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ISOLATION AND PURIFICATION OF *STAPHYLOCOCCUS AUREUS* SURFACE ANTIGEN B AND USING IT FOR THE DIAGNOSIS BY ELISA TECHNIQUE

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Summary:

Staphylococcus aureus is the most common cause of staphylococcal infections. It is a spherical bacterium, frequently lives on the skin or in the nose of a human.

Many tests were done for the diagnosis of *Staphylococcus aureus* isolated from blood culture, these include: culture, gram stain, catalase test, coagulase test and diagnosis by Vitek system. The results appear that 90% were (H7) isolates while 10% were other isolate.

Chromatography technique was used for extraction, purification and isolation of the antigen (enterotoxin B) from the cell wall of *Staphylococcus aureus*.

ELISA technique was used for the detection of enterotoxin B.

This antigen (enterotoxin B) was employed for the production of diagnostic ELISA kit against staphylococci (particularly for Iraqi peoples).

1.Introduction:

Staphylococcus aureus is the most clinically significant species of staphylococci. It can be recovered from almost any clinical specimen and is an important cause of nosocomial infection (hospital acquired infection). It is responsible for many infections both relatively mild and life-threatening (Coates *et al.*, 2014).

Staphylococcus aureus colonizes the skin readily and can lead to a wide variety of pathological conditions ranging from skin lesions to osteomyelitis, endocarditis, septicemia, necrotizing pneumonia and toxic shock syndrome (TSS) (Peterson *et al*., 2005; Bonar *et al*., 2015).

The ability of *S. aureus* to cause human disease depends on the production of various virulence factors including toxins, enzymes, adhesion proteins, cell surface proteins, factors that help the bacteria to evade the innate immunity, and antibiotic resistance that mediate bacterial survival and tissue invasion at the site of infection (Zecconi and Scali, 2013).

The most common toxins produced by *S. aureus* are cytolysins, exfoliativ toxins and superantigenes (enterotoxin and toxic-shock syndrome toxin-1) (Kong *et al*., 2016).

Enterotoxins (SEs) are potent gastrointestinal exotoxins produced by *S. aureus*. They are water soluble and are resistant to heat, acid, proteolytic enzymes and can resist inactivation by gastrointestinal proteases which include: pepsin, rennin, papain and trypsin. Thus, they can easily outlast the bacteria that produce them. (Argudín *et al.*, 2010; Oretga *et al*., 2010; Schelin *et al*., 2011).

Staphylococcal enterotoxins are made up of approximately 220–240 amino acids and have the same molecular weights of 25–30 kDa. Staphylococcal enterotoxin A and B (SEA and SEB) are the most common enterotoxins (Fooladi *et al*., 2010; Wu *et al.*, 2016).

Staphylococcal enterotoxin B (SEB) is the most studied and potent member of the enterotoxins secreted by *S.aureus* and it is responsible for staphylococcal food-poisoning (SFP). SEB easily produced in large quantities and at low concentrations, and can cause multi-organ system failure and death (Fries and Varshney, 2013).

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The primary targets and binding sites of SEB are the major histocompatibility complex class II molecules (MHC II) on antigen presenting cells (APCs) and the T-cell receptors (TcR) on T cells, resulting in a ternary complex structure. SEB binds to the MHC molecule outside the peptide-binding groove without prior processing. This binding cause stimulation, an immediate activation and proliferation of T-cells. T-cells will then release large amounts of cytokines including interleukin-2 (IL-2), tumor necrosis factor alpha (TNF-α), and gamma interferon (IFN-γ), and undergo hyperproliferation and ultimately depletion (Fries, and Varshney, 2013; Ahanotu *et al*., 2006).

Materials and Methods

1. Cell wall destruction and Antigen isolation

1. *Staphylococcus aureus* was cultured on blood agar plates and incubated at 36˚C overnight. The growth was harvested using microscopic slid.
2. Distilled water was added to the bacterial growth to make 5 ml suspension.
3. Bacterial suspension was treated with lyses buffer (50 ml of 2% SDS and 50 ml of 1% Acetate). The resulting suspension was incubated in boiling water for 20 minutes then vortexed vigorously.
4. One volume of the resulting suspension mixed with two volumes of chloroform. This suspension was vortexed vigorously and exposed to ultrasounds for 30 minutes at 50˚C.
5. The suspension was centrifuged at 4400 r.p.m. for 5 minutes, 10 volumes of the supernatant was precipitated using 1 volume of saturated ammonium sulfate (NH4)2SO4 then mixed on hot plate magnetic stirrer at 40˚C.
6. The mixture was dialyzed using dialysis bag (14000) against sucrose to obtain the stock (concentrated protein).

2. Purification of enterotoxin B by gel filtration chromatography

Fifty microliter of sephacryl S-300 was packeted in a column (2.5 cm diameter × 78 cm length, column volume: 379.9 ml). Five microliter of the sample that concentrated by ammonium sulfate was applied onto sephacryl S-300 column. Elusion was achieved at a flow rate of 5 ml fraction using D.W. as a buffer, the absorbance of each fraction was measured at 280ηm. The peaks were detected then concentrated by dialysis against sucrose.

3. Detection of Ag by ELISA kit

A) Ab. Coating

1. For capturing the Ab. in the bottom of the wells, 100 µl of diluted enterotoxin B Ab. (10 µg / ml) was added to the 96 wells of the microtitre plate.
2. The plate was covered and incubated at room temperature overnight.
3. The plate was washed 3 times with working wash buffer.

B) Blocking

1. For blocking the area in the bottom of the wells, 100 µl of blocking buffer was added to the wells.
2. The plate was covered and incubated at room temperature for 30 minutes.

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3. The plate was washed 3 times with working wash buffer.

C) Assay procedure

1. Serial dilutions for the Ag was prepared: 0.5 ml of the Ag was added to the first tube of 8 tubes each containing 0.5 ml D.W. 0.5 ml was taken from the first tube and added to the second tube and go on.
2. A l was left as blank well.
3. Fifty microliter of each dilution was added to the assigned wells and mixed gently. The microwell plate was covered and incubated at room temperature for 30 minutes.
4. The assigned wells were washed 3 times.
5. Fifty microliter of diluted enterotoxin B Ab was then added, the plate was covered and incubated at room temperature for 30 minutes.
6. The assigned wells were washed 3 times.
7. Fifty microliter of conjugate was added to the assigned wells except for the blank well, the plate was covered and incubated at room temperature for 30 minutes.
8. The assigned wells were washed 3 times.
9. Fifty microlitre of TMB was added to the assigned wells.
10. After 2-3 minutes, 50 µl of stop solution was added to the wells.
11. The absorbance was read at 450 ŋm. The concentration of the enterotoxin B Ag was determined by comparing the absorbance with the curve of Staphylococcus aureus (IgG Ab.) ELISA Kit.

4. Detection of Staphylococcal antibodies by ordinary ELISA kit

1. All reagents in the kit were allowed to be at room temperature and the required wells were assigned.
2. In the first well, 100 µl of patient serum infected with *Staphylococcus aureus* H7 was added.
3. In the second well, 100 µl of patient serum infected with *Staphylococcus aureus* of other isolate was added.
4. In the third well, 100 µl of monoclonal antibodies (standard) diluted with phosphate buffer saline (PBS) containing 10 IU/ml concentration of IgG antibodies against *Staphylococcus aureus* specific to isolate H7.
5. In the fourth well, 100 µl of antibodies (standard) diluted with PBS in ordinary ELISA kit containing 10 IU/ml concentration of IgG antibodies against *Staphylococcus aureus*.
6. The fifth well was left as control (blank).
7. The plate was covered and incubated at room temperature for 30 minutes.
8. The assigned wells were washed 3 times.
9. Fifty microliter of conjugate was added to the assigned wells except for the blank well, the plate was covered and incubated at room temperature for 30 minutes.

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10. The assigned wells were washed 3 times.
11. Fifty microlitre of TMB was added to the assigned wells and incubated at 37˚C for 15 minutes.
12. Fifty microliter of stop solution was added to the assigned wells.
13. The absorbance was read at 450 ŋm. The concentration of the antibodies was determined by comparing the absorbance with the curve of *Staphylococcus aureus* (IgG Ab.) ELISA Kit.

5. Detection of Staphylococcal antibodies by new ELISA kit

A) Ag Coating

1. For coating of microtiter well plate with the antigen, 100 µl of enterotoxin B antigen (10 µg / ml) was added to the 96 wells of the microtitre plate.
2. The plate was covered and incubated at room temperature overnight.
3. The plate was washed 3 times with working wash buffer.

B) Blocking

1. For blocking the area in the bottom of the wells, 100 µl of blocking buffer was added to the wells.
2. The plate was covered and incubated at room temperature for 30 minutes.
3. The plate was washed 3 times with working wash buffer.

C) Assay procedure

1. All the required wells were assigned.
2. In the first well, 100 µl of patient serum infected with *Staphylococcus aureus* H7 was added.
3. In the second well, 100 µl of patient serum infected with *Staphylococcus aureus* of other isolate was added.
4. In the third well, 100 µl of monoclonal antibodies (standard) diluted with phosphate buffer saline (PBS) containing 10 IU/ml concentration of IgG antibodies against *Staphylococcus aureus* specific to isolate H7.
5. In the forth well, 100 µl of antibodies (standard) diluted with PBS in ordinary ELISA kit containing 10 IU/ml concentration of IgG antibodies against *Staphylococcus aureus*.
6. The fifth well was left as control (blank).
7. The plate was covered and incubated at room temperature for 30 minutes.
8. The assigned wells were washed 3 times.
9. Fifty microliter of conjugate was added to the assigned wells except for the blank well, the plate was covered and incubated at room temperature for 30 minutes.
10. The assigned wells were washed 3 times.
11. Fifty microlitre of TMB was added to the assigned wells and incubated at 37˚C for 15 minutes.
12. Fifty microliter of stop solution was added to the assigned wells. The absorbance was read at 450 ŋm. The concentration of the antibodies was determined by comparing the absorbance with the curve of *Staphylococcus aureus* (IgG Ab.) ELISA Kit.

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Results and Discussion

1. Extraction and purification of enterotoxin B from *Staphylococcus aureus*

Enterotoxin B secreted from the cell wall of *S. aureus* was purified, three purification steps were used to obtain purified enterotoxin B used for further study of preparation of a new ELISA kit.

After precipitation of enterotoxin B with ammonium sulphate, it was dialyzed against sucrose to obtain the concentrated protein.

Gel-filteration chromatography technique (using sephacryl s-300) was nexa step in the purification of enterotoxin B.

A volume of 5ml of enterotoxin B concentrate was applied to the column. Its eluted with the D.W. as a buffer. A volume of 5ml fraction of the eluent was collected in plain tube and one peak resulted from elution of enterotoxin B on sephacryl s-300 column which appear high concentration in fraction number 4 – 6, fraction turbidity was measured by using spectrophotometer at [280 nm].

In figure (1), it was found that the second step "Isolation and purification of the cell wall (enterotoxin B)" treated with lysis buffer and vortexed to breaking the cell wall of Staphylococcus aureus and prepared for the addition to chromatography column for purification of enterotoxin B from other proteins were consistent with the study of (Muratovic *et al.*, 2015; Kientz *et al.*, 1997).

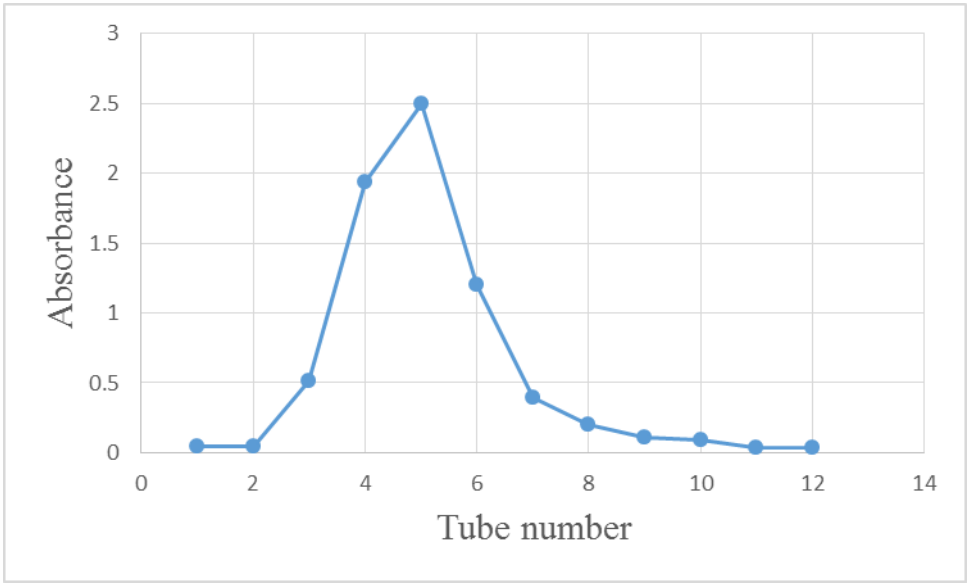

Figure (1): Gel filtration chromatography of enterotoxin B produced by *Staphylococcus aureus* using sephacryl s-300 column (2.5 ×78 cm). Fraction collected at a flow rate of 60 ml / hour.

2. Detection of enterotoxin B by indirect sandwich ELISA

After purification of enterotoxin B by chromatography, indirect sandwich ELISA was the next step to be used to confirm this protein and to detect its concentration.

In this method, anti-enterotoxin Ab. was captured in the bottom of a microtiter well plate, then purified diluted enterotoxin B was added and the plate was incubated for the formation of immune complex. Anti-enterotoxin B Ab. was added again, so the antigen was captured between two Abs. (thus named sandwich). Detecting Ab. (labelled with enzyme) was then added and followed by the addition of substrate which made the reaction visible to the naked eye through the development of a blue color that changed to a yellow color by the addition of stop solution.

The development of these colors indicates the formation of immune complex (anti-enterotoxin B Ab. – enterotoxin B Ag.) and the Ab. reacted specifically with the Ag. to be detected. The absorbance of each dilution was measured using spectrophotometer at 450 – 630 ηm. The

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concentration of the Ag. was obtained by blotting the absorbance on a standard curve. The results were shown in figure (2).

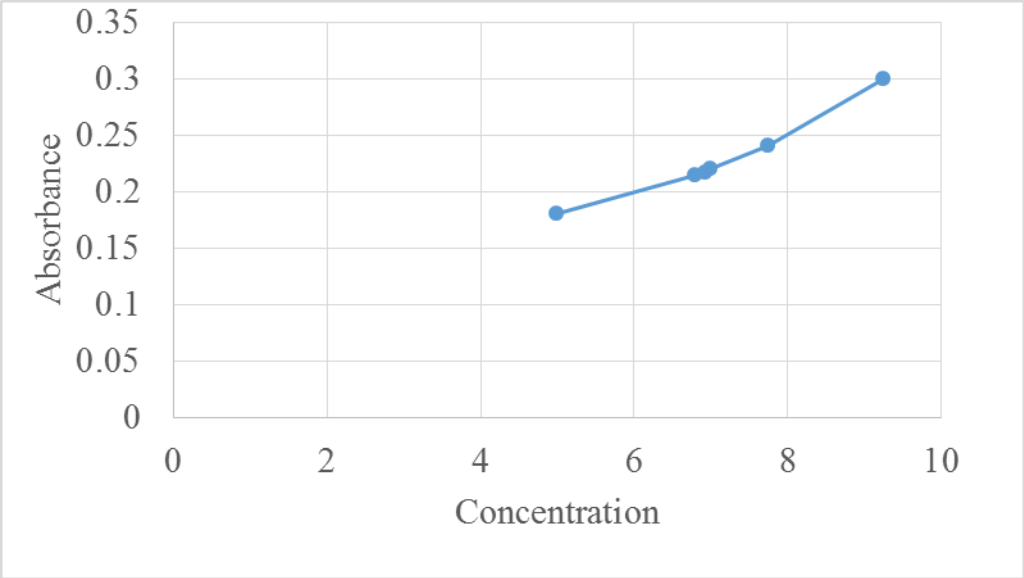

Figure (2): Absorbance and the concentration of enterotoxin B detected by sandwich ELISA.

4.4. Comparing new ELISA kit with ordinary ELISA kit

Comparison of new ELISA kit with ordinary one was achieved by investigation of many samples to new and ordinary ELISA Kit these samples were:

Sample 1: Serum of patient infected with *Staphylococcus aureus* isolate (H7)

Sample 2: Serum of patient infected with *Staphylococcus aureus* of other isolate.

Standard 1: Monoclonal antibodies diluted with PBS, contains (10 IU/ml) concentration of IgG antibodies against *Staphylococcus aureus* (specific to isolate H7).

Standard 2: Antibodies diluted with PBS in ordinary ELISA Kit, contains (10 IU/ml) concentration of IgG antibodies against *Staphylococcus*

aureus

Negative control: protein solution diluted with PBS, contains no IgG antibodies against *Staphylococcus aureus*.

Table and figure (3) showed that standard 1 (monoclonal antibodies specific to isolate H7) was more sensitive to new ELISA kit (absorption 2.8 at 450 nm) than ordinary ELISA kit (absorption 1.33 at 450 nm), per contra, the result of standard 2 (antibodies nonspecific to isolate H7) was less sensitive to new ELISA kit (absorption 0.8 at 450 nm) than ordinary ELISA kit (absorption 1.32 at 450 nm).

This result was similar to comparison of results in sample 1 (serum of patient was infected with *Staphylococcus aureus* isolate H7) and with sample 2 (serum of patient was infected with *Staphylococcus aureus* of other isolate), which showed that Sample 1 (absorption 2.5 at 450 nm) was more sensitive than sample 2 (absorption 0.6 at 450 nm) in new ELISA kit ,while in ordinary kit , opposite result was obtained which appear that sample 1 (absorption 1.03 at 450 nm) was less sensitive than sample 2 (absorption 1.12 at 450 nm) in ordinary ELISA kit.

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In standard 2 (antibodies nonspecific to *Staphylococcus aureus* isolate H7), new ELISA Kit had pure enterotoxin B, but it was specific to H7 only, therefore, standard 2 was more sensitive to antigen in ordinary ELISA kit than new ELISA kit.

Sample [1] was also taken from patients infected with *Staphylococcus aureus* isolate H7, it was shown that new ELISA kit was more sensitive than ordinary ELISA kit because it had specific antibody to *Staphylococcus aureus* (H7 isolate), whereas in sample 2 which was taken from patients who were infected with *Staphylococcus aureus* other isolate, the ordinary ELISA kit was more sensitive than new ELISA kit.

All these results in table (3) showed that new ELISA kit was successful for diagnosis of *Staphylococcus aureus* isolate (H7) compared with ordinary ELISA kit, and it was more sensitive and specific due to having pure enterotoxin B, and manufacturing of new ELISA kit, *Staphylococcus aureus* isolate H7 was used because 90 % of patient were infected in Baghdad and 10% were infected with other isolates of *Staphylococcus aureus*. These results agreed with studies of (Kuang *et al.*, 2013).

Table (3): comparison of new ELISA kit with ordinary ELISA kit

Sample	**Type of Antibody**	**The reading of the absorption New ELISA Kit**	**The reading of the absorption Ordinary ELISA Kit**
I	Serum of patient infected with *Staphylococcus aureus* isolate H7	2.5	1.03
II	Serum of patient infected with *Staphylococcus* aureus of other isolate.	0.6	1.12
III	Standard: Monoclonal antibodies diluted with PBS, contains (10 IU/ml) concentration of IgG antibodies against *Staphylococcus aureus* (specific to isolate H7).	2.8	1.33
IV	Standard antibodies diluted with PBS in ordinary ELISA Kit , contains (10 IU/ml) concentration of IgG antibodies against *Staphylococcus aureus*	0.8	1.32
Negative control	Without Ab	0.1	0.1

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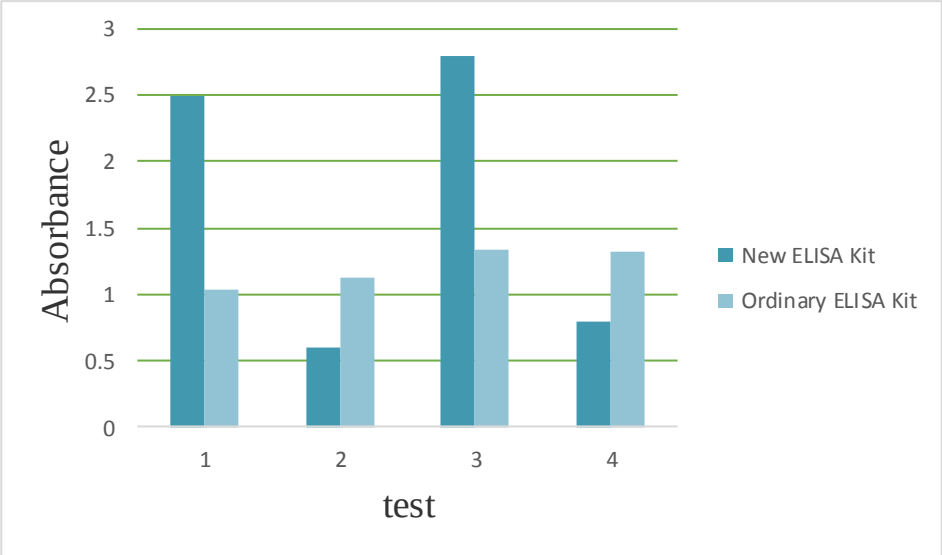

Figure (3): Comparison of new ELISA kit with ordinary ELISA kit

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