

Isolation, Purification and Identification of Staphylococcal Enterotoxin A from Pregnant Women have Urinary Tract Infections in AL-Kut City

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ABSTRACT

This study aimed to isolate, purify and identify staphylococcal enterotoxin A from *Staphylococcus aureus*. 200 clinical urine samples were collected from pregnant women with urinary tract infection aged from (20-40 years). Then, these isolates were identified as *Staphylococcus aureus* by biochemical and molecular methods. The percentage of positive culture of *Staphylococcus aureus* was 47%. One strain was selected to produce staphylococcal enterotoxin A and this enterotoxin was purified by chromatography techniques such as ion exchange and gel filtration. All purified fractions were collected and measured at 280 nm and then the presence of enterotoxin A was detected by ELISA kit. Finally, 50 mg/ml of SEA was extracted from 15 liters of brain heart infusion broth.

KEYWORDS: *Staphylococcus aureus*, Biochemical test, Molecular tests, Enterotoxin A, Ion exchange, Gel filtration

INTRODUCTION

Staphylococcus aureus is a gram-positive bacterium, causes many diseases in humans and usually lives on the skin and the upper respiratory tract (Le and Otto, 2015). *S. aureus* is a dangerous pathogen and its treatment is so difficult (Rasigade and Vandenesch, 2014). *S. aureus* has many types of virulence factors that include cytotoxins, enzymes and enterotoxins (Tong *et al*, 2015). The enterotoxin is a protein secreted by bacteria that lives in the intestines and these enterotoxins are encoded by plasmid or chromosome or prophage (Balaban and Rasooly, 2000). Cytotoxic effects of enterotoxins are by killing cells and changing the apical membrane permeability of the mucosal (epithelial) cells of the intestinal wall. They are mostly pore-forming toxins (mostly chloride pores) that cause the cells to die (Larkin *et al*, 2009). In addition, it resists to low pH, heat and proteolytic enzymes. Staphylococcal enterotoxins (SEs) have large roles in bacterial pyrogenic activity; it has a similar structure, sequence homology and function (Fleischer *et al*, 1991). These toxins could cause food poisoning, autoimmune diseases, toxic shock syndromes and several allergic diseases (Ortega *et al*, 2010). Many recent studies showed that staphylococcal enterotoxins, considered one of the virulence factors that found on mobile genetic elements, make them unstable and continuously change by horizontal transfer genes (Ahmad *et al*, 2017).

Material and Methods

Patients

This study included pregnant women had UTI infection with *S. aureus*. 200 Urine samples were collected from pregnant women aged from 20 to 40 years attending Al-Kut hospital for Gynecology and Obstetrics in Al-Kut \Iraq during the period from 1st March to 1st August 2018.

Biochemical Tests

Biochemical tests were used to identify *staphylococcus aureus* genus according to (Karmakar *et al*, 2016 and Suma *et al*, 2016).These samples were detected by different biochemical tests such as Oxidase test, Catalase test, Coagulase test, Manitol salt agar (MSA), Dnase agar, Chromogenic Staph Medium, Novobiosin sensitivity and API staph system.

Bacterial DNA Extraction

Bacterial DNA of *Staphylococcus aureus* was extracted by Presto™ Mini gDNA bacterial kit. The procedure of Gram positive bacteria was followed to extract *Staphylococcal* DNA.

Polymerase Chain Reaction and Real Time- PCR master mix

PCR and RT-PCR were used to detect *staphylococcal* enterotoxins genes. RT-PCR and PCR protocols were performed according to (Blaiotta *et al*, 2004) and (EI-Jakee *et al*, 2013) respectively . Primer sequence of *Staphylococcal* enterotoxins A was listed in table (1).

Table (1): primer of SEA

gene	Oligonucleotide sequence (5→3)	Size products (pb)	Primer Source
SEA	CCT TTG GAA ACG GTT AAA ACG TCT GAA CCT TCC CAT CAA AAA C	127	(Betley and Mekalanos, 1988)

Bacterial Fermentation and Toxin Production

Bacterial fermentation and enterotoxin production was carried out according to (Notermans and Heuvelman, 1983). After detection of *S. aureus* enterotoxins by PCR and RT-PCR, one selective strain was used for production of SEs. 15 liters of brain heart infusion broth were used in bacterial fermentation to increase the amount of *staphylococcal* enterotoxin A. NaCl also added to the fermenters medium as a humectant.

Precipitation of SEs by Ammonium Sulfate

Staphylococcus aureus was grown to the late stationary phase (as the purified *staphylococcal* enterotoxins released during exponential phase and stationary phase (Ghani *et al*, 2019). The supernatant was preparing for ammonium sulfate precipitation. Ammonium sulfate precipitation have be done by using the saturation value 60% according to (Niskanen and Lindroth, 1976; Green and Hughes, 1955).

Purification of SEs by chromatography using Anion Exchanger (DEAE-cellulose)

Purification of SEs by anion-exchange chromatography according to the procedure described by (Al-Shammary *et al*, 2012) with modification by using 1M NaCl to prepare a salt gradient used for eluting the bound proteins.

Purification of SEs by Gel Filtration (Sephadex G-100)

Sephadex-100 was used in SEA purification according to (Schutte *et al*, 1997). All fractions that were formed by gel filtration were assayed for presence of proteins (absorbance at 280 nm). Each peak of many fractions was dialyzed overnight.

Human *Staphylococcus aureus* Enterotoxins A ELISA Kit Procedure

This kit was utilized to detect the presence of SEA in purified and identified samples of *S aureus* isolates. This kit is based on sandwich enzyme –linked immune –sorbent assay technology. The O. D absorbance is measured spectrophotometrically at 450 nm in a microplate reader and the presence of SEA can be determined.

RESULTS AND DISCUSSION

In this presented study, only 94 clinical samples were characterized as *Staphylococcus aureus* which were isolated from 200 pregnant women patients with UTI by different biochemical and molecular tests. These female were aged from 20-40 years old (table 2).

Table (2): Positive culture results of *S. aureus* bacteria collected from pregnant women with UTI according to patient's sex and age.

Positive Culture of <i>S aureus</i>	Pregnant Females		Total
	21-30 years	30- 40 years	
Positive culture	50	44	94
of Positive culture	53.1%	46.8%	47%

Generally, women are more susceptible to UTI infection more than male due to the following reasons (Singh *et al*, 2014):-

- Hormonal effects and behavioral pattern.
- Anatomic differences.
- Hormonal effects and behavioral pattern and malnutrition.
- Poor hygiene, low socioeconomic status is associated with UTI.

Usually before DNA extraction, PCR and RT-PCR analysis, all the isolated colonies were identified by abundant biochemical tests. All these positive culture results of *S. aureus* were characterized and identified firstly by general biochemical test and finally were tested with molecular methods. Those methods and their percentage results were listed in table (3)

Table (3):- Biochemical and molecular tests and their percentage results

Bio-molecular tests	Positive results	% percentage
Manitol salt agar (MSA)	+94	47%
Novobiosin 5 µg	94	
Tube coagulase test	+94	
Chromogenic agar	94	
Dnase agar	+94	
API STAPH	+94	
PCR	94	
RT-PCR	94	

(+):- mean that more than 94 samples were give false positive results with this type of biochemical tests.

The results of this study concluded that many general and specific biochemical tests must be used and in duplicate for avoiding misidentification. MSA fermentation, tube coagulase test and Dnase agar must supported with other most developmental and specific assay such as Novobiosin sensitivity, API staph and chromogenic medium for reaching to the right identification.

Identification SEA gene by PCR and RT-PCR methods

PCR assay were recently more used to identify SEA due to it depended on the principle of amplification the target genes. PCR detection for encoded enterotoxins genes in *Staphylococcus aureus* is simple, low cost ,rapid and very specific. PCR assay supported with more specificity and sensitivity for identification (Saadati *et al*, 2011). The positive culture of *S. aureus* is 47% (94 samples out of 200), these results were depended on PCR result for its specificity and accuracy (figure 1).

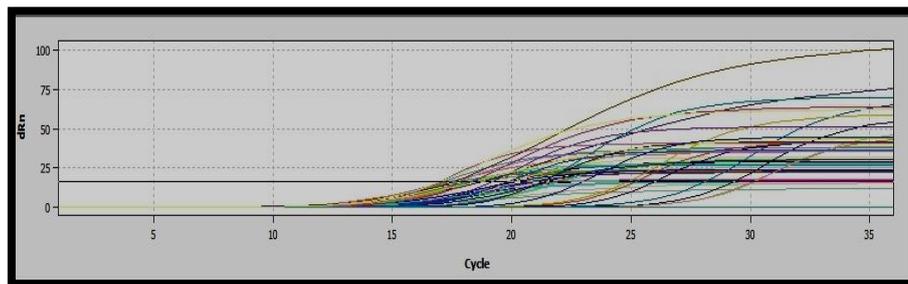


Figure (1):- Quality detection of SEA by RT-PCR

Ammonium sulfate precipitation

The saturation values (60 %) was used due to

- This saturation value act to precipitate our specific proteins (SEA).
- 60% saturation value of ammonium sulfate leading to quantitative precipitation of staphylococcal enterotoxin A.
- 60% saturation value is the best choice for SEA because it can be used for their protective of biological activity of proteins.

Table (4) showed the protein concentration (mg/ml) in each extraction steps. Protein concentration was (15.5) before dialysis and (13.2) after dialysis respectively, by using AS with 60% of saturation value. The following equation (According to Beer –Lambert) was used to determine protein concentration.

$$\text{ure of proteins} = 1.55 \times \text{absorbance in } 280 - 0.76 \times \text{absorbance in } 260$$

The purification by ion exchange was partially and must be followed by other types of purification such as gel filtration. The principle of using ion exchange in protein purification was depended on the molecules charge (positive or negative). On other hand, gel filtration was depended on the molecular weight (M.W). In gel filtration, the particles that have the same M.W will appear as a single peak while in ion exchange will give more than one peaks. In this studying an anion exchanger DEAE-cellulose (Gander and Thomas, 1986) was used for completing purification. All samples that were collected from ammonium sulfate precipitation at saturation values (60%) was dialyzed and concentration by dialysis tubes. The eluent fractions were checked for its absorbance at 280 nm manually in UV spectrophotometer. Then a plot was made between absorbencies and fractions number (figure 2, 3). All of these positive samples by ELISA kit were tested for proteins concentration, presence of SEA by using the following equation:-

$$\text{Protein concentration} = \left(\frac{\text{absorbance at } 280}{\text{Extinction coefficient}} \right) \times 10 \text{ mg \ ml}$$

The total protein that was obtained from 15 liters of BHI broth was 7 mg/ml and measured according the below equation:-

$$\text{Protein concentration} = \left(\frac{7}{1.4} \right) \times 10 \text{ mg \ ml} = 50 \text{ mg/ml from 15 liters}$$

* extinction coefficient for SEA is 1.4

In this study, 50mg/ml from 15 liters were obtained. 60% saturation value of ammonium sulfate was more effective due to 60% saturation value precipitated about 85% of SEA.

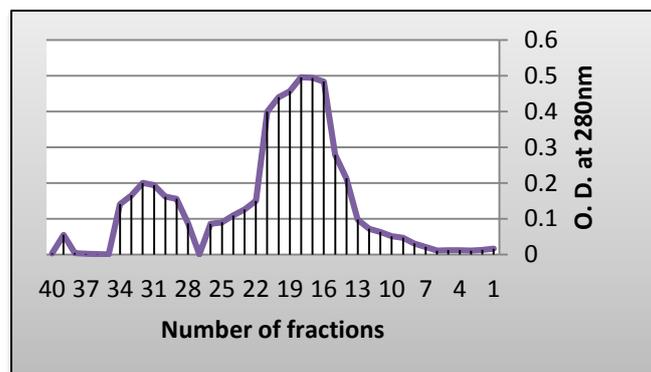


Figure (2): Purification of staphylococcal enterotoxin A by DEAE-cellulose ion-exchange chromatography (60 cm) from *Staphylococcus aureus*. The column was eluted with 1M of NaCl (100ml) to collect 40 fractions with pH 8.2

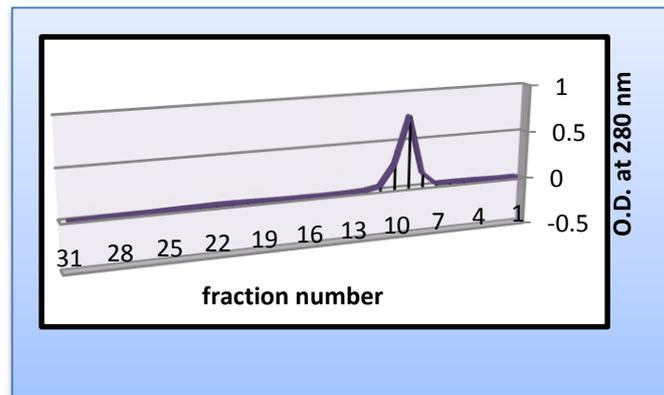


Figure (3): Gel filtration of purified enterotoxin on Sephadex G-100. The concentrated enterotoxin solution was passed through a Sephadex G-100 column equilibrated with phosphate buffer, pH 8.2; absorption at 280 nm, one peak represented by (8,9,10) fractions

Table (4):- illustrated steps of staphylococcal enterotoxin A purification from 15 liters

No.	Purification steps	Protein concentration (Mg/ml)	Notes
1	Ammonium sulfate (60%)		
A	Before dialysis with D.W	15.5	
B	After dialysis with D.W	13.2	
2	Ion exchange (DEAE Cellulose)		This value was calculated from different peaks from different group of fractions (40 fractions in every step). Each fraction contains 5ml.
A	Ion exchange at 60% saturation (after dialysis with sucrose for salt removing and increasing protein concentration)	11.3→washing with 200 ml of elution buffer (1M of NaCl) with pH 8.2	
3	Gel filtration (Sephadex-100)		
A	Gel filtration at 60% saturation (after dialysis)	10.2	

CONCLUSION

Biochemical and molecular methods must be used to identified the Staphylococcal spp. Chromatography was used to purified SEA. Ammonium sulfate at 60% saturation value was more effective to precipitate SEA. The fermentation broth must be suitable for SEA production.

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