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Research Paper

# EsxA might as a virulence factor induce antibodies in patients with *Staphylococcus aureus* infection

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## Abstract

*Staphylococcus aureus* (*S. aureus*) is an important human pathogen, which commonly causes the acquired infectious diseases in the hospital and community. Effective and simple antibiotic treatment against *S. aureus*-related disease becomes increasingly difficult. Developing a safe and effective vaccine against *S. aureus* has become one of the world's hot spots once again. The key issue of developing the vaccine of *S. aureus* is how to find an ideal key pathogenic gene of *S. aureus*. It was previously suggested that EsxA might be a very important factor in *S. aureus* abscess formation in mice, but clinical experimental evidence was lacking. We therefore expressed EsxA protein through prokaryotic expression system and purified EsxA protein by Ni-affinity chromatography. ELISA was used to detect the anti-EsxA antibodies in sera of 78 patients with S. aureus infection and results showed that the anti-EsxA antibodies were positive in the sera of 19 patients. We further analyzed the EsxA positive antibodies related strains by antimicrobial susceptibility assay and found that all of the corresponding strains were multi-drug resistant. Among those multi-drug resistant strains, 73.7% were resistant to MRSA. The results indicated EsxA is very important in the pathogenesis of *S. aureus*. We suggested that the EsxA is very valuable as vaccine candidate target antigens for prevention and control of *S. aureus* infection.

**Key words:** *S. aureus*, *esxA*, anti-EsxA antibodies, multi-drug resistant.

## Introduction

*Staphylococcus aureus* (*S. aureus*) is an important human pathogen that causes the acquired infectious diseases in the hospital and community (Lowy, 1998; Talia *et al.*, 2011). The increasing incidence of *S. aureus* infections through both the healthcare and community settings, are rapidly promoting *S. aureus* to acquire the antibiotic resistance to both first-line and more novel antibiotics. The number of antibiotic resistance isolates of *S. aureus* is rapidly increasing (Bal and Gould, 2005; Cunha and Pherez, 2009; Hidron *et al.*, 2008; Kirby *et al.*, 2009; Skiest, 2006). Of which, the methicillin-resistant *S. aureus* (MRSA) is the most important and the morbidity and mortality of these infectious diseases caused by MRSA is very high. Relying solely on the antibiotic therapy for *S. aureus*-related disease becomes increasingly difficult. Therefore, to develop effective and safe vaccine of *S. aureus* has once again become one of the world's hot spots. Thus far there is no very ideal *S. aureus* vaccine for clinical application. To seek the ideal target genes is the key to develop effective and safe vaccine of *S. aureus*.

*S. aureus* pathogenesis in the host relies on the secretion of virulence factor through the secretion system (Abdallah *et al.*, 2007; Jett and Gilmore, 2002; Sibbald *et al.*, 2006). It was reported that the recently named type VII secretion system(T7SS) was present in *S. aureus* (Abdallah *et al.*, 2007; Jett and Gilmore, 2002; Sibbald *et al.*, 2006).

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The T7SS was first found in the *Mycobacterium tuberculosis*, and the T7SS could secret ESAT-6 (early secreted antigen target 6 kDa) which could trigger cell-mediated immune response in host (Pallen, 2002; Stanley *et al.*, 2003). The T7SS of *S. aureus* has the ability to secret ESAT-6 like proteins EsxA and EsxB to the extracellular surroundings (Burts *et al.*, 2005). The locus of gene *esxA* and *esxB* is arranged with other six genes in the Ess gene cluster. Some genes in this gene cluster such as *essA*, *essB*, and *essC* are necessary for the synthesis and secretion of EsxA and EsxB. It was reported that the secretion of EsxA and EsxB was prevented in the absent of *essA*, *essB*, and *essC* (Burts *et al.*, 2005).

The abscess formation is the most important for the virules of *S. aureus* (Cheng *et al.*, 2009; Dinges *et al.*, 2000; Novick, 2003). There have been shown that *S. aureus* strains were reduced obviously capacity of the formation abscesses in the infection process of mice only when sortase mutants defective (Jonsson *et al.*, 2002; Mazmanian *et al.*, 2000). However it was recently reported that the *esxA* mutant strain show the obvious defect in the formation of abscess in infected mice (Burts *et al.*, 2005), and this suggests that EsxA may play an important role in the process of the pathogenesis for *S. aureus*. Therefor, EsxA was hopeful to become the candidate antigen for the development of *S. aureus* vaccine.

In this study, *S. aureus* was isolated from the clinical specimens of hospitalized patients who came from 10 different ward areas and the antimicrobial susceptibility assay was determined according to 2010 CLSI recommendations (Clinical and Laboratory Standard Institute, 2010). At the same time, the sera of the patients with *S. aureus* infection were collected. And then the protein EsxA of *S. aureus* was prepared and used as the antigen to detect the anti-EsxA antibodies in the serum of the patients with *S. aureus* infection by the indirect ELISA.

## Materials and Methods

### Collection of strains and serum

The Second Affiliated Hospital of Soochow University (1231 beds) is one of the largest hospital in Suzhou, China. Isolates of *S. aureus* were obtained from the clinical specimens of hospitalised patients from June 2010 to April 2011. Isolates were confirmed as *S. aureus* using a Staph SPA agglutination kit, Gram's stain and Phoenix System-100 BD Automated Microbiology analyser (BD Diagnostics, USA). At same time, 78 clinical sera were obtained from the coherent patients with *S. aureus* infection. The *S. aureus* isolates were mainly associated with lung infection and pyogenic soft-tissue infection and pyogenic post-operative wound surface infections in patients from 10 different ward areas such as the intensive care unit. Every *S. aureus* strain was isolated from different patients, one strain was corresponding to one patient. Fifty negative control sera were collected from the hospital medical center healthy people.

### Antimicrobial susceptibility

Isolates of *S. aureus* were inoculated onto the Phoenix panel according to the manufacturer's instructions and then the identification and antimicrobial susceptibility of these isolates were determined by Phoenix System-100 BD Automated Microbiology (BD Diagnostics, USA). Results of Minimum Inhibitory Concentrations (MICs) were recorded according to 2010 CLSI criteria (Clinical and Laboratory Standard Institute, 2010). Methicillin-resistant *Staphylococcus* aureus (MRSA) was confirmed if MIC of oxacillin > 4 μg/mL. *S. aureus* ATCC 29213 were used as a quality control strain for antimicrobial susceptibility testing.

### Preparation of the protein EsxA of *S. aureus*

The *esxA* gene was amplified with the following primer pairs: 5'-GCGGATCCATGGCAATGATTAAGATGAG-3' and 5'-AACTCGAGTTGCAAACCGAAATTATTAG-3'. The PCR products were cloned into the pGEM-T Easy vector to yield plasmids pGEM-*esxA*. The gene was cut out from the plasmid with the restriction endonucleases *Bam*H I and *Xho* I and cloned into the pET-28a vector to generate pET-28a-*esxA*, verified by sequencing *esxA* gene. Plasmid pET-28a-*esxA* was designed for the heterologous protein expression in *E. coli* Rosetta (Ros) to synthesize the N-terminal histidine-tagged recombinant proteins. *E. coli* Ros cells carrying pET-28a-*esxA* were grown in LB medium containing ampicillin (100 μg/mL) at 37 °C for 16 h. An aliquot (1 mL) of an overnight culture was used to inoculate 100 mL of the same medium and incubated at 37 °C with shaking (250 rpm) until the OD600 of culture reached 0.6. Expression of the recombinant proteins was induced for 3 h at 37 °C by the addition of IPTG to a final concentration of 100 μM. Cells were harvested by centrifugation at 6,000 *g* at 4 °C for 10 min, and resuspended in 10 mL of binding buffer (5 mM imidazole, 0.5 M NaCl, and 5 mM Tris-HCl; pH 7.9). The recombinant proteins $EsxA_{his6}$ carring 6 His tag were purified from cell lysate fraction by affinity chromatography with $Ni^{2+}$-NTA system (QIAGEN, Cologne, Germany) according to the manufacturer's protocol. After extensive washing, the bound proteins were eluted with 5 mM Tris-HCl buffer (pH 7.9) containing 0.2 M imidazole and 0.15 M NaCl. Purified proteins were identified by Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) in 12% polyacrylamide gels and stained with Coomassie brilliant blue. Protein concentration was determined by a protein assay reagent (Bio-Rad, California, USA) and with bovine serum albumin as the standard.

### Detection of the anti-EsxA antibody in serum by the indirect ELISA

The purified EsxA antigen was diluted to 20 ng/μL. The 96-well ELISA plates were coated with the above diluted EsxA antigen by 10 μLper well, and then put at 4 °C overnight. The ELISA plates were washed with washing Phosphate Buffered Saline Tween-20 (PBST) three times, each for min dried through pat, and then add 10 μL 10% fetal calf serum into each well. After incubated for 1 h at room temperature, the ELISA plates were washed as above, and then add 10 μL tested serum into each well. After incubated at 37 °C for 1 h by the water bath, the ELISA plates were washed as above too. Then, add 10 μL horseradish peroxidase labeled goat anti-human HRP-IgG into each well, and incubate the ELISA plates at 37 °C for 1 h by water bath. At last, wash the ELISA plates with buffer PBST five times, each for min then pat the plates for dry; add substrate buffer A, B the role of 5 μL into each well for 30 min, and then add 2 mol/L sulfuric acid 10 μL into each well to stop the reaction. Absorbance values ($OD_{450}$) were measured by Microplate Reader Model 680 BIO-RAD Japan). Each experiment was performed three times. The anti-EsxA antibodies in 50 healthy human sera were tested by indirect ELISA assay and calculated the average $OD_{450}$ value mean (M) and Standard deviation (SD). The value of M+2SD was defined as the threshold value. When the $OD_{450}$ value of the tested serum was greater than M+2SD, the sample was classified as a positive sample.

## Results and Discussion

*S. aureus,* one of the most important pathogens mainly spread in the community and hospital*,* can cause superficial infections, osteomyelitis, pneumonia, septic arthritis, endocarditis, meningitis, and even can lead to sepsis or other systemic infection (Lowy, 1998). In recent years, due to the overuse of antibiotics, a variety of new strains of antibiotic resistant have appeared, such as MRSA strain (Coia *et al.*, 2006; Haamann *et al.*, 2011; Muto, 2006; Salgado and Farr, 2006) and new-found vancomycin-insensitive *S. aureus* (Vancomysin-intermediate *S. ureus*, VISA) strain (Jones *et al.*, 2008; Woodford and Livermore, 2009). Those drug-resistant strains make it more difficult to treat *S. aureus* infection. There was reported that *S. aureus* whole-cell vaccine ineffective in clinical, and thus development of effective of vaccine should to find other antigens which could stimulate protective antibodies (Lee, 1998; Watson and Kennedy, 1981), but some clinical study results about *S. aureus* vaccine developed based on some antigens such as capsular polysaccharide antigen were not satisfactory (Fattom *et al.*, 1996; Rupp *et al.*, 2007; Shinefield, 2006). So far, there was no ideal of *S. aureus* vaccine.

This study constructed EsxA prokaryotic expression system successfully. *E. coli* Ros cells harboring pET-28a-EsxA was grown in liquid medium at 37 °C for 3 h in the presence of 0.1 mmol/L IPTG. One predominant band corresponding to the molecular mass of approximately 16 kDa was observed in the crude extract of IPTG-induced *E. coli* (Figure 1). The recombinant protein in the crude extract was purified by nickel-chelate column chromatography. The purified EsxA was apparently homogenous in the 16 kD protein bands as judged by SDS-PAGE (Figure 1). As shown in Figure 1, EsxA was purified to near homogeneity by $Ni^{2+}$-NTA, consistent with the experimental design. In order to understand EsxA might be as a virulence factor inducing the production of anti-EsxA antibodies in patients with *Staphylococcus aureus* infection. We further used the purified EsxA protein antigen to detect the anti-EsxA antibodies in serum of patients with *S. aureus* infection by indirect ELISA. The antibody level of EsxA in 50 healthy serum samples was tested and the mean $OD_{450}$ value (M) and standard deviation (SD) were 0.110 and 0.120, respectively. The value of M+2SD was defined as cutoff which was 0.350. It meant that the tested sample was anti-EsxA antibody positive when $OD_{450}$ value was greater than 0.350. Among the tested 78 clinical samples, 19 samples were positive and the positive rate was 24.35%. The result was shown in Table 1.

These results indicated that EsxA might be as a virulence factor inducing antibodies in patients with *Staphylococcus aureus* infection. To our best knowledge, this is the first study to show that EsxA might be a virulence factor and induce the production of anti-EsxA antibodies in patients with *S. aureus* infection. In addition, we further analyzed antimicrobial susceptibility with the EsxA serum antibodies related strains by Phoenix System-100 BD Automated Microbiology, and found that the corresponding

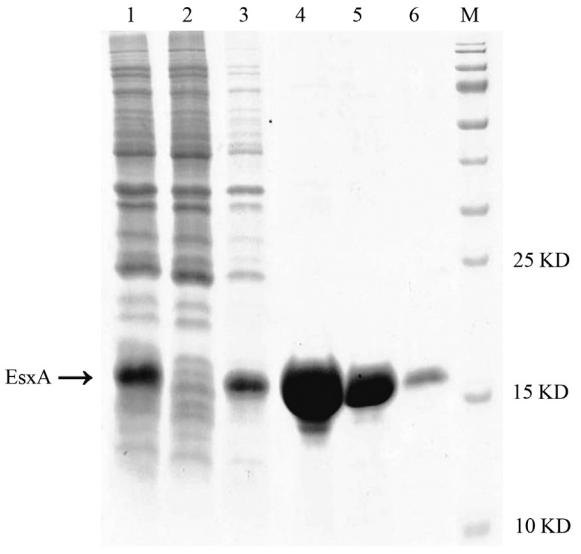


**Figure 1** - Analysis of the purified EsxA protein by SDS-PAGE. Lanes M: the standard molecular weight proteins; lane 1: the lysates of total cell; lane 2: the precipitates of cell by ultrasonic lysation; lane 3: the supernatants of cell by ultrasonic lysation; Lanes 4-6: the purified EsxA proteins with different concentrations.

**Table 1** - ELISA was detected to anti-EsxA serum antibodies in patients with *S. aureus* infection.

| Sample type | Total cases | Positive cases | Positive rate (%) | M | SD |
|---|---|---|---|---|---|
| Control | 50 | 0 | 0 | 0.110 | 0.120 |
| Test group | 78 | 19 | 24.35 | 0.546 | 0.244 |

Negative values of the sample co (M+2SD) = 0.350, greater than 0.350 judged as positive.
M: The average OD $_{450}$ value; SD: Standard deviation.

**Table 2** - Antimicrobial susceptibility analyses *S. aureus* with EsxA antibodies Positive.

| Number | Specimen | Ward | Antimicrobial susceptibility | | | | | | | | | | MRSA |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | C | R* | T | S | A | E | G | P | V | L | |
| 1 | Sputum | Respiratory | R | R | R | R | R | R | R | R | S | S | Y |
| 2 | Blood | ICU | R | R | S | R | R | R | R | R | S | S | Y |
| 3 | Sputum | ICU | R | R | R | R | R | R | R | R | S | S | Y |
| 4 | Urine | Renal | R | R | R | R | S | R | R | R | S | S | Y |
| 5 | Sputum | Haematology | R | R | R | R | R | R | R | R | S | S | Y |
| 6 | Abscess | Neurology | R | R | R | S | R | R | R | R | S | S | Y |
| 7 | Sputum | Cardiac | R | R | R | R | R | R | R | R | S | S | N |
| 8 | Sputum | Neurosurgery | R | R | R | R | R | R | R | R | S | S | Y |
| 9 | Abscess | Empyema surgery | R | R | R | R | R | R | R | R | S | S | N |
| 10 | Abscess | General surgery | R | R | S | R | R | R | R | R | S | S | Y |
| 11 | Sputum | Cardiac | R | R | R | R | R | R | R | R | S | S | Y |
| 12 | Sputum | ICU | R | R | R | R | R | R | R | R | S | S | Y |
| 13 | Sputum | Respiratory | R | R | R | R | S | R | R | R | S | S | N |
| 14 | Sputum | Empyema surgery | R | R | S | R | R | R | R | R | S | S | Y |
| 15 | Blood | Cardiac | R | R | R | R | R | R | R | R | S | S | Y |
| 16 | Sputum | Respiratory | R | R | R | R | S | R | R | R | S | S | Y |
| 17 | Abscess | Obstetrics | R | R | R | R | R | R | R | R | S | S | Y |
| 18 | Urine | Renal | R | R | R | R | R | R | R | R | S | S | N |
| 19 | Sputum | ICU | R | R | R | R | S | R | R | R | S | S | N |

R, resistant; S, susceptible; Y, yes; N, not.
C, Clindamycin; R*, Rifampicin; T, tetracyline; S, trimethoprim-sulphamethoxazole; A, Amoxicillin/ clavulanic acid; E, erythromycin; G, gentamicin; P, penicillin; V, Vancomycin; L, Linezolid.

positive strains all were multi-drug resistant, of which MRSA to 73.7% (shown in Table 2).

In summary, we successfully detected the EsxA antibody in the serum of clinical *S. aureus* infectious patients, and found that EsxA were present in multiple drug-resistant strains, especially MRSA strains. Above all, the results indicated that EsxA might as a virulence factor induce antibodies in patients with *Staphylococcus aureus* infection and suggested that EsxA maybe is a very valuable candidate target antigens of the new vaccine for prevention and control of *S. aureus* infection, but it needs further researches to clarify

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