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Detection of Staphylococcal protein A (SpA) in culture medium for developing sortase inhibitor screening method

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ABSTRACT

Staphylococcus aureus (S.aureus) is a bacteria that can cause infections disease in both of humans and animals. It has several virulence factors, most of which are toxins or enzymes that secrete to extracellular or exotoxin and can cause the disease to host. One of the virulence factors is a Staphylococcal SpA (SpA) that can be expressed at the cell wall and secreted into the culture medium by using enzyme Sortase (Srt) activity. This work studied the optimum condition for measuring the SpA in culture medium of S. aureus with anti-SpA antibody by Direct enzyme-linked immunosorbent assay (ELISA). In this study we used Methicillin-resistant S. aureus (MRSA)ATCC 43300 strain and Methicillin-sensitive S. aureus (MSSA) ATCC 25923strain which already examined that they have srtA gene by Polymerase chain reaction (PCR). The results showed that the highest SpA concentration in MRSA and MSSA were 41.79 and 63 ng/ml after 5-6 h cultivation, respectively. Moreover, MSSA treat with sortase A inhibitor show the reduction of SpA in the culture medium compare with non treatment ($p< 0.05$) The result may be developed as a sortase A inhibitor screening method further.

KEYWORDS: *Staphylococcus aureus, Staphylococcal protein A, SpA, Sortase, Sortase inhibitor*

1. INTRODUCTION

Cell surface protein of gram-positive pathogens plays an important role for host adhesion and invasionsuch as protein A, fibronectin binding protein, collagen binding protein[1], [2].

Surface proteins holding a C-terminal with an LPXTG motif are covalently linked to the cell wall peptidoglycan by a transamidase named sortase[3].Sortase (Srt) is the transpeptidase protein, encoded by srt gene can be classified into 3 isoforms which are A isoform found in all gram positive bacteria, B isoform found in *Bacillus spp.*, *Listeria spp.*, *S. aureus* and C isoform found in *Streptococcus spp.*, *Corynebacterium diphtheria*, *Enterococcus faecalis* and *Bacillus cereus*[4].

In *S.aureus*, the most virulent of many staphylococcal species, a mutation in the srtA gene results in defective anchoring of several proteins, including a number of surface-associated adherence factors [5]and also fail to process and display surface proteins which result to defective in the establishment of infections[6]. Moreover, inactivation of srtA gene caused a decreased in biofilm formation [7].Previous report also showed thatsortase A is important in anchoring of specific adhesive proteins which thesrtA knockout mutant of *S. agalactiae*showed the reduction in adherence to human and murine epithelial cells [8].Therefore, inhibitors of SrtA might consequently be promising candidates for the treatment and/or prevention of gram-positive bacterial infections.

Currently, the importance of sortase as a new target has recently been acknowledged. However, there have only been a few reports in the literature describing inhibitors of sortase. One of the reason is the assay for determining sortase activity such as fibronectin-binding assayand Fluorescence resonance energy transfer-based assay are time consuming and expensive[9].

Protein A of *S.aureus*or staphylococal SpA (SpA) is a cell wall component with molecular weight about 42,000 Da, becauseSpAinteract with the Fc fragment region of immunoglobulins from most mammalian species, these protein has been used extensively for quantitative and qualitative

immunological techniques[10].The biosynthesis of SpA occurs during the exponential growth phase of *S.aureus*. Some SpAis bound to the cell wall but in the stationary growth phase of the bacterium, some release probably occurs because of autolysis and release into the culture medium [11]. As sortase also play a crucial role for the covalently attachment of specific virulence protein including SpA to the cell wall of *S.aureus*[12], [13]therefore to determine the SpA in bacterial culture can be use as a tool for screening sortase A inhibitor we investigate the SpA level by ELISA technique comparing between with and without sortase inhibitor.

2. MATERIAL AND METHODS

2.1 Bacterial strains

Methicillin-sensitive *S.aureus* (MSSA)strain ATCC 25923 and Methicillin-resistant *S.aureus* (MRSA) strain ATCC 43300 isolated from a single colony, were sub-cultured on blood agar at 37°C for 24 h, and then transferred to 10 ml of nutrient broth in a 100 ml flask for 10 h at 37°C with continuous shaking at 150 rpm (GYROMAX™ 737, Amerex Instruments). The culture was centrifuged at 3000 rpm for 10 min and the pellet was resuspened in phosphate-buffered saline (pH 7.4) and optical density (OD) adjusted to 0.6 at 600 nm. The brain-heart infusion medium (pH 7.4) was inoculated with the bacterial suspension at the final concentration of 1 %(v/v) and incubated at 37°C in a shaking water bath at 150 rpm. A 5 ml sample was removed at 1 h intervals and centrifuged at 10,000 ×g for 5 min.

2.2 DNA extraction

The bacteria isolates were transferred to 2 ml of nutrient broth and incubated overnight at 37°C until reaching stationary phase. The cells were harvested by centrifugation at 12,000 rpm for 5 min and washed twice with phosphate buffered saline. The bacterial cells were extracted by NucleoSpin ®Tissue. After centrifugation at 12,000 rpm for 3 min, the supernatant was collected as a PCR template. The DNA concentration and purity were determined spectrophotometrically by measuring the A260 and A280 (UV-1601 Shimadzu). The genomic DNA samples were stored at -80 °C until use.

2.3 Amplification of srtA gene

The PCR primers were designed according to the S. aureussubsp.aureus N315 sequence (http://www.ncbi.nih.gov/gene/ 1125243srtA). The designed primer was used to amplify a 621bp DNA fragment carrying the srtA gene.

Forward primer
5'-GAGGATCCCAAAAAATGGACAAATCGATTAATG-3'

Reverse primer
5'-CGGTACCCGTTTGACTTCTGTAGCTACAAAG-3'

The PCR amplification was performed in a 50 ml total reaction volume. Using a Thermal cycler (GENEAMP PCR system 2400, Perkin Elmer), the samples were preheated at 94 °C for 3 min followed by amplification under the following conditions: denaturation at 94°C for 30 s, annealing at 55 °C for 30 s, and elongation at 72 °C for 30 s. A total of 35 cycles were performed and followed by a final elongation step at 72 °C for 5 min. Eight microliters of each amplified product was electrophoresed in 2% (wt/vol) agarose gel along with a molecular size marker (100bp Sharp DNA Marker, RBC Bioscience) in parallel. The Tris–borate–EDTA buffer electrophoresis was performed at 110 V for 0.5 h. The gel was stained with ethidium bromide and visualized under short wavelength UV light. The results were captured with digital imaging system (Alpha Innotech Corp., San Leandro, CA).

2.4 SpA determination by ELISA(Enzyme Linked Immunosorbent Assay)

Optimization of the ELISA method was developed with respect to the standard staphylococcal SpA (Invitrogen) concentration and incubation time for color development. Staphylococcal SpA was diluted in carbonate buffer (pH 9.6) at 200 ng/ml and then dispended at 100-200 ulper well of a 96 well microtitre plate (Nunc) and then two fold dilution. The plate was incubated overnight at 4°C and then washed three times with PBS containing 0.05% Tween 20 (Merk). The well were blocked with 100 ul of 3% bovine serum albumin (BSA) in PBS and incubated at room temperature for 1 h. Plate was emptied and wells washed as previous. 50 ul of chicken polyclonal to SpA (HRP) (abcam 18596) diluted in % bovine serum albumin (BSA) in PBS was added and incubated for 1 h at room temperature. Plates was emptied and washed as

previous and one more time with distilled water. 50 µl of enzyme substrate (TMB) was added followed by 2 M H_2SO_4. The absorbance was read on a microtiterplate reader (Sunrise™, Tecan Croup Ltd.) at 450 nm. The standard curve was established by plotting the OD values at Y axis and SpA at X axis.

For the assay, 100 µl of cell culture supernatant was prepared as described previously in 2.1 with or without sortase A inhibitor (Methanethiosulfonate)[14].

Negative controls (nutrient broth) with or without detective antibody were used to confirm technical reproducibility (results not shown). Experiments were repeated three times independently for each concentration.

2.5 Statistical evaluation

Data were analysis by Graph Pad Prism® 4.0.

3. RESULTS

3.1 PCR products

A 621 bp DNA fragment carrying the srtA gene was amplified from the chromosome of both MRSA and MSSA bacterial strains by PCR and analyzed by agarose gel electrophoresis. Both strains demonstrated clear banding patterns and presented a single positive band of 600–700bp in length. ***(Fig. 1)***

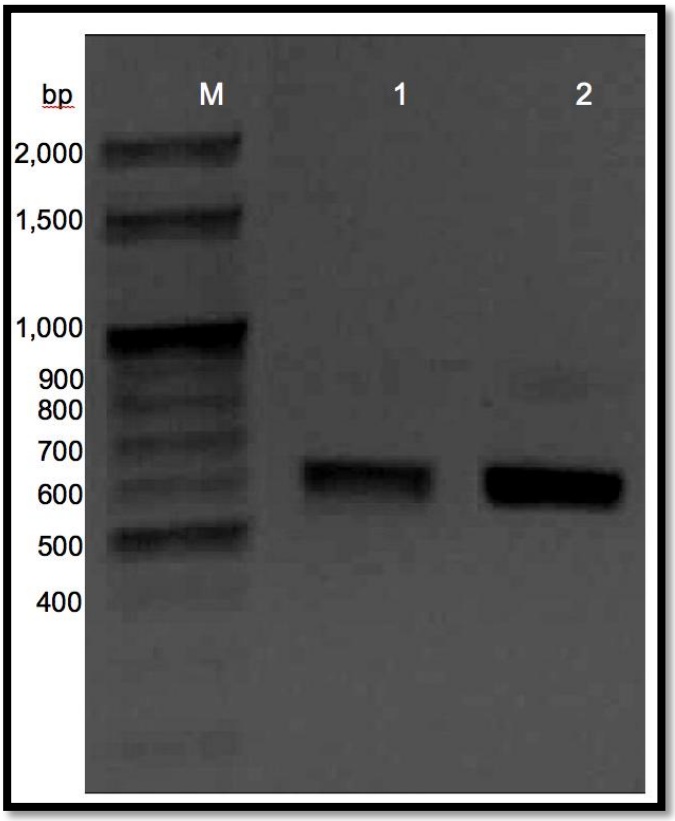

Fig 1. PCR products (600-700 bp) of srtA gene obtained from DNA extracted. Lane M: size marker (100 bp ladder), Lane 1:PCR product from MRSA, Lane 2: PCR product from MSSA.

3.2 SpA level

Fig. **2** shows the standard curve, which was plotted using absorbance values and the concentration range of 0.0977-200 ng/ml standard staphylococcal SpA (Invitrogen). The binding of chicken polyclonal to SpA (HRP) to the SpA molecules adsorbed to the wells was calculated from the standard curve.The production of SpA from methicillin-resistant *S. aureus*ATCC 43300 and methicillin-sensitive *S. aureus*ATCC 25923 cultured in brain-heart infusion medium in the shake flask was measured using the ELISA as described previously. The supernatant of samples was obtained every 1 h until7 h and the sample were then used for measuring the SpA concentration. Using this ELISA measurement, absorbance of the SpA standard and samples was performed in the same 96-well plate. *Fig.* 5 shows

concentration of SpA in the medium measured for a period of 7 h using ELISA technique. The concentration of SpA produced in this experiment was correlated with increase in cell number presented by growth curve (***Fig.* 3, 4**). The production increased exponentially during log phase and the results showed that the highest SpA concentration in MRSA and MSSA were 41.79 and 63 ng/ml at 6 hours cultivation, respectively. Thus, this technique is successful in measuring SpA concentration in *S. aureus* cultures.

We asked whether the decreasing of SpA concentration in bacterial culture supernatant was able to use as the indicator for sortase A inhibitors. ***Fig. 5*** showed the absorbance of ELISA indicated SpA level from MSSA cultured with or without sortase inhibitor (Methanethiosulfonate). This result revealed that the absorbance of MSSA treated with 5 mM sortase inhibitor were lower than MSSA without sortase inhibitor (Pair t test, $P<0.05$) but has no effect on cell growth (data not shown). This observation may suggest an inhibitory effect of sortase inhibitor on SpA production.

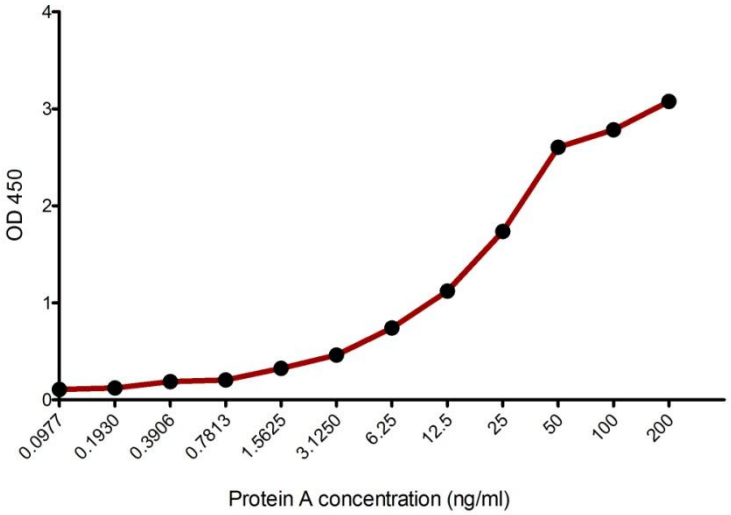

Fig2. Standard curve of SpA.

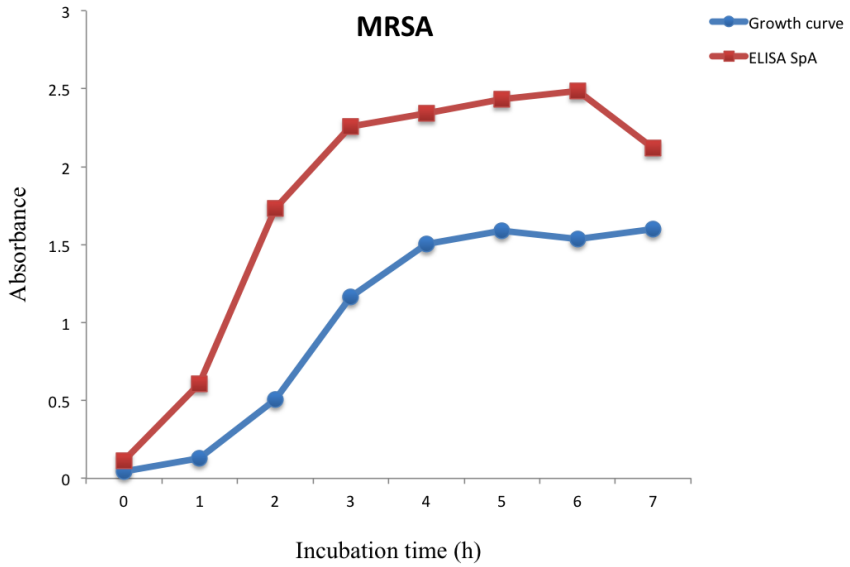

Fig 3. SpA from MRSA. Data represented as mean.

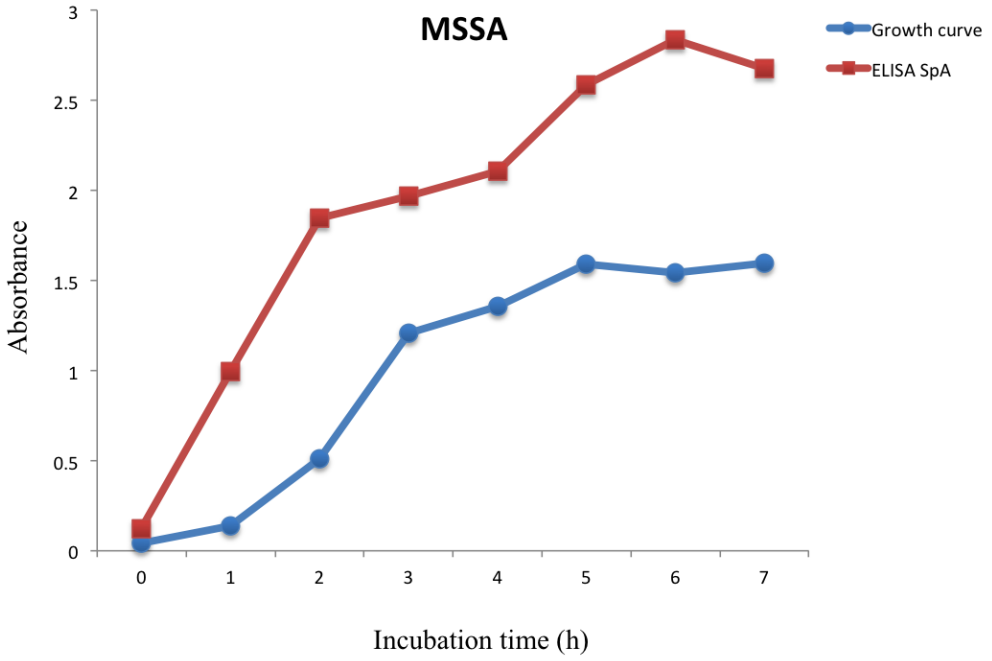

Fig 4. SpA from MSSA. Data represented as mean.

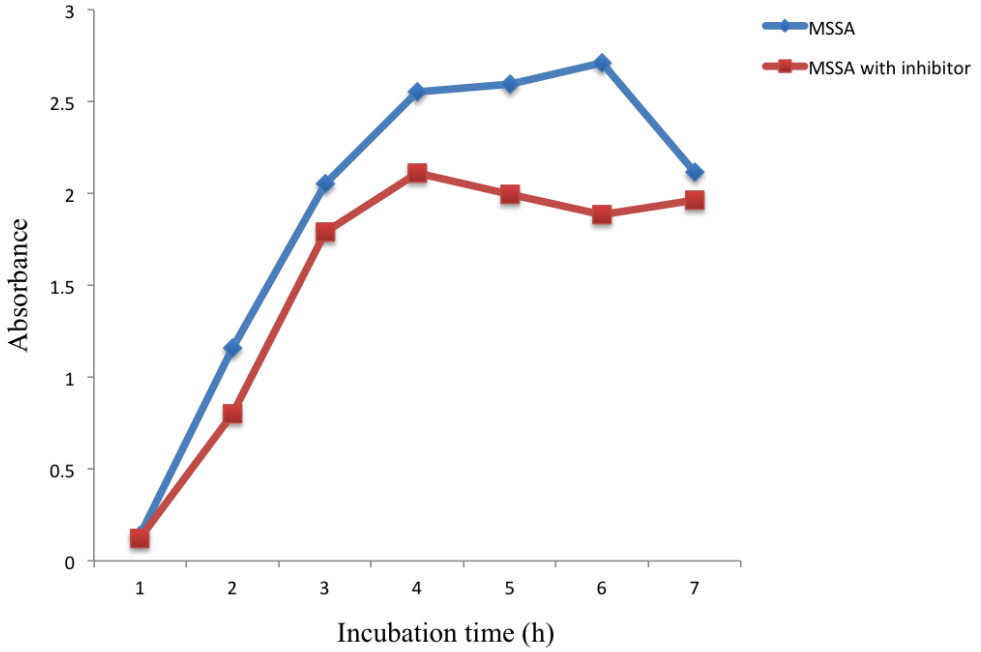

Fig 5. SpA from MSSA treated with sortase A inhibitor (Methanethiosulfonate, Sigma) 5 mM. Data represented as mean.

4. DISCUSSIONS

During the growth cycle, the biosynthesis of SpA occurs during the log phase of*S. aureus*[15], [16]. Most of SpA is covalently linked to the cell wall but some release into the culture medium [17]. Extracellular production of SpA is commonly observed in the methiclillin-resistant strains [18].It has been found that sortase also play a crucial role for the covalently attachment of specific virulence protein including SpA to the cell wall of *S.aureus* [12], [13] which sortase cleaves between the threonine and the glycine of the LPXTG motif,

resulting in the formation of a thioester enzyme intermediate [13].

To date sortase A inhibitor has been reported that it should act as anti-infective agents and interrupt the pathogenesis of bacterial infections without affecting their viability. Therefor the screening strategies for evaluate the potent inhibitors molecule could be crucial. Most of the assay to investigate SpA are expensive for example there were tested for their ability to inhibit sortase activity by measuring the enzyme activity [19-21].

5. CONCLUSIONS

Collectively, these data suggest that the simple direct ELISA can be monitoring the SpA level in culture medium and found the significance reduction of SpA when treat with inhibitor. This will allow for the development of strategies to simple screening for sortase inhibitors.

6. ACKNOWLEDGMENT

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