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Production Of Polyclonal Antibodies Against Exotoxin A Of Pseudomonas Aeruginosa

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Abstract:

Pseudomonas aeruginosa is Gram-negative opportunistic pathogen that causes chronic and acute infections at different sites within body mostly in hospitalized patients such as urinary tract infections, respiratory system infections, dermatitis, soft tissue infections andbacteremia.Exotoxin A inhibits protein synthesis in infected cells by ADP-ribosylation of eukaryotic elongation factor 2.ExotoxinA one of the most virulent factors of Pseudomonas aeruginosa which was concentrated and purified. Purification of toxin A produced by only two steps ion exchange chromatography and gel filtration chromatography which yield highly purified toxin. The molecular weight of exotoxin A is 66KDa. ELISAis used for diagnosis of exotoxin A which act as more rapid, sensitive and simple methods .SDS, indirect ELISA and Western blotting test performed to confirm extraction of toxin A.Special primers designed to detect presence of tox A virulence factor, 70.5% of isolated samples have the toxA gene.

Keywords: ELISA, Pseudomonas aeruginosa, SDS, Toxin A and Tox A

إنتاج الأجسام المضادة متعددة النسيلة ضد السموم الخارجية A من زانفه الزنجاريه.

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الخلاصة:

بكتريا الزائفه الزنجاريه هو نوع انتهازي سالب الجرام ويسبب التهابات مزمنة وحادة في مواقع مختلفة داخل الجسم ، ومعظمها في المستشفيات مثل التهابات المسالك البولية والتهابات الجهاز التنفسي والتهاب الجلد والتهابات الأنسجة الرخوة وتجرثم الدم. يتسبب السموم الخارجية Aفي ارتباط ADP- ribosylationلعامل استطالة حقيقيات النوى 2 الذي يسبب تثبيط تخليق البروتين في الخلية المصابة. السموم الخارجية :Exotoxin أحد أكثر العوامل فتكًا في Pseudomonasالذي تم تركيزه وتنقيته. تنقية السموم Aالناتجة عن خطوتين فقط كروماتوغرافيا التبادل الأيوني والكروماتوغرافيا بالترشيح الهلامي والتي تنتج سمًا عالي النقاء. الوزن الجزيئي للسموم الخارجيه هو 66 . يتم استخدام ELISAلتشخيص السموم الخارجية التي تعمل كطرق أكثر سرعة وحساسية وبسيطة. تم إجراء اختبار النشاف الغربي وELISA و SDS واختبار النشاف الغربي لتأكيد استخلاص السم أ. مواد أولية خاصة مصممة لاكتشاف وجود عامل ضراوة السموم 70.5٪ من العينات المعزولة تحتوي على جين المسئول عن انتاج السموم الخارجيه.

الكلمات المفتاحيه:اختبار الانزيم المرتبط بالمواد المناعيه- بكتريا الزائفه الزنجاريه- فصل كهربائي بطريقه صوديوم ديدوسيل سلفيت الخاصه بفصل البروتينات-سموم خاصه ببكتريا الزائفه الزنجاريه- الجين المسئول عن انتاج سموم الزائفه الزنجاريه.

Introduction

Pseudomonas aeruginosais an opportunistic pathogen.It is a flagellatedbacterial pathogen which cause dangerous disease especially to immunocompromised people, it was reported that Pseudomonas aeruginosaresponsible for 40% of acute ventilator induced pneumonia [1].Pseudomonas aeruginosa is the main producer of some second metabolites such as toxins, pyocyanin, hydrogen cyanide and exotoxin A. Quorum Sensing (QS) is the regulator of many of these second metabolites which shown to be important for P. aeruginosa fitness and biofilm formation. In addition,Pesudomonas aeruginosa produces two siderophores, pyoverdine and pyochelin, that used for scavenging iron in low iron condition [2].

Exotoxin A is a Pseudomonas aeruginosa extracellular enzyme that catalyzes (NAD) in a rabbit reticulocyte lysate, as well as the transfer NAD^+ to a protein in eukaryotic cells and this inhibit protein synthesis[3].

ELISA increases the ability for specific antigen-antibody reactions and enables qualitative and quantitative identification on the basis of common Ag-
Ab binding reactions.[4]Nowadays, ELISA is the trojan horse for surgical procedure work. This method, which has been widely used, provides a special identification in many different samples with a vast number of target test s.

ELISA has been able to find a variety of applications in food safety, economic, biotechnological and

chemical fields, among others, since the invention of the technique four decades ago [5]. The aim of this study is the purification and production of exotoxin A fromPseudomonas aeruginosa for production of polyclonal antibodies by animal injection and blood collection then followed by antibody titration using ELISA which considered being common and valuable screening method for diagnosis of bacterial toxins. Using specific primers was designed and used to detect presence of tox A virulence factor gene.

Materials and Methods:

Isolation of pathogenic bacteria from different agricultural localities in Giza

Different food sampleswere collected in sterilized bags. Samples surface rinsed with distilled water and diluted to tenfold dilution using nutrient broth medium for 24 hours at 37°C. Streak plate method was used on different selective media for isolation and identification.

Identification of isolated samples:

Morphological identification:

a. Gram -staining:

Gram- staining identification was performed on 48 hours old culture of purified species isolated from different localities in Egypt that shown in **Table 1.** The Gram –staining method based on reported protocol [6].

b. Biochemical tests

All selective media used shown in **Table 2** according to the biochemical identification method [7].Biochemical analyses were used to classify and describe the closetmatchwith the known bacterial genus according to Bergey's manual[8,9].

The tests are catalase test, starch hydrolysis, mannitol salt agar, oxidase test, urease broth test, carbohydrate fermentation testand MacConkeyagar method as shown in **Fig 2.**

Selection of the strongest organism for toxin production using well diffusion method

Nutrient agar plates inoculated with each oneof isolated organisms and wells contain 0.5ml of the other bacterial suspensions of isolated and purified bacteria, the control well containing distilled water in the middle. Isolate no. 3showing the highest clear zone in **Table3.**

Molecular identification ofisolate no. 3

a. DNA extraction:

DNA extraction was carried out using Propman ultra-Kit, U.K.Overnight culture (20 ml) of Pseudomonas aeruginosa was prepared in nutrient broth media. Bacterial cells harvested and 5 minutes centrifugation at 5000rpm and suspended in 200 ul of 1X TE buffer (pH8), 40mg/ml of lysozyme was added to cell suspension and incubated at 37°C for 2 hours. After incubation, 370ul, 1X TE (pH8) with proteinase K was added for 1 hour. After 30 minutes of phenol chloroform extraction, samples were centrifuged for 5 minutes at 6000 rpm with one equal volume of phenol/chloroform isoamyl alcohol (24/24/1). The aqueous phase was transferred to a clean Eppendorf tube, and the bacteria's genomic DNA was precipitated using isopropanol (one equal volume), followed by 10 percent ammonium acetate, and the pellet was dissolved in TE buffer.

b. Polymerase chain reaction (PCR):

The purified DNA stored in -20ºC for molecular studies. The PCR was done using Maxima Hot Start PCR Master Mix). The reaction contained 25µl master mix (2×), 1µl 16S rRNA forward primer, 1µl reverse primer, 5µl DNA and 18µl free water. The amplification reaction was carried out in a total volume of 50µl using Veriti thermal cycler, applied biosystem. PCR was performed under the following conditions:[initial denaturation / enzyme activation at 95ºC for 10 minutes, denaturation at 95ºC for 30 seconds, annealing at 65ºC for 1 minute, extension at 72ºC for 1minute and final extension at 72ºC for 10 minutes].

c. Nucleotide sequencing:

Washing Buffer (100µl) was added to the purification column, centrifuged for 60 seconds and the flow-through was discarded. To remove any remaining washing buffer, the purification column was centrifuged for a further minute. 25ul of elution buffer was added to the centre of the column membrane and centrifuged for 1 minute in a clean 1.5 ml micro centrifuge tube.

The purified DNA was sequenced using ABI 3730xl at Clinilab Scientific Services Company, Cairo, Egypt. The sequence was determined using the forward and reverse primers. Sequence comparisons were performed using BLAST. Multiple alignments with other sequences in NCBI were conducted using CLUSTALW to identify the organisms. The results show that the isolated and identified organism wasPseudomonas aeruginosa.

Production of pyocynine pigment:

Bacterial isolates were streaked on cetrimide media and incubated for 24 hours at 37° C to test production of pyocyanin pigment.

Ability to grow at 42°C:

To detect ability of bacteria to grow on nutrient agar media at 42°C for 24 hours. Presence of colonies indicates positive results.

P**roduction, concentration, precipitation of toxin A from theidentified organism:**
A dialyzed tryptic soy broth medium was produced by dissolving 45 g of powder in 1500 ml distilled water and dialysis overnight at 4°C against 0.15M Tris – OH buffer. For 15 minutes, the dialysate produced by the dialysis bag was autoclaved. Filtration via a Seitz filter sterilised monosodium glutamate 0.75 M. After the dialyzed media came out of the autoclave, 0.45mM was added aseptically to the dialysis medium with 1% glycerol, the purification process based on the protocol [10].

.The inoculum was cultured on blood agar plate for 18 hours at 37°C before being scraped off with a sterile loop to make a cell suspension of 10^{10} cells/ml. The cultures were initiated by adding 0.4 ml of freshly prepared bacterial suspension into 250 ml flasks and shaking at 150 rpm/min at 36°C for 22 hours. After incubation, cultures were centrifuged for 20 minutes at 10,000 xg.

a. Concentration of toxin A
The centrifuged culture was given a concentration of zinc acetate (1M). The mixture was placed overnight at 4°C before being centrifuged for 20 minutes at 14000 rpm to extract the precipitate, which was dissolved in 0.3 M sodium citrate solution. The mixture was dialyzed for 24 hours at 40°C against 0.01M Tris buffer pH 8.0 with numerous changes of buffer, then centrifuged to remove the insoluble material..

b. Precipitation with ammonium sulphate
Exotoxin A was precipitated by adding concentrated NaOH to $(NH_4)_2SO_4$ at 20%, 40%, 60%, and 80 % saturation. Centrifugation was used to collect the precipitate, which was then suspended in 20 mL Tris buffer and dialyzed once more. Without losing efficacy, the toxin was stored frozen at (4°C).

Purification of toxin A by Ion exchange chromatography
DEAE-cellulose column 1.5 x 35 cm washed multiple times with 0.01 M Tris-OH buffer pH 8. The column was injected with ten millilitres of toxin A. Toxin A was eluted with a NaCl linear gradient from 0.1 to 0.5 M in a 0.01M Tris-OH buffer pH 8 with 0.01M Tris-OH buffer. The toxin was collected at a rate of 3 ml/fraction, and the absorbance was measured at 280 nm. [11.]

Gel Filtration Chromatography with Sepharose 6B
Sepharose 6B gel column 1.5 x80. With 0.2 M Tris –OH buffer pH 8, the column was rinsed and equilibrated. On top of the Sepharose 6B column, two millilitres of concentrated toxin A purified using DEAE- Cellulose column were added. The 0.2 M Tris –OH buffer of equilibration was used to elute the fractions at a flow rate of 3ml/fraction, and the absorbance of each fraction was measured at 280 nm. The peaks were then collected and injected into BALB/C mice to see if they were cytotoxic.

Experimental animal

Toxicity in mice
A group of ten BALB/C mice was obtained from Egypt's Research Institute of Ophthalmology. Mice were cared for in compliance with the National Institute of Health's (NIH) animal care standards and policies. Following the approval of the study ethics committee, to detect the cytotoxicity of exotoxin A. (IACUC). The mice's ages ranged from (4-6) weeks to (28-30) grammes, with the last one serving as a control. Each group was housed in its own plastic cage, which was then kept in an animal house at a temperature of (23-25)^{o}C. Mice were given adequate amounts of water and a balanced food. The animals were given (50) ug crude exotoxin A emulsified in complete Freund's adjuvant, then four injections of (100) ug crude toxin A emulsified in incomplete Freund's adjuvant each. After 7 days, sera were collected for anti-toxin A testing, and mice were killed and their spleens were removed for RNA isolation.

SDS PAGE:
The Laemmli buffer system was used to perform sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The samples were heated for 10 minutes at 100°C in dissociating buffer containing 10% SDS and 5% 2-

mercaptoethanol before electrophoresis. Proteins were stained with Coomassie Brilliant Blue R-25 after SDS-PAGE using a page Ruler Prestained protein ladder (250) ul[12].

Western blotting test:

100 millilitres of crude toxin A was spun in two volumes of protein buffer extraction, centrifuged, and the supernatant was collected and billed for four minutes before being separated on a 10% SDS-PAGE. Proteins were electrotransferred onto a PVDF membrane (polyvinylidene difluoride). The membrane was cleaned and treated with anti-IBDV positive serum for 2 hours at room temperature after being transferred in block buffer (4 % skimmed milk). Anti-IgY labelled with alkaline phosphatase, Sigma Aldrich, USA, was added for 1 hour after being washed. After the final wash, the membrane was immersed in detection solution and left until bands visible [13].

Indirect ELISA:

To test the interaction between antigen and antibody:

Microtitre plates were coated overnight with 100 ul of antigen (purified toxin A) and 100 ul of (1X) coating buffer, resulting in a tenfold serial dilution in wells. The solution was discarded into a container when the microtiter plate was exposed. PBS and Tween 20 for two washes, then PBS just for three washes. By adding 200 l blocking buffer (4%) to the coated wells and incubating for 2 hours at room temperature, the protein-binding sites were blocked. Washing with PBS and Tween 20, then PBS only, before incubating for 2 hours at room temperature with a 100 ul mixture of (3ul first antibody + 3ml (1 percent) blocking buffer. Wash with PBS and Tween 20 again, then PBS just. Incubate for 2 hours with a 100 ul mixture of (1ul) 2nd antibody and 3ml (1 percent) blocking buffer. Rinse with 100 ul of substrate buffer and p-Nitro phenyl phosphate disodium salt tablets twice more. Then, at 405 nm, use an ELISA reader. [13].

Indirect ELISA:

To confirm the presence of tox A gene:

Microtitre plate was coated with 100 ul of antigen of the different samples, 100 ul of (1X) coating buffer making tenfold serial dilution in wells added and covered plates with foil for overnight. The plate was uncovered and the solution was discarded into a container. Washing with PBS and Tween 20 for 2 times then washed with PBS only for 3 times. Block the protein-binding sites in the coated wells by adding 200 µl blocking buffer (4%) and incubated for 2 hours at room temperature. Repeat washing with PBS and Tween 20 then PBS only, and then 100ul mixture of (3ul first antibody with 3ml (1%) blocking buffer was added and incubated for 2 hours at room temperature. Repeat washing with PBS and Tween 20 then PBS only. 100 ul mixture of (1ul) of 2nd antibody and 3ml (1%) blocking buffer was added and incubate for 2 hours. Repeat washing with 100 ul of substrate buffer and p-Nitro phenyl phosphate disodium salt tablets. Then use ELISA reader at 405 nm.

Detection of tox A using PCR

The PCR test was carried out on the base of the 16S rRNA genome, and genetic characterization was identified of the genes with virulence factors for P.aeruginosa,specific primers were developed using NCBI- GenBank and Primer3 for detection of tox A virulence factor gene [14].

Gently vortex and briefly centrifuge DreamTaq Green PCR Master Mix (2X) after thawing.

Place a thin-walled PCR tube on ice and add the following components for each 25 µl reaction:

DreamTaq Green PCR Master Mix (2X)	12.5 µl
Forward primer	1 µl
Reverse primer	1 µl

Template DNA	2 µl
Water, nuclease-free	8.5 µl
Total volume	25 µl

Gently vortex the samples and spin down.

Results and Discussion

Isolation and identification of bacteria from different spoiled food samples

It is very interesting to show the ubiquity of microbes in food samples. In this study,different food samples were investigated for bacterial isolation and identification.

Biochemical tests performed that there 8 Gram- positive bacteria and others are Gram- negative. Organisms were isolated on selective media such as mannitol salt agar which used to identify Staphylococcus aureus which ferment mannitol while Staphylococcus epidermidis not ferment mannitol. Also, MacConkey agar was used for identification of Salmonella which is non-lactosefermenter. Cetrimide and King's B media used to identify Pseudomonas aeruginosa which act as selective media for this type of bacteria. Among the various isolated pathogens Pseudomonas is predominant.

Isolate 4, 5,6, 8, 9, 15, 20 and 23 are Gram+ve bacteria

Isolate 1, 2,3, 7,10, 11,12,13,14, 16, 17, 18, 19, 21, 22, 24, 25 and 26 are Gram-ve bacteria

Our results for isolation of Pseudomonas aeruginosa on MaConkey, blood agar and King's B media [15,16]where, King's B media used for faster diagnosis of Pseudomonaspp. and for enumeration of greenish pigments produced by Pseudomonas aeruginosa.

Biochemical tests

Table 1,2 shown the selective media used for biochemical identification of bacterial isolates.

Table 1: Selective and differential media for isolated organisms.

No. of isolated organisms	Gram+ve	Gram-ve	Selective medium	MacConkey agar	Cetrimide and King's B medium
1	**-**	**√**	MacConkey-Sorbitol Agar	**√**	**-**
2	**-**	**√**	Xylose Lysine Deoxycholate agar	**√**	**-**
3	**-**	**√**	Cetramide and King's B medium	**√**	**√**
4	**√**	**-**	Mannitol salt agar	**-**	**-**
5	**√**	**-**	LB and Pepton yeast agar	**-**	**-**

6	**√**	**-**	Cereus Selective Agar Base	**-**	**-**
7	**-**	**√**	LB and blood agar	**√**	**-**
8	**√**	**-**	Mannitol salt agar	**-**	**-**
9	**√**	**-**	Lactobacillus selective media (LBS)	**√**	**-**
10	**-**	**√**	Cetrimide and King's B media	**√**	**√**
11	**-**	**√**	Cetrimide	**-**	**√**
12	**-**	**√**	MacConkey sorbitol agar		
13	**-**	**√**	Cetrimide and King's B media	**√**	**√**
14	**-**	**√**	Xylose Lysine Dexycholate agar	**√**	**-**
15	**√**	**-**	Starch nitrate media	**-**	**-**
16	**-**	**√**	Cetrimide and King's B media	**√**	**√**
17	**-**	**√**	Xanthomonas selective media	**√**	**-**
18	**-**	**√**	Cetrimide and King's B media	**√**	**√**
19	**-**	**√**	Minimal agar media	**√**	**-**
20	**√**	**-**	LB and peptone yeast agar	**-**	**-**
21	**-**	**√**	Cetrimide and King's B media	**√**	**√**
22	**-**	**√**	MacConkey	**√**	**-**

			sorbitol agar		
23	**√**	-	LB and peptone yeast agar	-	-
24	-	**√**	MacConkey sorbitol agar	**√**	-
25	-	**√**	Cetrimide and King's B media	**√**	**√**
26	-	**√**	Xylose Lysine Dexycholate agar	**√**	-

Table 2: Biochemical tests for bacterial isolates.

No. of isolate	Catalase	Starch hydrolysis	Oxidase	Urease	Carbohydrate fermentation
1	+ve	-ve	-ve	-ve	Glucose ,lactose, maltose and sucrose
2	+ve	+ve	-ve	-ve	Glucose and mannitol
3	+ve	-ve	+ve	-ve	Glucose, fructose and mannitol
4	+ve	-ve	-ve	+ve	Glucose, lactose, sucrose and mannitol
5	+ve	+ve	+ve	-ve	Glucose, sucrose, mannitol and xylose
6	+ve	+ve	-ve	-ve	Sucrose, mannitol and xylose
7	+ve	-ve	+ve	ve+	Sucrose, glucose, maltose and dextrose
8	+ve	-ve	-ve	+ve	Sucrose and maltose
9	-ve	+ve	-ve	-ve	Sucrose, glucose, ribose and galactose
10	+ve	-ve	+ve	-ve	Glucose, fructose and mannitol

11	+ve	-ve	-ve	+ve	Glucose, fructose and mannitol
12	+ve	-ve	-ve	-ve	Glucose ,lactose, maltose and sucrose
13	+ve	-ve	+ve	-ve	Glucose, fructose and mannitol
14	+ve	+ve	-ve	-ve	Glucose and mannitol
15	+ve	+ve	-ve	+ve	Sucrose, mannitol fructoseand
16	+ve	-ve	+ve	-ve	Glucose, fructose and mannitol
17	+ve	+ve	-ve	-ve	Sucrose, Fructose and mannitol
18	+ve	-ve	+ve	-ve	Glucose, fructose and mannitol
19	+ve	+ve	-ve	-ve	Glucose, lactose and mannitol
20	+ve	+ve	+ve	-ve	Glucose, sucrose, mannitol and xylose
21	+ve	-ve	+ve	-ve	Glucose, fructose and mannitol
22	+ve	-ve	-ve	-ve	Glucose ,lactose, maltose and sucrose
23	+ve	+ve	+ve	-ve	Glucose, sucrose, mannitol and xylose
24	+ve	-ve	-ve	-ve	Glucose ,lactose, maltose and sucrose
25	+ve	-ve	+ve	-ve	Glucose, fructose and mannitol

26	+ve	+ve	-ve	-ve	Glucose and mannitol

Selection of the strongest organism for toxin production using well diffusion method:

The results in **Table 3** showed that from the isolates obtained from different food samples, isolate no.3 was the strongestone which inhibit all other isolates and give the highest inhibition zone (53 mm).

Table 3: Measurement the inhibition zone of the isolated samples.

No. of sample	Inhibition zone diameter in (mm)
1	14
2	19
3	53
4	25
5	32
6	-ve
7	6
8	27
9	18
10	49
11	43
12	12
13	42
14	17
15	9
16	51
17	11
18	37
19	20
20	34
21	45
22	30
23	15
24	12
25	41
26	22

Molecular identification of isolate no. (3)PCR

PCR for isolate 3 was done using 16S rRNA gene showed in **Fig. 1**

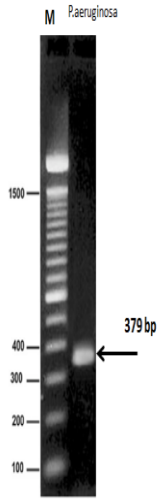


Figure1: Gel electrophoresis of 16S rRNA for Pseudomonas aeruginosa Lane M, Marker and Lane 3, Pseudomonas aeruginosa .

Sequencing

The results of morphological and physiological identification was confirmed by the sequencing using 16S rRNA gene which revealed that the organismis Pseudomonas aeruginosa using BLAST analysis in GenBank. The organism was approved by its accession number **MN 215468**, **Fig.2** showed the phylogenetic tree of organism on NCBI.

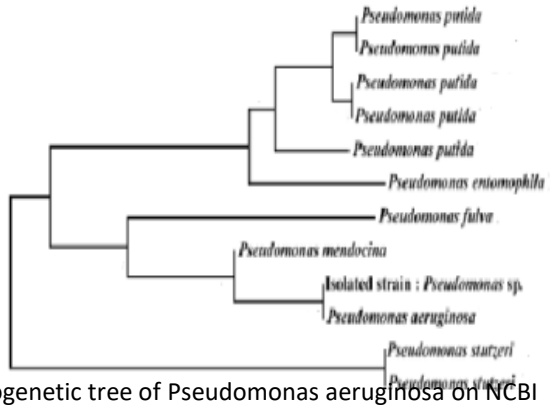


Figure 2: Phylogenetic tree of Pseudomonas aeruginosa on NCBI

Production of pyocyanin pigments,Ability to grow at 42°C and protease test

Only Pseudomonas spp. colonies, which inoculated on cetrimide agar for 24 hours at 37°C, were able to produce pyocyanin pigments.Pseudomonasspp cangrow at 42°C temperature. Also, Pseudomonas spp. isolates have the mildproteolytic activity and weakly produce protease enzyme.

Pseudomonas spp. isolates give positive results to protease enzyme;all isolates of Pseudomonas aeuginosa have proteolytic enzymes and have apr A gene which encoded to alkaline protease[17].Pseudomonas aeruginosa samples that have negative results with protease enzymes used for production of toxin A and give highly killing rate in mice where exotoxins destroyed by protease activity[18, 19].

All Pseudomonas aeruginosa produce pyocyanin pigment (blue green, non fluorescent pigment) when inoculated on King's B media. Sixty three isolates belonging to Pseudomonas isolated from different sources

able to produce pyocyanin pigment which act as a unique test for identification of Pseudomonas isolates[20].All Pseudomonas aeruginosa samples produce pyocyanin pigments that increased by toluene addition [21].

Extraction of toxin A from Pseudomonas aeruginosa

Dialyzed trypticase soy broth with supplements (glycerol and monosodium glutamate) act as powerful conditions to produce toxin A and increase killing rate when injected to mice.

Toxin A extracted from Pseudomonas aeruginosa one of the most virulence factors of this bacteria. Exotoxin A (PE), which has enzymatic activity and belongs to the mono-ADP-ribosyltransferase family [22] ·Pseudomonas aeruginosa act as causative agent of root softness especially in carrot, potatoes and celery. The most feature of organisms result in softening the plant tissue by secreting enzymes[23].

Toxin A was produced from Pseudomonas aeruginosa samples isolated from various food samples on tryptic soy dialysate enhanced with 1% glycerol in a shaker for 24 hours, which increased toxin A production and Pseudomonas growth. Trypticase soy agar media gave optimal toxin A synthesis [24].Toxin production In tryptic soy dialysate with 1% glycerol, A increased in a shaking rather than a static situation[25, 10].

Ammonium sulphate precipitation:

Different percentage of saturation range from 20% to80% was used[26]to increase toxin concentration When injected into mice, the concentration of ammonium sulphate was proven to increase the precipitation of toxin as well as the cytotoxicity.The 70% percent give the highest concentration of toxin A. Using ammonium sulphate is common method for protein precipitation due to high solubility and stability. Our results, showed that toxin A saturated at 60-75 % and give highest concentration of toxin A and maximum toxicity in mice. Both 60% and 70% of ammonium sulphate give highest concentration of toxin A [22,27].

Ion exchange and Gel filtration chromatography:

Fig.3demonstrated the two significant steps in the purification of exotoxin A: (A) revealed that following elution with a gradient concentration of sodium chloride and measuring the absorbance at 280 nm, two protein peaks will appear. In the first peak, the protein fraction ranges from 12 to 14, and in the second peak, it ranges from 20 to 24. Because both of the proteins in the two peaks were harmful to mice, they were collected and concentrated. After purifying exotoxin A by ion exchange chromatography with Sepharose 6B, which earlier equilibrated with (0.2) M Tris – OH buffer, pH 8, the next stage in the purification of exotoxin A was to use gel filtration chromatography. The results of gel filtration revealed that on peak was obtained from elution of toxin A that injected to mice showing cytotoxicity effect.

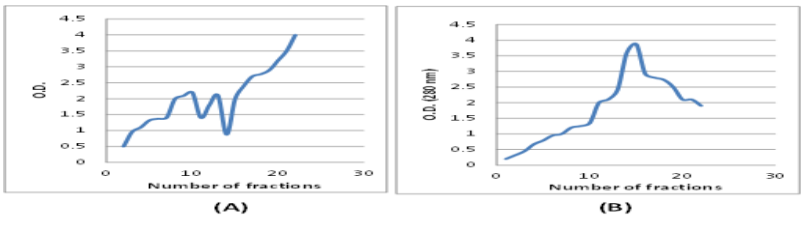


Figure 3: (A) Ion exchange chromatography using DEAE cellulose column and (B) Gel filtration chromatograpy

Using ion exchange chromatography with a gradient concentration of sodium chloride and measuring absorbance at 280 nm, a highly pure toxin was produced with two protein peaks. 0.01M elution in ion exchange chromatography first when a gradient concentration of NaCl is added to a Tris OH buffer, two peaks emerge that are very lethal when injected into mice[28,10]. Anther difficult method used to obtain purified toxin A with DNA fragment of Pseudomonas aeruginosa exotoxin to be cloned and expressed in E.Coli[28, 29].The fractions produced from ion exchange chromatography collected and applied to sepharose 6B and results showed one peak only from elution of toxin where toxin collected and concentrated in the fraction and

injected in mice showed protein applied to sepharose column and peak appeared after measuring fractions at 280nm. Peak 1 nontoxic but peak 2 contain toxin A and was toxic to mice when injected [25].

SDS –PAGE

Protein (toxin A) was analyzed by SDS-PAGErevealed high level expression of recombinant toxin A (PE protein) which appeared as a 66 KDa protein as single purified band usingCoomassie blue staining after induction samples, as shown in **Fig.4.**

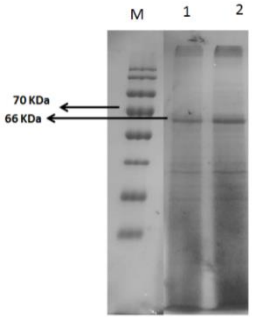
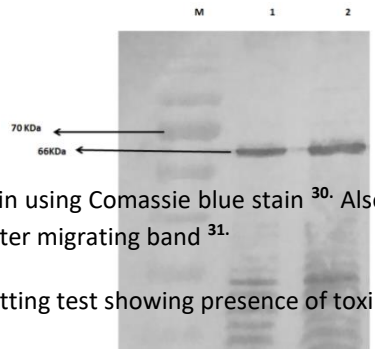


Figure 4: SDS-PAGE of toxin A that appeared at 66KDa, lane 1 and 2 are exotoxin A

Western blotting test:

Western blotting testwith anti-exotoxin A antibody (Sigma- serum of miceand universal antibody (Sigma-Immuno chemicals, antimouse IgG) showed in **Fig.5** confirmed the identity of toxinA andobserved at 66 KDa. The results showed that protein analyzed using SDS-PAGE appeared as single



band at 66 KDa protein using Comassie blue stain [30]. Also, 10%SDS gels showed single band or one major band and one very faint faster migrating band [31].

Figure 5: Western blotting test showing presence of toxin A band at 66 KDa lane 1 and 2 are exotoxin A

Application for extracted antibody (Sample collection)

According to the morphological appearance and staining 26 samples there are 17 identified the same characterization of Pseudomonas aeruginosa, and from the 17 samples only 12 samples have toxAgene. Indirect ELISA and PCR performed to confirm results.

In this study, Western blotting test showed that toxin A produced from Pseudomonas aeruginosa using Sigma-serum of mice and universal antibody where toxin A observed at 66KDa and with agreement with previous research which showed that Western blot analysis of serum samples against components of toxin A[32]. All antigens were typically observed and toxin A appeared at 66 KDa using Western blottingfor detection of pure toxin A which observed at 60 KDa [33].

Indirect ELISA :

Indirect ELISA results showed that only 12 samples gave positive results after 10 minutes using ELISA reader at 405nm.

The ELISA results which used for diagnosis the sensitivity of isolated antibodies toward the samples used showed that 12 samples give positive results as showed by PCR results that indicated the ELISA offers quick

and accurate results, highly sensitive, simple to performed and easily automated. Using of ELISA to establish an enzyme related monoclonal immunosorbent assay (ELISA),

for the quantification of raw meat products with porcine hemoglobin (PHb).Therefore, this immunoassay coul d measure PHb effectively in laboratories [34].Immunocapture ELISA (ICE) used for specific detection and quantification of Mycoplasma capricolum subsp. capripneumoniae antigen in the effective contagious Caprine pleuropneumoniae vaccine (used to increase productivity of healthy goats in cost effective manner),comparison between PCR and ICE gives good correlation between the two assays [35].

ScFvs consists of different regions of heavy and light chains. Where naive mousephage library with a view to generating scFvs against Cry1Ab toxin. In this study,positive phage ScFvs were isolated, sequenced and characterized by ELISA. New haptens have been engineered and synthesiz ed to generate antibodies to free histamine, but none has resulted inthe production of appropriate antibodies for ELISA [36].

The antisterium was nevertheless developed as a result of the reaction between the histamin and p- nitrobenzoic acid N-
hydroxysuccinimide ester (PNBAOSu)under mild conditions, having a high speciality and interaction with pnitro benzoic histamine (NPHA).A competitive indirect ELISA (ciELISA) was developed on the basis of rabbit polyclon al anticorps to assess histamine in milk [37].

A newly developed enzyme linked immunosorbant assay (ELISA)to detect anticorruptions against P. multocida dermonecrotic toxin were
designed to promote the regulation of progressive atrophic rhinitis (PAR) by pigs caused by toxic Pasteurella m ultocida[38].

ELISA can also be used to test the immune response in pigs after P. multocida toxoid vaccination. So, nowadays ELISA is considered to be the gold standard for immunoassay which act as very active test for detection and quantitative study of antibodies,antigens, hormones,enzymes and glycoproteins [39].

PCR to detect toxA gene

The presence or absence of toxA gene in P.aeruginosastrains showed through electrophoresis gel in **Fig.6,** where 12 strains only from 17 (70.5%)have tox A and 5 samples give negative results.

The results showed that tox A present in 12 isolates from 26 samples of Pseudomonas aeruginosa using gel electrophoresis, these results are in agreement with anther research[40]that showed tox A gene found in 72% of Pseudomonas aeruginosa gel electrophoresis showed that Mwt of toxA gene was 352 bp.

Among 200 Pseudomonas aeruginosa samples isolated from different farms in luxor government 142 isolates (71.42%) have different virulence genes such as toxA, exoS,lasB and las I for each of them [41,42].Pseudomonas disease and infections become more common over time, providing serious public health problems around world. As a result, a number of methods for detecting Pseudomonas pathogen quickly have been developed, as it is necessary in many food analyses. Nucleic acid-based, biosensor-based, and immunological-based methods are the three types of rapid detection techniques.The new strategies detect those Pseudomonas aerginosa toxins similar to most bacterial toxins that easily performed using ELISA technique [43, 44].

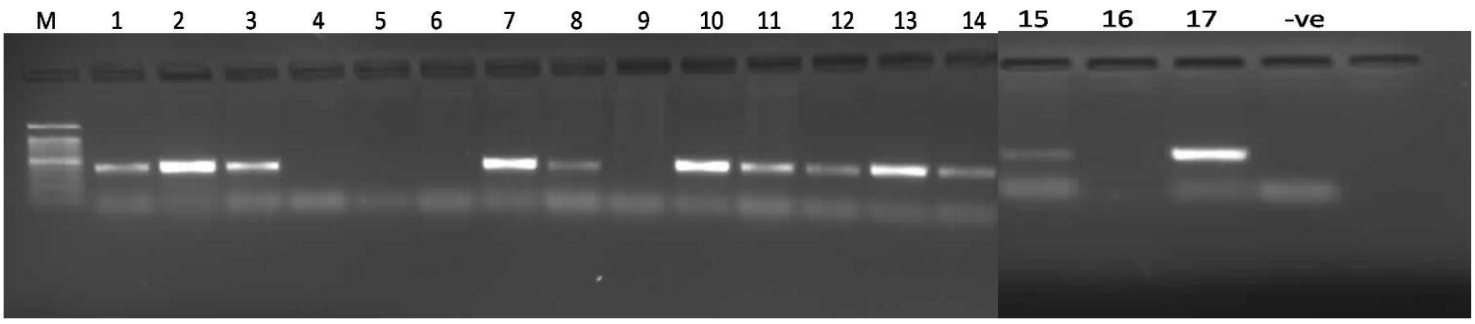


Figure6: The typical amplicon of the gene tox is visible on an agarose gel electrophoresis (1.5%). Isolates of P. aeruginosa produce this substance. A ladder-like pattern occurs when DNA is amplified. Lane (M) is a 100-bp DNA marker, and Lanes (1, 2, 3, 7, 8, 10, 11, 12, 13, 14, 15, and 17) are positive isolates. Negative isolates (lanes 4, 5, 6, 9, and 16)

Conclusion:

We produce and purify exotoxin A that injected to laboratory animals. Polyclonal antibodies produced against exotoxin A of Pseudomonas aeruginosa was performed using ELISAand Western blotting. Our results provide auseful data for designing a control strategy against exotoxin A.

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Author Contributions:

D. Mary Sobhy and D. Reda Salem choice the main topic and developed the theory and performed the computations.D. Mahsen Ismail gives her advice and help to design the experiments. D.Mary took the lead in writing the manuscript and conceived the study and was in charge of overall direction and planning.Doaa Zahran carried out the experiments. All authors discussed the results and contributed to the final manuscript.

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