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Full Length Research Paper

Prevalence of virulence genes and biofilm formation among *Staphylococcus aureus* clinical isolates associated with lower respiratory infection

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The aim of this study was to determine the frequencies of virulence genes and biofilm formation among *Staphylococcus aureus* clinical isolates, associated with lower respiratory infection. Different methods, from December 2007 to September 2009, were used to determine a total of 119 *S. aureus* clinical isolates, which were isolated from the sputum specimens of the patients with lower respiratory infections, verified by X-ray at a teaching hospital in Wenzhou, China. The present study has determined the frequencies of 9 virulence genes, including five classical major enterotoxin genes (sea to see), tst, eta, etb and lukS/F among these *S. aureus* isolates. Three multiplex PCRs and a single PCR were used for detecting the 9 virulence genes above. Microtiter-plate test was performed to semi-quantify biofilm formation of *S. aureus*. Results of all 9 tested virulence genes except see and etb, were detected among tested *S. aureus* isolates. All of 119 tested isolates, 79 isolates (68.1%) harbored at least one of the tested virulence genes, among which 76 isolates harbored only one gene tested and 3 isolates harbored two genes. The most prevalent virulence gene was sea (57.1%, 68/119). Only two isolates (1.7%) were positive for pvl. The prevalence of sea was significantly higher than those of other virulence genes ($p < 0.01$). The prevalence (62.8%) of sea among MRSA isolates was significantly higher than that (36.0%) among MSSA isolates ($p < 0.01$). Eighty-four (70.6%) of 119 isolates were biofilm producers. The prevalence of biofilm formation among MSSA isolates was significantly higher than that among MRSA isolates ($p < 0.01$). Conclusion, sea was the most prevalent virulence gene among the *S. aureus* clinical isolates associated with lower respiratory infection. Most of these isolates could form biofilm.

Key words: *Staphylococcus aureus*, virulence genes, biofilm formation, lower respiratory infection.

INTRODUCTION

Staphylococcus aureus, particularly methicillin-resistant *S. aureus* (MRSA), is associated with a wide range of diseases from superficial skin infections to life-threatening conditions such as bacteremia, endocarditis, pneumonia or toxic shock syndrome (Lowy, 1998). The ability of this clinically important species to successfully persist within the hosts, is largely due to the expression of a battery of virulence factors, which promote adhesion, acquisition of nutrients, and evasion of host immunologic responses (Bubeck et al., 2007; Dinges et al., 2000). However, some *S. aureus* isolates also produce one or more additional exoproteins, such as toxic shock syndrome toxin-1 (TSST-1), the staphylococcal enterotoxins (SEs), the exfoliative toxins (ETs) and

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leukocidins (Bubeck et al., 2007; Bunikowski et al., 2000; Dinges et al., 2000).

TSST-1 and the SEs are known as pyrogenic toxin superantigens (PTSAgs) which have effects such as superantigenicity, pyrogenicity, toxicity and direct damage to the endothelial (Dinges et al., 2000). TSST-1 is involved in staphylococcal toxic shock syndrome, especially in menstrual cases and ETs are responsible for the staphylococcal scalded-skin syndrome (Dinges et al., 2000). The SEs are one cause of food poisoning in humans (Thomas et al., 2007). Apart from causing food poisoning; SEs are also associated with other diseases such as allergy sensitization, asthma, chronic obstructive pulmonary disease, scarlet fever, glomerulonephritis or vasculitis (Semic-Jusufagic et al., 2007). The presence of sea gene was associated with food poisoning. The positive rate of se genes for *S. aureus* isolates originated in food poisoning was 100%, among which 58% of isolates were associated with the sea gene and the majority of these isolates possessed other se genes (Omoe et al., 2002).

Recently, Panton-Valentine leukocidin encoded by two contiguous and co-transcribed genes (lukS-PV and lukF-PV) is considered as an important virulence factor for community-acquired MRSA (Gillet et al., 2002). Biofilm formation of *S. aureus* isolates is associated with human infections (Souza et al., 2009). The principal component of biofilm formation of *S. aureus* isolates is a polysaccharide intercellular adhesion encoded by the ica operon (icaA, icaB, icaC, and icaD) (Gad et al., 2009). The aim of this study was to determine the frequencies of virulence genes and biofilm formation among *S. aureus* clinical isolates, associated with lower respiratory infection.

MATERIALS AND METHODS

Bacterial strains

From December 2007 to September 2009, a total of 119 *S. aureus* clinical isolates were isolated from sputum or bronchoalveolar samples from the patients with lower respiratory infections verified by X-ray and other physical examinations, at a teaching hospital in Wenzhou, China. The sputum samples with more than 25 white blood cells and less than 10 epithelial cells, determined by microscopic examination amplifying 100 folds, were qualified for culturing bacteria. The *S. aureus* isolates from the patients with clinical signs and symptoms of infection, including increased white blood cell counts and fevers, were isolated more than two times and considered for inclusion. The *S. aureus* isolates from the patients without clinical signs and symptoms of infection were considered to be colonization and excluded. All the isolates were confirmed as *S. aureus* by using a Staph SPA agglutination kit (bioMe´rieux, Marcy l'Etoile, France), Gram's stain and a Vitek-60 microbiology analyzer (bioMe´rieux, Marcy l'Etoile, France).

DNA isolation procedures

S. aureus isolates were cultured on blood agar overnight. Clones were re-suspended in 150 µl of TE buffer with 5 µl of recombinant lysostaphin (1 mg/ml; Sagon, China), incubated at 37°C for 1 h and then boiled at 100°C for 15 min. After centrifugation at 15000 rpm for 15 min at 4°C, the supernatant was removed and stored at -20°C for further assays.

Multiplex PCR procedures

Both the multiplex PCR for detecting simultaneously the classical SE genes (sea, seb, sec, sed, and see) and the multiplex PCR for detecting the ET genes (eta and etb) and the TSST-1 gene (tst) were performed as previously described (Becker et al., 1998). mecA gene and PVL genes were simultaneously detected by another multiplex PCR procedure described previously (McClure et al., 2006). Previously characterized clinical *S. aureus* isolates were used as positive control.

Biofilm formation

S. aureus isolates were grown overnight and diluted until 108 CFU/ml in TSB containing 0.25% glucose. 200 µL aliquots of the cultures were inoculated into individual wells of polystyrene, flat-bottomed 96-well microtiter plates. After 24 h of incubation at 37°C, the wells were washed three times with 200 µL sterile 0.9% NaCl solution. Subsequently, 100 µl crystal violet solution (0.3% wt/vol) was added to all wells. After 5 min, the excess crystal violet was removed by washing three times with water.

Staphylococcus epidermidis (ATCC 35984) was used as positive control for biofilm formation. *S. epidermidis* (ATCC 12228) was used as negative control for biofilm formation. To quantitate the biofilms, bound crystal violet was released by adding 100 µl 70% (vol/vol) ethanol after drying the plates. Absorbance was measured spectrophotometrically by a microplate reader at 570 nm (A570). The isolates with A570 values above the mean A570 values plus three standard deviations of the negative control were considered biofilm formation. All assays were performed in triplicate and repeated on three occasions.

Statistical analysis

Differences between groups were assessed by using the chi-square test. Software SPSS 11.0 was used to perform calculations. P values of < 0.05 were considered statistically significant.

RESULTS

Frequencies of virulence genes among *S. aureus*, associated with lower respiratory infection

The tested virulence genes except see and etb were detected among 119 isolates associated with lower respiratory infection. Seventy-nine (68.1%) of 119 isolates harbored at least one virulence gene. Among the 79 isolates above, 76 isolates harbored only one gene tested and 3 isolates harbored two genes tested among which one isolate with seb and eta, one isolate with sea and tst and another isolate with sea and sec. The most prevalent virulence gene was sea (57.1%, 68/119). The remaining virulence genes were seldom detected. Only two isolates (1.7%) were positive for pvl. The prevalence

of seb, sec, sed, tst and eta were 2.5 (3/119), 0.8 (1/119), 0.8 (1/119), 2.5 (3/119) and 0.8% (1/119). The prevalence of sea was significantly higher than those of other virulence genes tested ($p < 0.01$).

Comparison of sea in MRSA and MSSA isolates

Among 119 isolates, 94 isolates (79.0%) harboring mecA detected by PCR were determined as MRSA. The prevalence (62.8%) of sea among MRSA isolates was significantly higher than that (36.0%) among MSSA isolates ($p < 0.01$).

Biofilm formation

Eighty-four (70.6%) of 119 isolates were biofilm producers. Twenty-two (88.0%) of 25 MSSA isolates were biofilm-forming isolates, whereas the prevalence of biofilm formation among MRSA isolates were 66.0% (62/94). The prevalence of biofilm formation among MSSA isolates was significantly higher than that among MRSA isolates ($p < 0.01$).

DISCUSSION

Seven of 9 virulence genes tested were detected among *S. aureus* clinical isolates associated with lower respiratory infections. sea was the most prevalent virulence gene. In our previous study, sea was found to be prevalent among 36.0% of the *S. aureus* isolates associated with skin and soft tissue infections (SSTIs) and 30.0% of the *S. aureus* isolates associates with bloodstream infection whereas only 8.5% of colonizing *S. aureus* isolates from the nasal specimens of healthy college students without clinical signs and symptoms of infection were found to carry sea (Yu et al., 2010). Only 11.4% of *S. aureus* isolates causing continuous ambulatory peritoneal dialysis (CAPD)-associated peritonitis carried sea (Barretti et al., 2009). Becker et al. reported that sea was found in 17.4% of blood isolates and 15.9% of nasal isolates (Becker et al., 2003).

Compared with the data given in the literatures and our previous study (Barretti et al., 2009; Becker et al., 2003; Yu et al., 2010), a higher prevalence of sea (57.1%) was found among the isolates associated with lower respiratory infections in the present study. The data reported here indicated that, presence of sea gene was associated with lower respiratory tract infection. The presence of sea gene was also found to be associated with septic shock in patients with *S. aureus* bloodstream infection and the severity of *S. aureus* sepsis was positively associated with sea (Ferry et al., 2005). The reason why sea might cause *S. aureus* sepsis was that it triggers the over-expression of inflammatory mediators associated with shock (Ferry et al., 2005). In our previous report, the frequencies of seb, sec, sed and tst among the isolates associated with SSTIs were 22.5, 12.6, 10.8 and 19.8%, respectively (Yu et al., 2010). However, these virulence genes were seldom found in the present study.

Infections caused by PVL positive isolates are predominantly represented by skin and soft-tissue infection and necrotizing pneumonia (Gillet et al., 2002). Community-acquired methicillin-resistant *S. aureus* (CA-MRSA) clones usually contain the Panton Valentine leukocidin (PVL) genes (Gillet et al., 2002). In our previous studies, high prevalence of PVL genes was found among the *S. aureus* isolates associated with SSTIs (Yao et al., 2010). Surprisingly, low prevalence of PVL genes was found among *S. aureus* clinical isolates associated with lower respiratory infections. Hu et al. reported that the MRSA isolates harbored more superantigenic toxin genes than the MSSA isolates, among which possession of the sec, sell and tst-1 genes in MRSA isolates (77.1 %) was significantly more than that in MSSA isolates (2.1%) (Hu et al., 2008).

In the present study, we also found that the prevalence of sea MRSA isolates was significantly higher than that of MSSA isolates. However, Peck, et al. reported that sea was more prevalent among MSSA isolates compared with MRSA isolates (Peck et al., 2009).

The pathogenesis of bacteria was closely related to biofilm formation. Bacteria can easily adhere to the surface of biological materials or live tissues/organs and form biofilms to cause persistent infection. In the present study, the majority of the isolates tested could form biofilm. The reason is probably that the *S. aureus* isolates, can easily adhere to pulmonary tissues. Biofilm formation is a multistep process. The principal component of biofilm formation of *S. aureus* isolates is a polysaccharide intercellular adhesion encoded by the ica operon (icaA, icaB, icaC and icaD).

In conclusion, sea was the most prevalent virulence gene among the *S. aureus* clinical isolates associated with lower respiratory infections. Most of these isolates could form biofilm.

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