Identification of putative virulence-associated genes of
*Streptococcus pyogenes* by genomic subtractive hybridization

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The fragments of virulence genes in *Streptococcus pyogenes* were isolated with suppression subtractive hybridization (SSH). Then these fragments were directly inserted into T/A cloning vector to set up subtractive library, and the amplification of the library was carried out after transferring into *Escherichia coli* TOP10. Dot blot was used to screen the subtracted library, and the differentially expressed cDNA fragments were sequenced and analyzed with basic local alignment search tool (BLAST) search. As a result, a smear with dots ranging from 100 to 2000 bp was obtained. Partial positive clones in the library were randomly selected and successfully sequenced. Five-twelfth (5/12) of sequences showed no homology and presumably represented unknown genes, and 7/12 had a high similarity to the known genes. In a conclusion, virulence genes subtracted library of *S. pyogenes* is constructed successfully with SSH and T/A cloning techniques. The library is efficient and lays solid foundation for screening and cloning new and specific virulence genes of *S. pyogenes*.

Key words: *Streptococcus pyogenes*; suppression subtractive hybridization; virulence gene; dot blot.

INTRODUCTION

Group A streptococcus, also known as *Streptococcus pyogenes*, usually cause fester inflammation, upper respiratory tract infection or puerperal infection, such as tonsillitis, pharyngitis, cellulitis, subacute bacterial endocarditis, etc (Cole, 2011). Based on the fact that streptococcus M protein contains common antigens with myocardial and glomerular basement membrane, *S. pyogenes* can also produce rheumatic fever, acute glomerulonephritis and other hypersensitivity diseases. Additionally, scarlet fever, toxic shock syndrome and some viral diseases may be related with the infection of group A streptococcus. Therefore, *S. pyogenes* has serious threat to human health (Holm, 1996).

Some strains of group A streptococcus have stronger toxicity than others. Thus some researchers thought that the virulence variation may correlate with strains concerned (Zhang et al., 2000). By comparing the genetic variations of virulent strain genomic DNA with that of standard strains, we could identify the virulent genes of infectious diseases and further understand the molecular pathogenesis of these diseases, which will provide theoretical basis for clinical diagnosis and treatment. This study aims to identify the peculiar genetic sequences of virulent strains, for better understanding the development of the disease, developing the effective treatment and producing the prevention vaccines.

MATERIALS AND METHODS

Materials

*S. pyogenes* strain was extracted from the patient with *S. pyogenes* infection in Nanjing First Hospital, and the standard strain purchased from the China Center of Industrial Microbiology Culture Collection (CICC), with strain number 10373. In general, the stand-
ard strain has weaker toxicity and usually plays a non-pathogenic role. Suppression subtractive hybridization kit was purchased from the CLOTH Company, and bacterial genomic extraction kit from Bi-Yun-Tian Company. Isopropyl-β-D-thiogalactoside (IPTG) and X-Gal were purchased from SIGMA Corporation, and ampicillin (AMP) from PROMEGA. Escherichia coli TOP10 and PMD18T vector were purchased from TAKARA Corporation. Polymerase chain reaction (PCR) product purification kit was purchased from OMEGA Company.

Growth condition of bacterial strain

The bacterial cells were grown in the solid medium with blood agar and cultured at 37°C, 5% CO₂ overnight.

Extraction of bacterial genomic DNA

The genomic DNA of the two strains, S. pyogenes strain and the standard strain, were extracted according to the instructions for a Bacterial DNA Mini kit. The concentration and purity of the extracted DNA were determined by ultraviolet (UV) spectrophotometer.

Suppression subtractive hybridization

The genomic subtraction between virulent strain and standard strain was performed using a Clontech PCR-Select Bacterial Genome Subtraction Kit with some modifications.

Rsα I digestion

The digestion system, including 1.5 μl of Rsα I (10 U/μl), 5 μl of 10×buffer, 2 μg of genomic DNA and sterile water supplemented to 50 μl, was incubated at 37°C for 16 h. Then, the digestion reaction was stopped by adding 2.5 μl of 0.2 mol/L ethylenediamine tetra acetic acid (EDTA), 5 μl of digestion products was identified with agarose gel electrophoresis and the remaining was precipitated with 95% ethanol and 4 mol/L NH₄OAc. The precipitate was dissolved in 5.5 μl of sterile water with a final concentration of 300 ng/μl. The digested genomic DNA from virulent strain was named Tester and that from standard strain for Driver.

Ligation of adaptors

Tester DNA was divided into two aliquots, one for connecting with Adaptor 1 (named as Tester1) and the other with Adaptor 2R (named as Tester2). The sequences of Adaptor 1 were 5'-CTAATACGACTCACTATAGGGTGATCTCGAGGCGGCCGCGGCCGAGGT3' and 3'-GGCCGCCGCTCA-5', and those of Adaptor 2R were 5'-CTAATACGACTCACTATAGGGTGATCTCGAGGCGGCCGCGGCCGAGGT3' and 3'-GGCCGCCGCTCA-5'. Of Tester DNA, 2 μl of Adaptor and 7 μl of master mix were mixed and incubated at 16°C overnight for ligation reaction. After that, 1 μl of EDTA was added and incubated at 68°C for 5 min to terminate the reaction. Meanwhile, Tester1 and Tester2 were mixed as an unsubtracted control.

Two rounds of selective PCR amplification

The hybrid products were dissolved in 200 μl of diluted buffer, and 1 μl was taken out as template. Then the first round of PCR was carried out with the primer (5'-CTAATACGACTCACTATAGGGC-3') and 94°C 30 s, 66°C 30 s, 72°C 1.5 min for 25 cycles as the amplification condition. The acquired product was diluted for 40-fold as the template for the second PCR, which was performed with the nested primer1 (5'-TCGAGCGGCCGCGCCGAGGT3') and nested primer2R (5'-AGCCTGTCGCGGCCGAGGT3'), and 94°C 30 s, 68°C 30 s, 72°C 1.5 min for 15 cycles as the amplification condition. The unsubtracted controls were operated under the same condition. The final products were identified with 2% agarose gel electrophoresis.

Analysis of subtraction efficiency

The subtracted and unsubtracted products were diluted for 10-fold as templates. Then the 23S rRNA forward primer (5'-CTA-CTTACTAGCGCCGTTATTAGTAC-3') and 23S rRNA reverse primer (5'-GAAGAACTAGCCAAATGTGCTCC-3') were added for PCR reaction with 94°C 30 s, 60°C 30 s, 68°C 2 min as condition. After amplifying for 18, 23, 28 and 33 cycles, each of 5 μl of products was taken out for agarose gel electrophoresis.

Dot-blot screening of subtracted fragments

The secondary PCR products were cloned into a PMD18T vector and transferred into E. coli TOP10 competent cells, followed by plating onto agar plates containing AMP, X-Gal, and IPTG. White clones were selected randomly and grown at 37°C overnight in an lysogeny broth (LB) medium containing ampicillin (100 μg/ml), and the plasmids were extracted using the alkaline lysis method. The TOP10 containing plasmids was placed into LB medium with 100 μg/ml ampicillin in the plate with 96 wells and cultured at 37°C overnight. 1 μl of the culture was inoculated in nylon membrane (Roche Corporation) placed onto the agar plate containing 100 μg/ml ampicillin, which was incubated at 37°C for 18 h. After denaturation, neutralization, and stability at 80°C for 2 h, the plaque on nylon membrane was prepared. 80 ng (about 3 μl) of the secondary PCR products of forward and reverse subtractive hybridization were purified and labeled with 5 μl of [α-32P] d-ATP, which was pre-hybrid with the plaque on nylon membrane at 42°C for 1 h. Then the nylon membrane was further hybrid for 18 h, washed two times for each of 20 min with 2 × SSC (containing 0.5% sodium dodecyl sulphate, SDS) at 58°C and 0.2 × SSC (containing 0.5% SDS) at 58°C, respectively, and finally performed autoradiography at -70°C for 12 h.

Clone sequencing and analysis

After the dot-blot positive clones were screened, PCR amplification was performed on these clones with nested primer 1 and 2. Then the PCR products were identified with 2% agarose gel electrophoresis and confirmation of inserted fragment by sequencing with PE ABI PRISM 3700 sequencer (Shanghai Invitrogen Biotechnology Co.). The acquired DNA sequences were edited manually to remove the vector sequences and analyzed using BLASTN in GenBank (http://www.ncbi.nlm.gov/BLAST).

RESULTS

Extraction of bacterial genomic DNA

By detection with UV spectrophotometer, the concentra-
Figure 1. Agarose gel electrophoresis for bacterial genomic DNA. 1, Genomic DNA from Streptococcus pyogenes strain; 2, genomic DNA from standard strain; M, DNA marker DL2000.

Figure 2. Isolation of virulent strain DNA fragments not present in standard strain using subtractive hybridization. 1, PCR products amplified using Tester1 as the template and 23S rRNA forward primer and PCR primer1 as primers; 2, PCR products amplified using Tester1 as the template and 23S rRNA forward and reverse primers as primers; 3, PCR products amplified using Tester2 as the template and 23S rRNA forward primer and PCR primer1 as primers; 4, PCR products amplified using Tester2 as the template and 23S rRNA forward and reverse primers as primers; M, DNA marker MD101.

Analysis of ligation efficiency

The results on the analysis of ligation efficiency were shown in Figure 2. The band gray-scale analysis of agarose gel electrophoresis showed that the intensity of PCR products amplified using PCR primer1 and 23S rRNA forward primer was less for 2-fold than that using 23S rRNA forward and reverse primers, indicating that the ligation was efficient.

Analysis of subtraction efficiency

Electrophoresis analysis was performed on subtracted and unsubtracted PCR products of 18, 23, 28 and 33 cycles and the results are shown in Figure 3, indicating that subtractive hybridization was efficient.

Screening of subtracted fragments

The result of dot-blot screening of subtracted fragments is shown in Figure 4. After dot-blot screening and searching for homology in GenBank, 12 DNA fragments were identified (Table 1). Among them, seven had a high degree of homology with known genes, and the other five without. These fragments may be new virulent genes of S. pyogenes.

DISCUSSION

The first premise of understanding microbial pathogenic mechanism is to identify and separate virulence genes (Liang and Pardee, 1992). A kind of technology such as suppression subtractive hybridization (SSH) (Diatchenko et al., 1996) put forward by Diatchenko in 1996 was originally used to analyze cDNA differences between tumor cells and normal cells genome and then further extended to microbial fields. SSH has been applied to identify virulence genes of many pathogenic bacteria, including uropathogenic E. coli strains 536 (Janke et al., 2001), Aeromonas hydrophila (Diatchenko et al., 1996), Haemophilus parasuis (Zhou et al., 2010), Salmonella heidelberg (Bronowski and Winstanley, 2009), Streptococcus pneumonia (Suzuki et al., 2005), and Salmonella pullorum (Qiuchun et al., 2009), and got the ideal effect. SSH was also applied to identify bacteria (Radnedge et al., 2001), evaluate virulent island (Walker and Verma, 2002), confirm insert sequences (Sawada et al., 1999) and construct detection probe (Parsons et al., 2002), etc., showing that the technique is a simple and effective method for identifying genetic differences between bacteria.

This was the first time that SSH technology was applied to identify the virulence genes of S. pyogenes. By strictly controlling the quality of genomic DNA extracted from S. pyogenes and standard strains, enzyme digestion of genomic DNA, conjunction of the adaptors, two rounds of
Figure 3. Evaluation on the subtraction efficiency of subtracted DNA. PCR for 23S rRNA was performed on the unsubtracted (lanes 1 to 4) and subtracted (lanes 5 to 8), DNA for 18 (lanes 1 and 5), 23 (lanes 2 and 6), 28 (lanes 3 and 7) and 33 cycles (lanes 4 and 8), respectively. M, 1 kb plus DNA ladder.

Figure 4. Dot-blot screening of subtracted fragments. I, The membrane was screened by hybridization with genomic DNA from virulent strain; II, The membrane was screened by hybridization with genomic DNA from standard strain. A1, A2, A6, B5, C5, C7, D3, E4, E8, F5, G5 and H2 were the positive clones.

Table 1. Basic information for 12 subtracted DNA fragments.

<table>
<thead>
<tr>
<th>Fragment number</th>
<th>Fragment size (bp)</th>
<th>Homology with BLAST</th>
<th>Intensity of hybrid signal</th>
</tr>
</thead>
<tbody>
<tr>
<td>A1</td>
<td>518</td>
<td>DNA gyrase subunit A (gyrA)</td>
<td>Enhanced</td>
</tr>
<tr>
<td>A2</td>
<td>238</td>
<td>Streptokinase gene</td>
<td>Enhanced</td>
</tr>
<tr>
<td>A6</td>
<td>481</td>
<td>Unknown sequence</td>
<td>Enhanced</td>
</tr>
<tr>
<td>B5</td>
<td>573</td>
<td>Unknown sequence</td>
<td>Enhanced</td>
</tr>
<tr>
<td>C5</td>
<td>1740</td>
<td>Macrolide efflux gene (mefE)</td>
<td>Enhanced</td>
</tr>
<tr>
<td>C7</td>
<td>298</td>
<td>Unknown sequence</td>
<td>Enhanced</td>
</tr>
<tr>
<td>D3</td>
<td>584</td>
<td>Pyrogenic exotoxin C (speC)</td>
<td>Enhanced</td>
</tr>
<tr>
<td>E4</td>
<td>443</td>
<td>Complement inhibitor (SIC gene)</td>
<td>Enhanced</td>
</tr>
<tr>
<td>E8</td>
<td>317</td>
<td>Unknown sequence</td>
<td>Enhanced</td>
</tr>
<tr>
<td>F5</td>
<td>400</td>
<td>Collagen-like surface protein 1 (SCL1)</td>
<td>Enhanced</td>
</tr>
<tr>
<td>G5</td>
<td>916</td>
<td>C5a peptidase (scpA)</td>
<td>Enhanced</td>
</tr>
<tr>
<td>H2</td>
<td>206</td>
<td>Unknown sequence</td>
<td>Enhanced</td>
</tr>
</tbody>
</table>
subtractive hybridization and PCR, the common gene fragments were subtracted and virulence genes were amplified specifically. Amplified PCR products were purified and connected with vector T to construct the virulent gene library of *S. pyogenes* successfully. After sequencing with M13 two-way primers and comparing with BLAST data in Genbank, 12 virulence genes were obtained, seven highly homology with known genes including DNA gyrase subunit A, streptococcus kinase, macrolide efflux gene, pyrogenic exotoxin C, complement inhibitor, collagen-like surface protein 1 and C5a peptidase, and the other five without homologous sequence in Genbank. Although some sequences showed homology to certain hypothetical proteins or putative proteins, their function was unknown. Therefore, the biological function of these virulent genes and the relationship between these genes and diseases are still further investigated.

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**REFERENCES**


