Polyclonal antibody production against recombinant Staphylococcal Enterotoxin A (rSEA) derived from a dairy mastitis isolate

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Abstract. Staphylococcal enterotoxin A (SEA) is one of the most prevalent toxins associated with staphylococcal food poisoning. However, only a few diagnostic methods provide adequate sensitivity and specificity for SEA detection. In this study, *Staphylococcus aureus* isolated from a dairy mastitis cow (strain FKH 182) was analyzed by polymerase chain reaction (PCR) and confirmed to carry the *sea* gene (127 bp). The SEA gene fragment encoding the mature peptide was amplified, cloned, sequenced, and inserted into a pET22b expression vector. The recombinant SEA (rSEA) protein was expressed as a hexahistidine-tagged fusion protein in *Escherichia coli* BL21 (DE3). Purified rSEA was then used to generate polyclonal antibodies (pAb) in Balb/c mice. Indirect enzyme-linked immunosorbent assay (ELISA) demonstrated that the anti-rSEA pAb reached a titer of 1:3200 at 59 days post-immunization. Western blotting confirmed the specificity of the antibody with a distinct band at approximately 30 kDa. Furthermore, the polyclonal antibody was applied in antigen capture-ELISA (AC-ELISA) to detect SEA in 50 human clinical *Staphylococcus aureus* isolates, with diagnostic sensitivity and specificity of 0.80 and 0.91, respectively, compared with PCR. These findings indicate that recombinant SEA is a reliable immunogen for pAb production and that the developed pAb can be applied as a practical tool for large-scale detection of SEA in clinical and food safety investigations.

1 Introduction

Mastitis is one of the most important diseases in dairy industry and potentially reduces the quality and quantity of milk [1]. *Staphylococcus aureus* is recognized as main bacterial causative agents of both clinical and sub-clinical mastitis in dairy ruminants [2][3]. *S. aureus* predominantly producing a variety of extracellular protein, that help the bacterium evade host

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defenses, hence allowing the microorganism to colonize the mammary glands of ruminants, including staphylococcal enterotoxins (SEs) [4][5].

Twenty-four SEs have been describe based on their antigenicity from staphylococcal enterotoxin A to Y (SEA to SE*l*Y) [6][7]. Staphylococcal enterotoxin A (SEA) is the most important serotype involved in staphylococcal food poisoning, although Staphylococcal enterotoxin G to Q (SEG to SEQ) were frequently found in *S. aureus* strains isolated from bovine mastitis [8][9]. Low quantities of SEA in food are sufficient to cause food poisoning symptom include nausea, vomiting, weakness and diarrhoea [10]. In particular, classical SE were observed to be most frequently associated with staphylococcus food poisoning outbreaks in more than 80 % of any countries [8]. Limited methods are sufficiently sensitive and specific to confirm the existence of SEs [11]. This study aimed to produce polyclonal antibodies in vivo using the recombinant protein of Staphylococcal enterotoxin A (rSEA) antigen to develop immunoassay detection methods.

In this study, rSEA was produced through cloning and expression of the *sea* gene in a prokaryotic system. Balb/c mice were subcutaneously immunized with rSEA antigen to produce polyclonal antibody against rSEA protein. Polyclonal antibody anti-rSEA protein was produced for developing a sensitive and reliable diagnostic using indirect enzyme-linked immunosorbent assays (ELISA) for SEA detection. Implementation using polyclonal antibodies remain valuable in many applications because their ability to recognize multiple epitopes provides broader antigen coverage, greater tolerance to antigen variability, and more robust detection in complex or heterogeneous biological samples, especially in the rapid test detection.

2 Materials and methods

2.1 Ethical Approval

All procedures performed on animals in this study complied with the ethical clearance issued by the Animal Ethics Committee of Universitas Gadjah Mada with registration number 048/EC-FKH/Int./2023.

In this study, two groups of Balb/c mice mice (female, 7-8 weeks old) were used to produce polyclonal antibodies against rSEA at Animal Testing Services, University of Gadjah Mada, were used in this experiment.

2.2 DNA extraction and amplification of *sea* gene

In this study, *Staphylococcus aureus* strain FKH 182 was derived from bovine milk (concentration 34.1 ng/µl) for amplify the *sea* gene at cloning and expression stages. Genomic DNA was extracted and purified using a DNeasy mini kit (Qiagen, Hilden, Germany) according to the manufacturer's guideline. The bacterial strains were cultivated on blood agar base (Oxoid, Germany) for 24 hours at 37°C. A total 5-10 *S. aureus* colonies were suspended with 180 µl TE buffer (10 mM Tris-HCl and 1 mM EDTA pH 8.0) in 2 ml microfuge tubes containing 5 µL lysostaphin (1.8 U/µl; Sigma, USA). The suspension was incubated for 1 hour at 37°C, and was added 25 µl of proteinase K (14.8 mg/ml; Sigma, USA). Microtubes were filled with 200 µl of AL buffer (containing reagents AL1 and AL2; Qiagen, Germany). Furthermore, incubated the suspension for 30 minutes at 56°C and 10 minutes at 95°C. The suspension being spun at 6,000 × g for a few seconds. A total of 420 µl ethanol was added to each sample and placed in a spin QIAmp column. Centrifugate the suspensions at 6,000 × g for 1 min, the spin columns were placed in a clean collection tube and the sample was washed twice with 500 µL of AW buffer (Qiagen, Germany). After the second wash and

centrifugation at 6,000 × g for 3 min, the QIAamp spin columns were placed in a clean 2 ml microfuge tube, and the DNA was eluted twice with 200 µl and 100 µl of AE buffer (Qiagen, Germany). DNA was stored at -20ºC.

The PCR condition was optimized to an initial denaturation at 95ºC for 1.05 min followed by 35 cycles of denaturation (95ºC/30s), annealing (55ºC/30s), extension (72ºC/15s); and final extension at 72ºC for 5 min. The amplified fragment of the *sea* gene separated using 1,5% agarose gel electrophoresis (Invitrogen, USA) stained by RedSafe® nucleic acid staining solution (iNtRon Biotechnology, Korea). PCR program was run in Mastercycler® PCR thermocycler (Eppendorf, USA). The PCR amplified product was detected by electrophoresing in 1x TBE buffer at 100 volts for 10 minutes. The resulting bands were visualized on a transilluminator.

2.3 Cloning and sequencing *sea* gene into *Escherichia coli (E. coli)*

The PCR product was amplified using KAPA HiFi HotStart ReadyMix (KAPA system, USA) to predicted the mature form of SEA peptide with the forward sequence primer (5'GGGGCCATATGCACCACCACCACCACCACAGCGAAGAAATAAATGAAAAAG -3') and reverse (5’-GTGGCATGGATCCTTACAAATTATTGAGATC-3'). The PCR products were digested with *Nde*I and *Bam*HI before ligating the restriction site of expression pET 22b+ vector (Novagen, EMD Millipore, USA). The plasmid was transformed into the *E. coli* bacterial strain DH5α using standard procedures. The transformed cells were plated on Luria-Bertani broth (10 g NaCl, 10 g Trypticase peptone, and 5 g yeast extract per liter [pH 7.2]) containing ampicillin (100 µg/ml) and incubated overnight at 37°C. The culture plates were stored at 4°C. The insertion was verified by DNA sequencing (ABI 3100xl; Applied Biosystems, CA, USA).

2.4 Expression of *sea* gene

The recombinant plasmid harbouring *sea* gene was introduced into *E. coli* BL21 (DE3) cell for protein expression. A single transformed (DE3) cells carrying the recombinant SEA plasmid were cultured in 300 ml Luria-Bertani medium supplemented with 100 µg/ml ampicillin to an optical density (OD) at 600 nm of 0.5 to 0.6 with aeration at 37°C. The expression of fusion was induced by adding 0.5 mM of isopropyl-β-D-thiogalactopyranoside (IPTG; Sigma, USA), followed by incubation with continuous shaking at 30°C for 24 h constantly for final concentration. Cultures were centrifuged at 4,000 × *g* for 15 min at 4°C and the resulting pellets were stored at -80°C until further use.

The cell pellets were suspended in lysis buffer containing 5 mM imidazole, 50 mM NaH2PO4, and 300 mM NaCl [pH 7.0]. The suspension was sonicated on ice to prevent degradation of the recombinant protein. Cellular debris was removed by centrifugation (9,000 × g at 4°C for 20 min). The resulting supernatant was collected for the isolation of N-terminally 6×His-tagged SEA fusion proteins by using CO^{2+} affinity chromatography (Clontech Laboratories, Inc.) according to the manufacturer's procedures. The purity of the eluted proteins was analyzed on 12% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and to stained the proteins was used Coomassie brilliant blue R250 (Nacalai Tesque, Kyoto, Japan). The protein concentration was measured using a Bio-Rad protein assay (Bio-Rad, Hercules, CA, USA) with bovine serum albumin (BSA; Sigma-Aldrich, St. Louis, MO, USA). Purified proteins were dialyzed against a 1,000× volume of Dulbecco's phosphate-buffered saline (DPBS) (2.68 mM KCl, 1.46 mM KH2PO4, 136.9 mM NaCl, 8 mM Na2HPO4·12H2O pH 7.4) at 4°C for 24 h.

2.5 Polyclonal antibody anti-rSEA production

Female Balb/c mice were immunized intraperitoneally with rSEA emulsified in complete Freund's adjuvant at an interval 14 days with dose 10 µg. Three additional boosters with 14 days interval were followed with same doses in incomplete Freund's adjuvant. Blood samples were collected three time through retro-orbitalis plexus route before primary injections, after second booster (d 35) and third booster (d 49) injections. The blood was incubated at 37ºC for 1 h and a low centrifuge (4000 rpm) at room temperature. Blood serum was collected and stored at -20ºC until required. Antiserum titre was measured by indirect ELISA to recognize anti-rSEA immune responses. The specificity and sensitivity of the produced IgGs against rSEA protein were determined by Western blot analysis.

2.6 Development of AC-ELISA for SEA detection at laboratory-scale test

A total 50 human clinical isolates collection from Clinical Pathology Laboratory, Faculty of Veterinary Medicine, Gadjah Mada University was performed using phenotypic and genotypic tests of *S. aureus* identification. A total 36 negative isolate was used to calculate the cutoff point for the assay. The upper limit of mean + (2×standard deviation) value was determined as the cutoff point. All of *S. aureus* isolates were further tested for the presence of SEA using AC-ELISA method.

The test results were confirmed by identifying the presence of the enterotoxin virulence factor through PCR amplification of the *sea* gene. The accuracy of the test results was determined through sensitivity and specificity and Cohen's Kappa test .

3 Results and Discussions

In this study, the *sea* gene of *S. aureus* FKH 182 was amplified by PCR. The PCR-amplified gene was ligated into the pET 22b+ vector. PCR-positive clones were analysed for expression, and a thick band of recombinant SEA toxin was observed at approximately 27 kDa on SDS–PAGE gel (Figure 1A). The position of recombinant SEA protein was also confirmed by SDS-PAGE (Figure 1B).

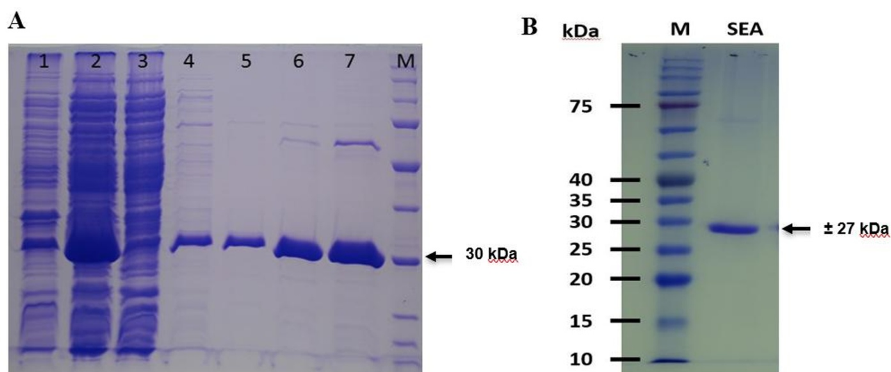

Fig. 1. (A) SDS–PAGE of affinity purified recombinant SEA. Pellet (line 1); Supernatant (line 2) ; Column flowthrough (line 3) ; equilibration with 10 ml imidiazole 5 mM, 10 mM, 20 mM respectively (line 4-6) ; Eluate imidiazole 200 mM (line 7) ; M (molecular weight marker). (B) Confirmation of purified recombinant proteins of the SEA subtype by SDS-PAGE (± 27 kDa).

Furthermore, the purified rSEA protein was used to raise polyclonal antibodies in Balb/c mice models. A polyclonal antibody for SEA was produced in Balb/c mice were immunized

with rSEA using a series of four doses. Before being injected, the serum was titrated. The antibody titer represents the last serum dilution in which the antibody is detected. The level of antibodies in blood serum indicated the antibody titer's existence. It is defined as the lowest serum concentration, which an antibody assay still produces a detectable positive result. The higher the antibody concentration in the blood, the more significant dilution that will produce a detectable signal. Indirect ELISA observed antibody titers of the obtained serum with rSEA antigen coated microtiter plates. Different dilutions of antibody anti-rSEA were added in the rSEA antigen coated wells and added by the secondary conjugate goat anti-mouse IgG alkaline phosphtase. The polyclonal antibody anti-rSEA titer, as revealed by indirect ELISA, was 1:3200 at 59 days.

The specificity of the polyclonal antibody anti-rSEA was assessed using Western blot analyses. A rSEA protein (concentration 2.1 µg/µl) was applied to 12% SDS-PAGE in 10x dilution. Following electrophoresis, the proteins were incubated with the polyclonal antibody anti-rSEA at a 1:3500 dilution. After extensive washing, a goat anti-mouse IgG alkaline phosphatase (1:5000, Sigma, USA) was applied as secondary antibody. Reactions were visualized using a 1-stepTM NBT/BCIP (Thermo Scientific, Waltham, MA, USA). The antibody anti-rSEA detected a specific band of approximately 27 kDa characteristic of SEA .

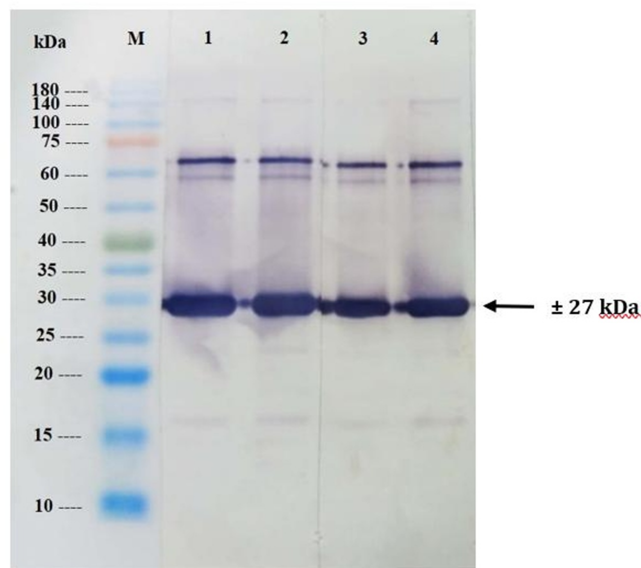

Fig. 1. Analysis of the specificity of polyclonal antibody against rSEA by Western blot. (Lane 1) M (molecular weight marker) ; (lane 2-5) antibody anti-rSEA. The arrow indicates specific gene of SEA (± 27 kDa).

To determine the cut off point for the AC-ELISA assay was used 36 human clinical isolates were PCR detected unamplified sea gene. Negative samples were analysed in the assay and the upper limit of mean + (2.SD) value was determined as the cut-off point. The data shows the cut-off point of SEA was 1.141. All the samples value ≥ 1.141 was considered positive of SEA and all the samples value < 1.141was examined negative of SEA. The AC-ELISA assay shows a total of 50 human clinical's isolates, 15 samples were estimated positive SEA by showing value ≥ cut-off point of SEA. The remain 35 isolates were shown OD lower than the cut-off value was estimated SEA negative. The result of the ELISA assay in comparison to the established of PCR test of *sea* gene (Table 1). According to the test

result, the performance of the AC-ELISA of SEA was calculated. The AC-ELISA shows a sensitivity and specificity of SEA at 0.750 (95% CI: 47.62 to 92.73) and 0.9118 (95% CI: 76.32 to 98.14).

The predictive value considered as a positive-test probability revealed a positive predictive value (PPV) of AC-ELISA 80.0 %. On the other hand, the proportion of true-negative among all samples with a negative test considered as the negative predictive value (NPV) were calculated of 88.57 %. The accuracy data of the AC- ELISA of SEA was detected 86.00 %. The AC-ELISA assay show a sensitivity score above 70 % would be considered developing and specificity score above 80 % is good, while accuracy score above 70 % is considered acceptable for diagnostic measures. The AC-ELISA analysis was compared with the established of *sea* gene of PCR methods with a Kappa's index. The Kappa's index showed value of 0. 67, These results exhibited the Kappa value fall under the interpretation of substantial agreement (0.61-0.80), where Kappa index of 1 is a perfect agreement, suggesting that the AC-ELISA assay is promising method for detecting the presence of enterotoxin of SEA in raw cow milk.

Table 1. Relative performance of AC-ELISA and PCR *sea* gene for detection SEA in clinical isolates

AC-ELISA	PCR *gen sea* (number of isolates) Positive (+)	Negative (-)	Total
Positive (+)	12[a]	3[b]	15
Negative (-)	4[c]	31[d]	35
Total	16	34	50

SN (*Sensitivity/Se*) = 75.00 % (true positive/true positive + false negative)
SP (*Specificity/sp*) = 91.18 % (
PPV (+ *Predictive value*) = 80.00 %
NPV (- *Predictive value*) = 88.57%
Accuracy = 86.00 %

[a]True positive (TP); [b]false positive (FP); [c]false negatives (FN); [d]true negative (TN)
Se = [TP/(TP + FN)], Sp = [TN/(FP + TN)], LR (+) = [Se/(1 - Sp)], LR (-) = [(1 - Se)/Sp]

4 Conclusion

The rSEA could be applied to produce polyclonal antibodies and was confirmed using indirect ELISA and Western blot analysed. The laboratory-test scale was conducted to detect SEA from human clinical isolates and compared the result with the established of *sea* gene of PCR methods. Although molecular technique such as PCR method have been used widely to detect the existence of the staphylococcal enterotoxins, this method required a sophisticated equipment, high-cost laboratory settings and cannot directly detect enterotoxins and assess its levels in food. In conclusion, the polyclonal antibody raised against rSEA demonstrated promising potential for application in the detection of staphylococcal enterotoxins A (SEA), further refinement and validation with larger sample sizes are necessary to enhance their accuracy and specificity.

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