Simultaneous isolation ofemm89-type* Streptococcus pyogenes* strains with a wild-type or mutated covS gene from a single streptococcal toxic shock syndrome patient

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Streptococcal toxic shock syndrome (STSS) is a re-emerging infectious disease in many developed countries. Recent studies have suggested that mutations in CovRS, a two-component regulatory system in *Streptococcus pyogenes*, play important roles in the pathogenesis of STSS. However, in vivo evidence of the significance of CovRS in human infections has not been fully demonstrated. We investigated five *S. pyogenes* strains isolated simultaneously from the pharynx, sputum, knee joint, cerebrospinal fluid and blood of a single STSS patient. All were emm89-type strains, and multilocus sequence typing (MLST) analysis revealed that the strains of pharynx and blood were isogenic. The growth rates of the strains from pharynx and sputum were faster than those of the other strains. Protein profiles of the culture supernatants of strains from the pharynx and sputum were also different from those of the other strains. Sequence analyses revealed that strains from the knee joint, cerebrospinal fluid and blood contained a single nucleotide difference in the covS coding region, resulting in one amino acid change, compared with the other strains. Introduction of a plasmid containing the covS gene from the pharynx strain to the blood strain increased the production of SpeB protein. This suggests that the one amino acid alteration in CovS was relevant to pathogenesis. This report supports the idea that mutated CovS plays important roles in vivo in the dissemination of *S. pyogenes* from the upper respiratory tract of human to aseptic tissues such as blood and cerebrospinal fluid.

INTRODUCTION

*Streptococcus pyogenes* is a Gram-positive bacterium that causes mild (pharyngitis, scarlet fever), invasive [bacteraemia/septicaemia, necrotizing fasciitis, streptococcal toxic shock syndrome (STSS)] and non-suppurative (rheumatic fever, glomerulonephritis) infections (Cunningham, 2000). The pathogenesis of STSS is not completely understood, but quantitative and qualitative changes in the expression of virulence factors are believed to be contributing factors. There are several proteins in *S. pyogenes* that regulate the production of virulence factors. Among them, CovRS (CsrRS) (Levin & Wessels, 1998; Federle *et al.*, 1999), one of the thirteen two-component regulatory systems (Ferretti *et al.*, 2001), has been mostly analysed. CovR was reported to influence transcription of 15% of the *S. pyogenes* genome, including many genes involved in virulence (Graham *et al.*, 2002). Mutation of CovRS was first reported to result in enhanced virulence in murine infection models (Engleberg *et al.*, 2001). In addition, the mutation of covS was suggested to be a very important step in the transition from a pharyngitis strain to an invasive strain (Sumby *et al.*, 2006). Other reports also indicated a highly frequency of CovRS mutations in STSS isolates (Ato *et al.*, 2008; Ikebe *et al.*, 2010). One possible explanation is that a mutation within CovRS promotes resistance to neutrophil-mediated killing by altering the production of proteins such as Sda1, M protein and hyaluronic acid capsule (Walker *et al.*, 2007; Cole *et al.*, 2010). However, in vivo human evidence of the significance of CovRS has not been fully demonstrated. In this work, we investigated *S. pyogenes* isolates from five different tissues of a single STSS patient, and validated the relevance of a CovS mutation in pathogenesis.

Abbreviations: 2-DE, two-dimensional gel electrophoresis; MLST, multilocus sequence typing; STSS, streptococcal toxic shock syndrome.
METHODOLOGY

Strains. Five *S. pyogenes* strains were isolated from the pharynx, sputum, knee joint, cerebrospinal fluid and blood of single patient admitted to the Saitama Medical Center with STSS.

Culture conditions. Bacteria were cultured in brain heart infusion broth (Eiken Chemical) supplemented with 0.3% yeast extract (Becton Dickinson) (BHI-YE) at 37°C without agitation. The growth of the bacteria was monitored using a colorimeter (Asahi Science) at OD 660 nm.

Determination of M-type. Typing of the M protein coding gene (*emm*) of the streptococcal isolates was performed as previously described (Hasegawa et al., 2010b).

Multilocus sequence typing (MLST). Internal fragments of seven housekeeping genes (*gki, gtr, murl, mutS, recP, xpt* and *yqiL*) were amplified and sequenced according to the method of Enright et al. (2001). The sequence data were queried via the Internet (www.mlst.net).

Two-dimensional gel electrophoresis. Each bacterial isolate was cultured in BHI-YE at 37°C overnight without agitation. Proteins from the culture supernatant of the pharynx and sputum were prepared and subjected to two-dimensional gel electrophoresis (2-DE) as previously described (Hasegawa et al., 2010b).

Sequence analysis of *covR/covS*. The coding region of *covR/covS* was amplified by PCR using PrimeSTAR HS DNA polymerase (Takara Bio). The primers for amplification were *covR*-n3 (5'-CGGTACAGGTCTTGACAGAG-3') and *covS*-c5 (5'-GCACCTTGAAACCCCAT-3'). PCR products were purified and directly sequenced with several sequencing primers (Hasegawa et al., 2010b).

Establishment of *covRS* complemented strains. DNA fragments encoding *covRS* were amplified by PCR using oligonucleotide primers *covR*-n2 (5'-CTTTAGAATATGTTACT-3') and *covS*-c2 (5'-GTAATTACATTTTGGACAAC-3'), genomic DNA from the pharynx and blood strains as templates, and PrimeSTAR HS DNA polymerase. The fragments contained *covR, covS*, and their 5'-noncoding region, which possibly contained the promoter region. These fragments were treated with T4 polynucleotide kinase (Takara Bio) and ligated into the SphI site of the pLZ12-Km2 plasmid (Okada et al., 1998). The transformation was performed as previously described (Hasegawa et al., 2010a; Ichikawa et al., 2011).

RESULTS AND DISCUSSION

Based upon their antibiotic resistance patterns, all the *S. pyogenes* strains isolated from the five different tissues were initially considered to be the same clone (data not shown). We analysed the *emm* type of all strains and found that all were *emm89*. However, when we examined the growth rate of each strain using a colorimeter, the growth rates of the strains from pharynx and sputum were faster than those of the strains from knee joint, cerebrospinal fluid and blood. In addition, the final densities of the strains from pharynx and sputum were higher than those of the strains from knee joint, cerebrospinal fluid and blood (Fig. 1). We then analysed the proteins from the culture supernatant by SDS-PAGE and found the considerable differences in protein production. In particular, a very intense band was detected from the culture supernatant of the pharynx and sputum strains (Fig. 2, lanes 1 and 2). To further identify the protein differences, a 2-DE analysis was performed. The readily apparent difference involved the production of streptococcal pyrogenic exotoxin B (SpeB) proteinase (Bohach et al., 1988; Tanaka et al., 2005). The abundant production of SpeB was detected in the culture supernatant of the pharynx strain (Fig. 3a); however, SpeB was not clearly detected in the supernatant of the blood strain (Fig. 3b).

The differences in growth rate and protein production prompted us to examine the sequence of the *covRS* gene because we have noted similar data differences between wild-type and *covS*-mutated *emm1* strains (Tatsuno et al., 2013). Sequence analyses of the pharynx and sputum strains showed that one and four nucleotides in the *covR* and *covS* coding region, respectively, were different from the sequences of these coding regions in the *emm1* genome strain (Ferretti et al., 2001). However, no amino acid changes resulted from these mutations. The sequences of strain from knee joint, cerebrospinal fluid and blood had an additional nucleotide change at position 575 (C → G) of the *covS* gene, and this mutation resulted in the alteration of aa 192 from leucine to arginine.

To rule out that all strains were not isogenic, we performed an MLST analysis. We analysed the strains of pharynx and blood. The sequences of seven housekeeping genes were completely identical, and the sequence types of these strains were 101; the allele numbers were 16 for *gki, 2* for *gtr, 8* for *murl, 3* for *mutS, 1* for *recP, 13* for *xpt* and *3* for *yqiL*. These data suggested that the strains were isogenic.

To examine the significance of this *covS* mutation in an *emm89* strain, the *covRS* gene from the pharynx strain was cloned into plasmid pLZ12-Km2 (Okada et al., 1998), and introduced into the blood strain. The reason for using the
The covRS gene, not the covS gene alone, was that we had data from our previous experiments that the introduction of the covRS gene was more effective than the covS gene alone. We found that SpeB production was clearly increased (Fig. 2, lane 6). The introduction of the control plasmid did not increase SpeB production (Fig. 2, lane 7). These results suggested that the covS gene from the pharynx strain complemented the mutated covS of the blood strain. We also introduced covRS gene from the pharynx strain into the emm1 type covS knockout S. pyogenes strain. In the covS knockout strain, the level of SpeB was decreased, and the level of the streptococcal inhibitor of complement (SIC) (Akesson et al., 1996) was increased (Hasegawa et al., 2010b). We confirmed that SpeB and SIC productions were restored (data not shown), suggesting that the covRS gene from the pharynx strain had also the same function even in the emm1 type S. pyogenes. Regarding the growth differences, we checked several independent blood strains introduced with the covRS gene of the pharynx strain or pLZ12-Km2 control plasmid, respectively. The growth rates of the strains containing the pLZ12-Km2 control plasmid differed, possibly due to the emergence of fast-growing mutant strains. However no statistically significant difference in growth rates of blood strains containing the covRS gene and those containing the control plasmid was observed (data not shown). Hence we could not conclude that the growth deterioration of the blood strain was caused by the covS mutation.

Many recent studies have described the significance of covS mutations in the pathogenesis of STSS. However, to the best of our knowledge, only one study analysed isolates from several tissues of a single STSS patient. Garcia et al. (2010) reported that an insert in the covS gene distinguishes a pharyngeal isolate from a blood isolate obtained from the same individual. In that study, the isolates were obtained 13 days apart. In addition, the patient was administered penicillin, and the bacteria were exposed to this antibiotic for 5 days. The authors worried that a lack of compliance was a contributing selection pressure for the pharyngeal isolate to enter the blood. In our study, the patient was admitted to the hospital in a very bad condition, and the isolates were taken at the same time. Only a single emm89 clone was isolated from several tissues. This suggested that the emm89 clone had grown dominantly in both pharynx and the upper respiratory tract, and that the covS mutated clone emerged unpredictably and disseminated into the

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**Fig. 2.** SDS-PAGE analysis of the culture supernatant proteins. Strains were incubated in 4 ml BHI-YE broth overnight and 400 μl of culture supernatant was precipitated with trichroloacetic acid, washed with acetone, and subjected to SDS page. Lanes: M, size marker; 1, pharynx strain; 2, sputum strain; 3, knee joint strain; 4, cerebrospinal fluid strain; 5, blood strain; 6; blood strain following introduction of the covRS gene of the pharynx strain; 7, blood strain following introduction of the pLZ12-Km2 control plasmid.

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**Fig. 3.** 2-DE analysis of the culture supernatant. Pharynx strain (a) and blood strain (b) were cultured in 15 ml BHI-YE broth overnight, and the proteins in the culture supernatants were subjected to 2-DE analysis by using 7 cm Immobiline DryStrip gels (pH 3–10, Bio-Rad).
blood and other aseptic tissues. Advantages for the covS mutant to grow in aseptic tissues such as the blood have been suggested. This study strongly supports this idea. Studies that investigate the mechanisms underlying the emergence of covS mutants should be performed in the future.

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