streptococcal infections associated with toxic shock-like syndrome. N Engl J Med 321: 1-7, 1989
5) Stevens DL: Invasive group A streptococcus infections. Clin Infect Dis 14: 2-13, 1992
6) Stevens DL: Streptococcal infections of skin and soft tissue. In Atlas of Infectious Diseases (Stevens DL, Mandell GL, ed). 3.1-3.11, Churchill Livingstone, New York, 1995.
7) Centers for Disease Control and Prevention Homepage. *Streptococcus pyogenes* database. http://www.cdc.gov/ncidod/biotech/strep/strepindex.html.
8) Horstmann RD, Sievertsen HJ, Knobloch J, *et al.*: Antiphagocytic activity of streptococcal M protein: selective binding of complement control protein factor H. Proc Natl Acad Sci USA 85: 1657-1661, 1988
9) Fischetti VA: Streptococcal M protein: molecular design and biological behavior. Clin Microbiol Rev 2: 285-300, 1989
10) Inagaki Y, Konda T, Murayama S, *et al.*: Serotyping of Streptococcus pyogenes isolated from common and severe invasive infections in Japan, 1990-5: implication of the T3 serotype strain-expansion in TSLS. Epidemiol Infect 119: 41-48, 1997
11) Ikebe T, Murai N, Endo M, *et al.*: Changing prevalent T serotypes and emm genotypes of *Streptococcus pyogenes* isolates from streptococcal toxic shock-like syndrome (TSLS) patients in Japan. Epidemiol Infect 130: 569-572, 2003
12) Hidalgo-Grass C, Dan-Goor M, Maly A, Eran Y, Kwinn LA, *et al.*: Effect of a bacterial pheromone peptide on host chemokine degradation in group A streptococcal necrotising soft-tissue infections. Lancet 363:696-703, 2004
13) Ato M, Ikebe T, Kawabata H, Takemori T, Watanabe H : Imcompetence of neutrophils to invasive group A *streptococcus* is attributed to induction of plural virulence factors by dysfunction of a regalator. PLoS ONE 3 : e3455, 2008

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Highly Frequent Mutations in Negative Regulators of Multiple Virulence Genes in Group A Streptococcal Toxic Shock Syndrome Isolates

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Abstract

Streptococcal toxic shock syndrome (STSS) is a severe invasive infection characterized by the sudden onset of shock and multiorgan failure; it has a high mortality rate. Although a number of studies have attempted to determine the crucial factors behind the onset of STSS, the responsible genes in group A *Streptococcus* have not been clarified. We previously reported that mutations of *csrS/csrR* genes, a two-component negative regulator system for multiple virulence genes of *Streptococcus pyogenes*, are found among the isolates from STSS patients. In the present study, mutations of another negative regulator, *rgg*, were also found in clinical isolates of STSS patients. The *rgg* mutants from STSS clinical isolates enhanced lethality and impaired various organs in the mouse models, similar to the *csrS* mutants, and precluded their being killed by human neutrophils, mainly due to an overproduction of SLO. When we assessed the mutation frequency of *csrS*, *csrR*, and *rgg* genes among *S. pyogenes* isolates from STSS (164 isolates) and non-invasive infections (59 isolates), 57.3% of the STSS isolates had mutations of one or more genes among three genes, while isolates from patients with non-invasive disease had significantly fewer mutations in these genes (1.7%). The results of the present study suggest that mutations in the negative regulators *csrS/csrR* and *rgg* of *S. pyogenes* are crucial factors in the pathogenesis of STSS, as they lead to the overproduction of multiple virulence factors.

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Introduction

Streptococcus pyogenes (group A *Streptococcus*; GAS) is one of the most common human pathogens. It causes a wide variety of infections, ranging from uncomplicated pharyngitis and skin infections to severe and even life-threatening manifestations, such as necrotizing fasciitis (NF) and bacteremia. Several streptococcal virulence factors, including pyrogenic exotoxins, streptokinase, and streptolysins, are reportedly involved in these diseases. Streptococcal toxic shock syndrome (STSS) is a severe invasive infection that has been recently characterized by the sudden onset of shock and multiorgan failure; it has a high mortality rate, ranging from 30% to 70% [1]. There is controversy as to whether the cause of STSS largely depends on host factors or bacterial factors. Although many studies have sought to determine the crucial factors behind the onset of STSS, the responsible GAS genes have not been clarified.

Recently, we and others have reported that mutations in the *csrS* (*covS*) gene—a sensor gene of a two-component regulatory system—were detected in a panel of clinical isolates from severe invasive streptococcal infections, but not in non-STSS isolates [2–4]. Mutations in the gene caused an increased expression of various virulence genes; the upregulation of streptolysin O (SLO) induced necrosis of neutrophils and prompted the escape of *csrS* mutated strains from being killed by neutrophils, resulting in increased virulence in lethality in the mouse model [2]. Complementation of the wild *csrS* gene into *csrS*-mutated STSS isolates dramatically decreased their virulence in lethality [2]. Similarly, *csrR* (*covR*) mutations were found in the clinical isolates of STSS patients [5]. Such results suggest that *csrS/csrR* mutations are closely associated with the onset of STSS.

However, several study groups that investigated the *csrS/csrR* gene sequence in each STSS isolate [3,4,6,7] also report that there is no mutation in the *csrS/csrR* gene of STSS isolates [4]. These results raise questions as to how frequently STSS isolates have mutations in the *csrS/csrR* genes in a mass of isolates, and what mutations other than *csrS/csrR* genes may be responsible for the onset of STSS.

In this study, we sequenced the *csrS/csrR* genes of 164 GAS strains that have been isolated from STSS patients in Japan since 1992. Almost one-half of the STSS isolates had a mutation in the *csrS/csrR* genes. In addition, we found a mutation in the *rgg* (*ropB*) gene, instead of the *csrS/csrR* genes, in the clinical isolates of patients with STSS. Since the *rgg* gene negatively regulates various virulence genes in a manner similar to that of the *csrS* gene, a mutation of the *rgg* gene in STSS clinical isolates increased the