STUDY THE BIOLOGICAL AND BIOCHEMICAL CHARACTERIZATION OF STAPHYLOCOCCUS AUREUS ENTEROTOXIN

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ABSTRACT

The aim of this study was to investigate the purification of Staphylococcus aureus A20-L2 enterotoxin which induced clinical and sub clinical mastitis in dairy cattle in Sulaimani province. The production of enterotoxin types A, B, C, D, E, G, H, I and J were also tested by PCR technique. Among 246 CoPS isolates, 125 (50.8%) were found to be positive for production of one or more of enterotoxin types A, B, C and E, while none of the CoNS isolates had the ability to produce enterotoxin. Ion exchange-chromatography and gel filtration chromatography were used for the purification of enterotoxin from one isolate of S. aureus which was A20-L2. The molecular weight of the purified enterotoxin was approximately equal to 28.2 kilo Dalton determined by gel filtration chromatography whereas sodium dodecyle sulfate-polyacrylamide gel electrophoresis revealed a single band which was approximately equal to 26 kilo Dalton. Isoelectric focusing was used to determine the isoelectric point (pI) value and it was estimated as 7.

Key words: Staphylococcus aureus, Enterotoxin, CONS isolates, Purified enterotoxin, molecular weight, Isoelectric point.
INTRODUCTION

Many strains of \textit{S. aureus} express a plasminogen activator called staphylokinase. This factor lyses fibrin. The genetic determinant is associated with lysogenic bacteriophages\cite{1}. A complex formed between staphylokinase and plasminogen activates plasmin-like proteolytic activity which causes dissolution of fibrin clots. The mechanism is identical to streptokinase. As with coagulase there is no strong evidence that staphylokinase is a virulence factor, although it seems reasonable to imagine that localized fibrinolysis might aid in bacterial spreading \cite{2}.

Staphylococcal enterotoxins are a group of single-chain, low-molecular weight, 27-34 kilo Dalton, produced by some species of staphylococci, primarily \textit{Staphylococcus aureus}, but also by \textit{S. intermedius}, \textit{S. hyicus}, \textit{S. xylosus} and \textit{S. epidermidis}. To date, 14 distinct enterotoxins have been identified based on their antigenicity and they have sequentially been assigned a letter of the alphabet in order of their discovery (SEA to SEO). There is no enterotoxin F as this letter was assigned to a protein that proved not to be an enterotoxin. Also, several SECs have been recognized and, while they all react with the same antibody, three to five residue differences in their amino acid sequences differentiate them \cite{3}.

They are similar in composition and biological activity, but are identified as a separate proteins due to their differences in antigenicity, it means that they have been identified based on their relation to specific antibodies prepared against each of the purified SEs \cite{4}. It had been shown that they are of high toxicity and relatively low antigenicity \cite{5}.

Studies on staphylococcal enterotoxins (SEs) started from the analysis of \textit{S. aureus} strains involved in staphylococcal food poisoning. For the first SEs identified, the peptide sequence was available before the nucleotide sequence. This was the case for SEA, SEB, and SEC\cite{6}. The abundance of literature on SEs varies considerably among the types, according to the chronology of their identification and their importance in staphylococcal food poisoning. Subsequent translation leads to the generation of a precursor protein, containing a N-terminal leader sequence that is cleaved during export from the cell to form the mature enterotoxin protein. Slight variations in processing or post-translational modification may occur as evidenced by the existence of three SEA isoforms with three different isoelectric points \cite{7}. They are rich in lysine, aspartic acid, glutamic acid, and tyrosine residues. Most of them possess a cystine loop required for proper conformation and which is probably involved in the emetic activity. They are highly stable, resist most proteolytic enzymes, such as pepsin or...
trypsin, and thus keep their activity in the digestive tract after ingestion. They also resist chymotrypsine, rennin and papain. Nevertheless, SEB and SEC1 have been cut in the cystine loop by mild trypsin digestion. The two properties of enterotoxins mitogenicity and emetic activity are located on different sections of the protein. Whereas the toxin's mitogenic activity is postulated to be on the N-terminal segment (approx. 6000 MW), the C-terminal and the central portion of the molecule contain the sites for emetic activity [8,9].

The aims of this study to estimate the level of toxins among Staphylococcus species isolates from bovine mastitis and the biological and biochemical characterization of Staphylococcus aureus enterotoxin.

MATERIALS AND METHODS

Milk Sampling

Two hundred and fifty lactating cows (985 quarters) of which 15 animals had lost a quarter each was examined from dairy herds and individual cows in different smallholder farms in six regions during March to August 2007. Milk samples were collected using aseptic technique from individual quarters of the cows that were suspected to have subclinical mastitis as detected by high Somatic cell counts (SCC greater than 200,000 cells/ml), and clinical observation, the milk was immediately transported cooled (4°C) to the Microbiology Laboratory, Veterinary Medicine/ University of Sulaimaniyah for samples cultured according to standard protocols suggested by the National Mastitis Council.

Determination of Staphylococcal Virulence Factors

Urease Production Test : Urease test was performed according to Colle and Mass [10]on Christensen’s urea agar. Purified bacterial colonies recovered from brain heart infusion agar were inoculated on the urea agar. The medium was incubated for 24 hours at 37 ºC. A positive result was indicated by appearance of pink color (breakdown of urea).

Lipase Production Test : Lipase test was performed according to Baird-Parker [11] on Baird – Parker agar medium. Purified bacterial colonies recovered from brain heart infusion agar were streaked on the Baird – Parker agar medium. The medium was incubated for 48 hours at 37 ºC. A positive result was indicated by appearing of a pearly layer of an iridescent film in and immediately surrounding the colonies visible by reflected light (iridescent) sheen or oil in water.
Caseinase Test (Casein Hydrolysis Test) was performed according to Colle and Mass[10] on skim milk agar. Purified bacterial colonies recovered from brain heart infusion agar were streaked on the skim milk agar medium. The medium was incubated for 48 hours at 37 ºC. A positive result was indicated by clearing of the agar around the bacterial growth indicating casein hydrolysis.

Deoxyribonuclease (DNase) Production Test was performed according to Schreier [12] on DNase agar. A positive result was indicated by appearing of clear zones around the bacterial colonies.

Slime Production Test was performed according to Freeman et al [13] on Congo red agar. A positive result was indicated by black colonies on the surface around the bacterial growth indicates slime production.

Haemolysin Production Test was performed according to Ryan and Ray[14] on blood agar media. A positive result was indicated by appearing of a wide zone of complete haemolysis around the bacterial colonies indicating Beta-haemolysin, or a wide zone of incomplete haemolysis around the bacterial colonies indicating Alpha-haemolysin.

Enterotoxin Types Detection
Staphylococcal enterotoxin type- reversed passive latex agglutination (SET-RPLA) kit was used for the detection of staphylococcal enterotoxin types A, B, C and D in culture filtrates and can also be used for detection of enterotoxins in food. It is dependent on reversed passive latex agglutination. The reversed passive latex agglutination (RPLA) method was performed according to Rose et al., (1989). In this method the V shaped wells micro dilution plates were used with a culture supernatant-to-reagent dilution ratio of 1:2. A diffuse layer at the bottom of the V-well was regarded as a positive result; a tight button of latex particles was regarded as a negative result.

Purification of Enterotoxin
Purification of Enterotoxins by Ion Exchange Chromatography
A total of 25 ml of the concentrated enterotoxin was purified in a DEAE-cellulose column, 2.5 x 20 cm, according to Yi-Cheng et al.[15]. Twenty five milliliters of the concentrated enterotoxin were separately passed after loading onto the column carefully. One hundred milliliters of 0.05M phosphate buffer pH 7.5 was added. Elution of proteins was performed by
dispensing 200 ml of a gradient from 0.05- 0.3 M phosphate buffer pH 7.5. The protein content of the fractions was estimated by measuring the absorbance at 280 nm wavelength by a spectrophotometer. The enterotoxin activity was determined for the fractions of each major peak.

**Purification of Enterotoxins by Gel Filtration Chromatography**

A Sepharose– 6 B, 80 x 2.0 cm, column was prepared and packed according to the instructions of the manufacturing company (Pharmacia- Sweden). Three milliliters of the concentrated enterotoxin were carefully added into the Sepharose - 6B column using a Pasture pipette. Elution of proteins was performed by dispensing 200 ml of 0.2 M phosphate buffer (pH 7.5) through the column with a flow rate of 40 ml / hour. The protein content of the fractions was estimated by measuring the absorbance at 280 nm wavelength by a spectrophotometer.

**Determination of Molecular Weights of Enterotoxin :**

Molecular weights of staphylococcal enterotoxin can be determined by two methods including gel-filtration through Separose-6B and sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

**Standardization of the Enterotoxin Molecular Weight**

Different standard proteins, as show in Table blow, were dispended through a Sepharose- 6B column, and then eluted by a 0.2 M phosphate buffer (pH 7.5) with a flow rate of 50 ml / hour.

<table>
<thead>
<tr>
<th>Standard protein</th>
<th>Molecular weight (kilo Dalton)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Catalase</td>
<td>232</td>
</tr>
<tr>
<td>Bovine serum albumin</td>
<td>67</td>
</tr>
<tr>
<td>Ovalbumin</td>
<td>43</td>
</tr>
<tr>
<td>Lysozyme</td>
<td>14.4</td>
</tr>
</tbody>
</table>

**Table: Low molecular weights of standard proteins**

**Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE)**

The purity of the proteins and the apparent masses of their variants were estimated by SDS-PAGE which was performed according to the Laemmli procedure described by Garfin [16]. Standard proteins used for standardization of the enterotoxin molecular weight by SDS-PAGE.
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<table>
<thead>
<tr>
<th>Standard protein</th>
<th>Molecular weight (kilo Dalton)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bovine serum albumin</td>
<td>67</td>
</tr>
<tr>
<td>Catalase</td>
<td>60</td>
</tr>
<tr>
<td>Ovalbumin</td>
<td>43</td>
</tr>
<tr>
<td>Trpsin</td>
<td>24</td>
</tr>
</tbody>
</table>

**Isoelectric Focusing**

The toxin was focused by using a constant current of 50 mA/gel slab at 240 volts for 4 hours. After focusing was completed, the gels were removed from glass and the gel was cut in 20 pieces, each one 0.5 cm long and put in tube which contained 1 ml of distilled water, to determine pH and acidity.

**RESULTS AND DISCUSSION**

**Determination of Staphylococcal Virulence Factors**

The distribution of virulence factors of *Staphylococcus aureus* and coagulase negative staphylococci (CoNS) are summarized in Table 1, it was found that the ability of *S.aureus* isolates to produce virulence factors such as coagulase, clumping factor, urease, DNase and γ haemolysin were higher than coagulase negative staphylococci isolates, while the ability of coagulase negative staphylococci isolates to produce slime production, α and β haemolysin were higher than *S.aureus*. The ability of both *S.aureus* and CoNS to produce lipase and caseinase were close.

**Table 1: Results of virulence factors tests of 246 *S.aureus* and 95 CoNS isolates.**

<table>
<thead>
<tr>
<th>Tests</th>
<th>Results</th>
<th>Positive Isolates</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>*CoPS isolates (%)</td>
</tr>
<tr>
<td></td>
<td>no.</td>
<td>(%)</td>
</tr>
<tr>
<td>Tube coagulase test</td>
<td>246</td>
<td>100%</td>
</tr>
<tr>
<td>Slide coagulase or clumping test</td>
<td>228</td>
<td>92.7%</td>
</tr>
<tr>
<td>Urease test</td>
<td>233</td>
<td>94.7%</td>
</tr>
<tr>
<td>Lipase test</td>
<td>186</td>
<td>75.6%</td>
</tr>
<tr>
<td>Caseinase test</td>
<td>139</td>
<td>56.5%</td>
</tr>
<tr>
<td>Slime production</td>
<td>55</td>
<td>22.4%</td>
</tr>
<tr>
<td>Leucocidin test</td>
<td>*</td>
<td></td>
</tr>
<tr>
<td>Enterotoxin test</td>
<td>*</td>
<td></td>
</tr>
<tr>
<td>DNase test</td>
<td>201</td>
<td>81.7%</td>
</tr>
<tr>
<td>Haemolysis tests</td>
<td></td>
<td></td>
</tr>
<tr>
<td>α</td>
<td>13</td>
<td>5.3%</td>
</tr>
<tr>
<td>β</td>
<td>39</td>
<td>15.9%</td>
</tr>
<tr>
<td>γ</td>
<td>139</td>
<td>56.5%</td>
</tr>
</tbody>
</table>

*Leucocidin test, enterotoxin test are tested by PCR

*CoPS : coagulase positive staphylococci  *CoNS : coagulase negative staphylococci
Enterotoxin Assay

Bacterial Isolates and Culturing Technique used in Enterotoxin Assay

All *Staphylococcus* species isolates obtained in this study were investigated for their ability of enterotoxin production by using culture technique on BHIA with phenol red at pH 5.4 as indicator. Among 246 isolates of *S. aureus*, 125 isolates had the ability to produce enterotoxin. None of CoNS had the ability to produce enterotoxin.

*Staphylococcus aureus* is a bacterium that colonizes and causes disease in mammalian hosts. It produces a wide variety of exoproteins that contribute to both colonization and the disease process [17]. Nearly all isolates secrete cytoxins and enzymes (alpha, beta, gamma and delta haem-lysins; nuclease; lipases; hyaluronidase; and collagenase) whose main functions are to convert local host tissues into nutrients required for bacterial growth and to promote spreading of the pathogen throughout the host body. Some isolates secrete one or more additional toxins, comprising leukocidin (*Luk*) and pyrogenic toxin superantigens (PTSAgs), such as toxic shock syndrome toxin 1 (TSST-1), staphylococcal enterotoxins (SEs), and exfoliative toxins. PTSAgs have profound effects on the host immune system, both acute and long term [18].

A number of biochemical activities are considered to contribute to the virulence of pathogenic staphylococci isolated from this study for both *S. aureus* and CoNS isolates, see Table 2. The coagulase test has been considered to be specific to coagulase positive *Staphylococci* species[19]. All *S. aureus* isolates were positive for coagulase, whereas all the isolates of CoNS were negative for this test. These findings concur with those of Devries and Oeding [20], who reported that production of coagulase remained a constant property of bovine strains of *S. aureus*. Erasmus [21] also found that coagulase-positive staphylococcal species fermented mannitol and produced catalase.

DNase is not entirely reliable as an indicator of pathogenicity. It has been reported that CoNS also have DNase activity[22]. In this study, the rate of DNase positivity was 81.7 % (201) and 45.3 % (43) for CoPS and CoNS, respectively. These results are parallel with the other studies [23].

With respect to DNase production, the results agree with those of Langlois et al [24], who found that 42 % of *S. epidermidis* isolates were positive for DNase activity. For mannitol fermentation, the results are in disagreement with those of Oyekunle and
Adetosoye[25]. These workers reported that a higher percentage (100%) of coagulase-negative isolates rather than coagulase-positive isolates (88.2%) of bovine staphylococci fermented mannitol.

Fournier et al.[26], reported that coagulase-positive staphylococci were mostly positive to DNAase and mannitol fermentation reaction. These tests along with the test for hyaluronidase production were found satisfactory for differentiation of \textit{S.aureus} and coagulase-negative staphylococci. According to Devries and Oeding [20], characteristics of staphylococci derived from certain hosts can vary between geographic regions and strains recovered from different animal species may also differ from one another. In addition, the biochemical reactions of staphylococci have been shown to vary within the same gland over time [27].

Haemolysis of sheep erythrocytes is one of the most common characterization techniques in the identification of \textit{S. aureus} strains. In this study, 56.5% (139) of the isolates were non-haemolytic strains. These results differ from other studies which described a high percentage of the isolates identified with haemolysis of different types[27]. This difference could be explained by the initial identification in this study. Damage to host cells is in part mediated by staphylococcal haemolysins, which contribute importantly to virulence in \textit{S. aureus}. Alpha haemotoxin assembles into transmembrane pores in many nucleated cells, and readily lysed sheep or rabbit erythrocytes, while horse or human red cells are less susceptible. Beta haemolysin has sphingomyelin-specific phospholipase activity, resulting in partial cell lysis [28]. In the present study, it was determined that 5.3% (13) of the \textit{S. aureus} and 35.8% (34) of CoNS strains had alpha haemolysis and 15.9% (39) of the \textit{S. aureus} and 22.1% (21) of CoNS strains had beta haemolysis characteristics. In general, beta haemolysis was seen in the \textit{S. aureus} strains more often than in the CoNS while alpha haemolysis was detected in the CoNS strains more often than in the \textit{S. aureus} [29]. This showed that the incidence of beta haemolysis was higher than that in another study previously performed [29]. Slime production may reflect the microorganismes capacity to adhere to specific host tissues and thereby to produce invasive microcolonies [19]. In this study, the rate of congo red agar (CRA) method positiveness was 22.4% (55) and 49.5% (47) for \textit{S. aureus} and CoNS respectively. These results showed that slime formation in staphylococci was an important virulence factor, which was an reported in previous studies [30].

Although the ability of CoNS to cause chronic mastitis is well recognized, little is known about the staphylococcal virulence factors that contribute to their pathogenesis. For instance,
factors that may influence the ability of coagulase negative staphylococci to colonize host tissues, or their survival in grossly different host environments, are poorly understood.

In this study, the ability of *S. aureus* and CoNS to produce lipase, caseinase and urease was recorded and it was found that the *S. aureus* and CoNS isolated from bovine mastitis in the Sulaimani region had the ability to produce lipase this agrees with results of Younis *et al* [31].

CoNS strains also produce several toxins and enzymes that could contribute to virulence such as hemolysin, leukocidin, lipase, proteases, and DNase [32]. Many of the CoNS strains isolated from the mastitis samples had higher protease, DNase, and lecininase activity than those of CoNS from normal cows [33]. However, the roles of these enzymes on the pathogenesis of CoNS are unclear.

**Enterotoxin Types Detection**

Staphylococcal enterotoxins (SE) were extracted from 125 isolates of *S. aureus* which had the ability to produce enterotoxin, and then the enterotoxin types were detected by using reverse passive latex agglutination (RPLA) kit.

Out of 125 *S. aureus* enterotoxin producer, it found 66 isolates enterotoxin type A producer (52.8 %); 27 isolates enterotoxin type B producer (21.6 %) and 20 isolates enterotoxin type C producer (16 %). None of the isolates tested produce enterotoxin type D. Enterotoxins of these isolates A13-R2, A20-L2, A4-L1, T5-L2, T10-R1, Tg8-L2, Tg9-L2, B15-R2, B16-R2, B21-L1, P1-L2, P3-L1 and K9-L2 were produced in a similar concentration (320ng/ ml). In spite of these similarities, the scores of agglutination in different dilutions of the cell-free extracts of these isolates enterotoxin type A and type B were more obvious than enterotoxin type C, which indicated that enterotoxin type C was produced in smaller amount than enterotoxin type A and type B.

**Enterotoxin Activity Test**

The detection of enterotoxin activity from thirteen *S. aureus* isolates with 320ng/ ml enterotoxin production was performed by rabbit ligated ileal loop test using 13 laboratory rabbits. According to the results in Table 2, it was found that the enterotoxigenic activity ranged between 0.23- 0.50 ml while the negative control value was 0.18 ml.

A20-L2 isolate showed the maximum enterotoxin activity, which was 0.5 ml. Tg9-L2, B21-L1 and P1-L2 showed the minimum enterotoxin activity which was 0.23 ml.
Table 2: Toxicity tests for *Staphylococcus aureus* cell-free filtrate.

<table>
<thead>
<tr>
<th><em>Staphylococcus spp.</em></th>
<th>Isolate No.</th>
<th>Enterotoxin V/L*</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>S. aureus</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A13-R2</td>
<td>0.25</td>
<td></td>
</tr>
<tr>
<td>A20-L2</td>
<td>0.50</td>
<td></td>
</tr>
<tr>
<td>A4-L1</td>
<td>0.30</td>
<td></td>
</tr>
<tr>
<td>T5-L2</td>
<td>0.25</td>
<td></td>
</tr>
<tr>
<td>T10-R1</td>
<td>0.32</td>
<td></td>
</tr>
<tr>
<td>Tg8-L2</td>
<td>0.24</td>
<td></td>
</tr>
<tr>
<td>Tg9-L2</td>
<td>0.23</td>
<td></td>
</tr>
<tr>
<td>B15-R2</td>
<td>0.44</td>
<td></td>
</tr>
<tr>
<td>B16-R2</td>
<td>0.48</td>
<td></td>
</tr>
<tr>
<td>B21-L1</td>
<td>0.23</td>
<td></td>
</tr>
<tr>
<td>P1-L2</td>
<td>0.23</td>
<td></td>
</tr>
<tr>
<td>P3-L1</td>
<td>0.32</td>
<td></td>
</tr>
<tr>
<td>K9-L2</td>
<td>0.40</td>
<td></td>
</tr>
<tr>
<td>Negative control</td>
<td>-</td>
<td>0.18</td>
</tr>
</tbody>
</table>

V/L*: Volume/Length

**Purification of Enterotoxins from Staphylococcus aureus**

The purification of the enterotoxin from *S. aureus* isolate A20-L2 was performed by ion-exchange chromatography using DEAE-cellulose column as described in 3.10.6/1 which was carried out by the concentration the sample. Twelve milliliters of the concentrated partially purified enterotoxin was added to the DEAE-cellulose column followed by washing of protein with 150 ml of 0.05 M phosphate buffer (pH 7.5) through the column.

As shown in Figure 1, the gel filtration procedure had created three peaks of protein which represented to other proteins without any toxin activity. Then after elution of proteins by dispensing 150 ml of a gradient from 0.05 M to 0.3 M phosphate buffer (pH 7.5) through the column. As shown in Figure 2, the gel filtration procedure had created three peaks represented by fractions 34 -39, 41 -45 and 46– 56 However, the second peak, 41- 45, was obtained high enterotoxin activity. Accordingly, enterotoxin was obtained after purification with ion-exchange chromatography. Partially purified enterotoxin was passed through an Amicon-filtrate P50 in a Ultrafiltration-cell in order to concentrate it to 5 ml.

**Purification of Enterotoxin by gel-filtration chromatography**

The purification of the concentrated enterotoxin from *S.aureus* isolate A20-L2 was performed by gel-filtration chromatography using Sephorase - 6 B column. Five milliliters of the concentrated purified enterotoxin was added to the column followed by the elution of protein
by dispensing 200 ml of 0.2 M phosphate buffer (pH 7.5) through the column. As shown in Figure 2, the gel filtration procedure had created three peaks represented by fractions 21 -25, 27 -30 and 31 -35, respectively. However, to determine the enterotoxin activity of the major peaks has revealed that the enterotoxin activity was found only in the second peak which represented purified enterotoxin collected from four fractions (fractions 27 - 30). The purification of the enterotoxin from *S. aureus* isolate A20-L2 was performed by ion-exchange chromatography using a DEAE-cellulose column which was carried out by concentrating the sample. As shown in Figure 4.5, the gel filtration created three peaks of protein which represented to other proteins without any toxin activity. Then, after elution of proteins through the column, the gel filtration created three peaks represented by fractions 34 -39, 41 -45 and 46– 56. However, the second peak, 41- 45, was showed high enterotoxin activity.

![Figure 1](image1.png)

**Figure 1:** Purification of entrotoxin from *S. aureus* by ion-exchange chromatography using a 2.5 x 20 cm DEAE-cellulose column. The column was washed by using a 0.05 M phosphate buffer of pH 7.5, then eluted by using a gradient of 0.05 M to 0.3 M of a phosphate buffer of pH 7.5.

![Figure 2](image2.png)

**Figure 2:** Purification of enteroxin form *S. aureus* by gel filtration chromatography using an 80 x 2.0 cm Sepharose - 6B column. The eluent was a 0.2 M phosphate buffer of pH 7.5 with a flow rate of 40 ml / hour.
The results illustrated in Table 3 and Figure 3 showed that the elution volume to the void volume ratio \((Ve/Vo)\) of the purified enterotoxin was equal to 2.3 since it was located between the ovalbumin and lysozyme standard proteins. Accordingly, the molecular weight of the purified enterotoxin was estimated to be equal to 28.2 kilo Dalton.

The purification of the concentrated enterotoxin from *S.aureus* isolate A20-L2 was performed by gel-filtration chromatography using Sephorase-6B columns. As shown in Figure 2, the gel filtration created three peaks represented by fractions 21-25, 27-30 and 31-35. The major peak of enterotoxin activity was found only in the second peak which represented purified enterotoxin collected from four fractions (fractions 27-30).

Staphylococcal enterotoxin molecular weight of 28.2 k Dalton determined by SDS-PAGE is in good agreement with the molecular weight of 26 k Dalton determined by Sepharose – 6 B gel filtration. Both values are close to those of the known SEs (26,000 to 29,600) [34]. In this study *Staphylococcus aureus* A20-L2 gave only one band by SDS-PAGE under both reducing and nonreducing conditions.

The SDS-PAGE results, illustrated in Figure 4, clearly showed a protein band representing the purified enterotoxin obtained from *Staphylococcus aureus* isolate A20-L2. The relative mobility of each standard protein and of the purified enterotoxin was measured after being subjected to the SDS-PAGE experiment followed by construction of a standard curve, see Figure 5, wherein the X axis stands for the relative mobility for each standard protein while the Y axis represents the logarithm of the molecular weight of standard proteins. By the aid of this standard curve, it was possible to estimate the molecular weight of the purified enterotoxin.

### Table 3: Standardization of enterotoxin molecular weight based on the elution volume / void volume * ratio (Ve / Vo) ratio.

<table>
<thead>
<tr>
<th>Standard protein</th>
<th>Molecular weight (kilo Dalton)</th>
<th>((Ve/Vo)) ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>Catalase</td>
<td>232</td>
<td>2.15</td>
</tr>
<tr>
<td>Bovine serum albumin</td>
<td>67</td>
<td>2.2</td>
</tr>
<tr>
<td>Ovalbumin</td>
<td>43</td>
<td>2.25</td>
</tr>
<tr>
<td>Entrotoxin</td>
<td>28.2</td>
<td>2.3</td>
</tr>
<tr>
<td>Lysozyme</td>
<td>14.4</td>
<td>2.4</td>
</tr>
</tbody>
</table>

* The void volume of the column used in gel filtration chromatograph.
Figure 3: Standardization of the enterotoxin molecular weight based on the elution volume to the void volume ratio (Ve / Vo) by gel filtration chromatography.

which was approximately equal to 26 kilo Dalton. Figure 4 and one band by isoelectric focusing, see Figure 5. All the identified SEs are neutral to basic proteins with isoelectric points ranging from 7.0 to 8.6 [35]. However, *S. aureus* A20-L2 isolate has a neutral isoelectric point of 7.

Figure 4: SDS- polyacrylamide gel electrophoresis of the purified enterotoxin obtained from *Staphylococcus aureus*.

1: Purified enterotoxin
2: Standard Proteins

1

Bovine serum albumin 67 kDa
Catalase 60 kDa
Ovalbumin 43 kDa
Trypsin 24 kDa
Figure 5: Determination of the molecular weight of the enterotoxin of *Staphylococcus aureus* isolate A20-L2 by SDS-polyacrylamide gel electrophoresis using standard proteins of different molecular weights.

The isoelectric point (pI) of the enterotoxins from *S.aureus* isolate A20-L2 was determined by isoelectric focusing on Ampholyte plate gels, pH 3.5 to 10. When staphylococcal enterotoxin was examined by isoelectric focusing, the enterotoxin was shown to consist of one isoelectric point 7, (Figure 6).

Figure 6: Isoelectric point (IEF) of enterotoxin from *S.aureus* isolate A20-L2.

According to the results of molecular weight and isoelectric point of *S.aureus* A20-L2 isolate enterotoxin which agreed with Su and Wong [36] to enterotoxin type B molecular weight
28,366 Dalton and isoelectric point of 8.6. These differences may be because the *S. aureus* A20-L2 isolate enterotoxin amino acid content was neutral and may because it’s locally.

We can concluded from this study that *Staphylococcus aureus* and CoNS strains had the ability to produce at least two types of virulence factors, including enzymes and toxins, which increase the pathogenicity of these organisms to cause intramammary infections. The results indicated that *S. aureus* isolated from bovine mastitis has the ability to produce enterotoxin, while CoNS did not have the ability to produce enterotoxin.

**REFERENCES**


