Extraction and purification of alpha- hemolysin which producing by *Staphylococcus aureus* isolated from different Clinical samples.

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الخلاصة

جمعت أربعمائة وستون عينه مرضيه للفترة من الأول من حزيران 2003 الى نهاية مايس 2004 وجد إن 163 مرضيه كانت بسبب المكورات العنقودية الذهبية وكانت كالأتي : الإدرار 52 عزلة ، مسحات الجروح 38 عزلة، مسحات الإذن 21 عزلة، مسحات اللوزتين 17 عزلة ، مسحات الخراج 11 عزلة بالإضافة إلى 24 عزلة قد تم جمعها من مسحات الأنف. تم التحري عن قابلية عزلات المكورات العنقودية الذهبية على إنتاج الهيمولايسين بواسطة مقايسة الحالة الدموية ووجد إن 70 عزلة شكلت نسبة 42% منتجه للهيمولايسين واستخلص الهيمولايسين من مرشح السائل للعزلة المنتجة المختبرة بالنبذ المركزي المبرد وتم تنقيته بالترسيب بكبريتات الامونيوم والترشيح بالهلام كما حددت فعالية الهيمولايسين إذ كانت فعاليته 5260 وحده تحلل/مل . تم تحدي درجه حرارة الحضن المثلى وتأثيرها على فعالية الهيمولايسين ووجد إن الهيمولايسين ذو فعاليه في مدى حراري يتراوح بين 20-40 فقد كانت الحضانة المثلى فقد كانت 14 ساعة والأس الهيدروجيني الأمثل لفعالية الهيمولايسين هو 7. ددت سمية الهيمولايسين المنتج من قبل المكورات العنقودية الذهبية على خلايا الدم البيضاء للإنسان إذ وجد إن هنالك تناقص في عيوشية هذه الخلايا إلى 38% د معاملتها بتركيز 15 ملغم/مل بعد مرور 30 دقيقه فيما انعدمت عند تركيز 30ملغم مل بعد مرور 30 دقيقه أيضا.

Summary

Four hindered seventy six clinical samples were collected from patients since June 2003 to May 2004 the number of *Staphylococcus aureus* isolated from the samples were 163 isolates as follows : urine 52 isolates , wound swab 38 isolates , ear swab 21 isolates , tonsillar swab 17 isolates. Boil swab 11 isolates and Nasal swab 24 isolates Alpha hemolysin *of Staphylococcus aureus* isolates was designated by hemolysin assay it was found that 42% (70) of isolates were alpha- hemolysin producers. The extraction of alpha- hemolysin from liquid media from producing isolates was done by cooling centrifugation and purified by ammonium sulphate

precipitation and gel - filtration chromatography. Activities of purified alpha-hemolysin were detected, it had 2560 HU/ml the optimum temperature of alpha - hemolysin activity was 20 - 40 C and the optimum incubation period was 14hr. The optimum pH of alpha-hemolysin activity was 7. Cytotoxic activity of alpha - hemolysin against human leukocytes were determined and found the survival ratio of human leukocytes was decreased (38%) after treatment with 15 mg/ml for 30 min. while it lost after treatment with 30 mg/ml for 30 min. too.

Introduction

Staphylococcus aureus have been reported as the causative agents many diseases, it's causes a variety of supportive(pus-forming) infections and toxinose in human, it cause superficial skin lesions such as boils ,sties and furnculosis; more serious infections such as pneumonia , mastitis, phlebitis ,meningitis and urinary tract infections and deep-seated infections such as osteomyelitis and endocarditis. *S. aureus* is a major cause of hospital acquired (nosocomial) infections, of surgical wounds and infections associated with indwelling medical devices.[1,2,3] *S.aureus* csuses food poisoning by releasing enterotoxines into food and toxic shock syndrome by release of superantigens into the blood stream.[3,4]

The importance of *Staphylococcus aureus* was increased since it produces a variety of toxins and other virulent factors which plays a significant role in its pathogenesis.[4]

A among theses virulence factors was production of hemolysin and the best characrized and most potent membrane damaging toxin of *Staphylococcus aureus* is alpha toxin.[4]

Its expressed as a monomer that binds to the membrane of susceptible cells Subunits then oligomerize to form heptametrical rings which a central pore through which cellular contents leak.[5] In human , platelets and monocytes are particularly sensitive to alpha – toxin . Susceptible cell have a specific receptor for alpha- toxin which allows the toxin to bind causing small pores through which monovalent cations can pass. The mode of action of alpha – hemolysin is likely by osmatic

lyses.[6,7,8]. The aim of this study was undertaken due to the production of hemolysin by local of *Staphylococcus aureus* isolates and investigate some of the characteristics of alpha-hemolysin.

Materials and methods

Source of *Staphylococcus aureus*

This study deal with the isolation and identification of *Staphylococcus aureus* bacteria from Al- Nassiria hospital since June 2004 to May 2005, which includes the collection of different clinical samples obtained from 476 patients as follows , urine 115,wound swab 111,ear swab 81,tonsillar swab 67 , boil swab 55 , and nasal swab 47 .

Culture media

The samples were cultured on blood agar and nutrient agar, then incubated at 37 C for 24 hrs. The isolated bacteria were identified by using many different biochemical tests to be *Staphylococcus aureus* while the others were neglected. Brain heart infusion broth was used to extraction and purification of alpha – hemolysin after incubated on tryptone soya agar at 37 C for 18 hrs. [9]

Detection of hemolytic strain

According to [10] and after incubated of isolates on brain heart infusion broth at 37C for 18 hrs.The supernatant were collected in sterile test tube by cooling centrifuge 7000\ min. for 15 min. then hemolysin assay were done for each sample to detected the producer isolates by using rabbit blood .Protein concentration for each sample was measured according to [11].

Detection of *Staphylococcus aureus* isolate to this study

According to hemolysin assay (hemolytic activity) and protein concentration, one strain of Staphylococcus *aureus* was chosen to study alpha-hemolysin.

Extraction and purification of *Staphylococcus aureus* alpha-hemolysin

The Extraction and purification were accomplished by procedure which includes: cooling centrifuge, ammonium sulphate precipitation, gil-filtration chromatography (sephadex G-100) [12].

Effect of incubation temperature on hemolysin activity

Purified hemolysin was taken place in three different temperature (30, 37, 40) C were used for obtain the optimum temperature for hemolysin activity; it was determined from correlation between temperature and hemolysin activity.

Effect of incubation period on hemolysin activity

Purified hemolysin was incubated at 37C for (2, 4, 6, 8, 10, 12, 18, 24) hrs. Samples were assayed for hemolysin activity for each incubation period.In order to obtain optimum pH for hemolysin activity different pH values of were used (2,4,6,8,10,12) added to purified α–hemolytic then incubated for one hour at 37 C and assayed for activity the optimum pH was determined from the relation between the pH and hemolysin activity.

Alpha-hemolysin effect on white blood cells of human

Cytotoxic effect of α-hemolysin on leucocytes of human was done according (Cech and Lehrer 1984) [13] and the effect of hemolysin on Leukocytes (viable cells) according to (Nonomama et al,1979).[14]

Results

Source of *Staphylococcus aureus* isolates

Staphyloccus aureus isolates were collected from different clinical samples obtaind from 476 patients and found 163 cases were caused by *Staphylococcus aureus* as follows UTI 52 isolates; wound swab 38 isolates;ear swab 21 isolates ; tonsilar

swab 17 isolates ; boil swab 11 isolates; and nasal swab 24 isolates . Table (1).

Table (1):No. of S. *aureus* isolates from different clinical samples.

Type of samples	No. of total samples	No.of *S. aureus* isolates	Percentage
urine	115	52	45.2
Wound swab	111	38	34.2
Ear swab	81	21	25.9
Tonsilar swab	67	17	25.3
Boil swab	55	11	20.0
Nasal swab	47	24	51.0
Total	476	163	34.2

Assay for hemolytic activity

Alpha-hemolysin of *Staphylococcus aureus* isolates was designated by hemolysis assay it was found that 42.0% (70) of isolates were hemolysin producers as follows UTI 22(42.3%) isolates; wound infection 18(47.3%) isolates; otitis media 7 (33.3%) isolates; tonsilitis9(52.9%) isolates ;boil infection 2 (18.18%) isolates; and nasal infection 12(50.0%) isolates .Table (2) .

Table (2): Relationship between hemolytic strain of *S. aureus* and the source of isolates

Source of samples	No.of *S. aureus* isolates	No. of alpha hemolytic strain	Percentage
urine	52	22	42.3
Wound swab	38	18	47.3
Ear swab	21	7	33.3
Tonsilar swab	17	9	52.9
Boil swab	11	2	18.1
Nasal swab	24	12	50.0
Total	163	70	42.9

Extraction and purification of *Staphylococcus aureus* alpha- hemolysin

Alpha – hemolysin were extracted and purified by cooling centrifugation. Precipitation by ammonium sulphate and Gil – filtration (sephadex G 100). Table (3) shown the hemolytic activity by steps: crude hemolysin precipitation by ammonium sulphate and gel- filtration as follows 320, 640, 2560 respectively. The specific activity of crude hemolysin was 18.4 while the specific activity of alpha hemolysin after precipipitetion with ammonium sulphate and Gil filtration were 290.9, 8258 respectively .Table (3)

Table (3): Activity of alpha-hemolysin during purification steps.

Steps	Protein concentration	Hemolytic activity(HU/ml)	Specific activity
Crude hemolysin	17.3	320	18.4
Precipitation by ammonium sulphate	2.2	640	290.9
Gel filtration by (Sephadex G100)	0.31	2560	8258

Effect of incubation temperature on hemolysin activity

Table (4) shown the relationship between incubation temperature on hemolysin activity of alpha hemolysin, our result show the optimum temperature for higher alpha – hemolysin activity measured by hemolytic activity (HU/ml) was 1280 at (20-40) C.

Table (4): Relationship between incubation temperature and hemolytic activity.

temperature (C)	Hemolytic activity (HU/ml) after				
	15 min.	30 min.	60 min.	90 min.	120 min
20	1280	1280	1280	1280	1280
40	1280	1280	1280	1280	640
60	640	320	320	80	-
80	-	-	-	-	-
100	-	-	-	-	-

120	-	-	-	-	-

(-): non hemolytic activity

Effect of incubation period on hemolysin activity

Fig. (1) Shows that the typical incubation period of hemolysin activity was 14 hrs. For better hemolysin activity measured by the higher activity 1280 (HU/ml).

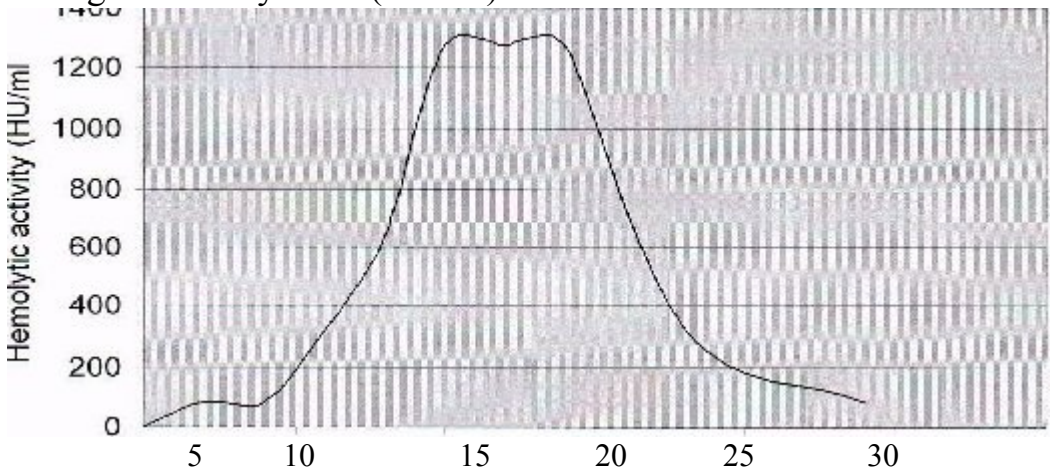
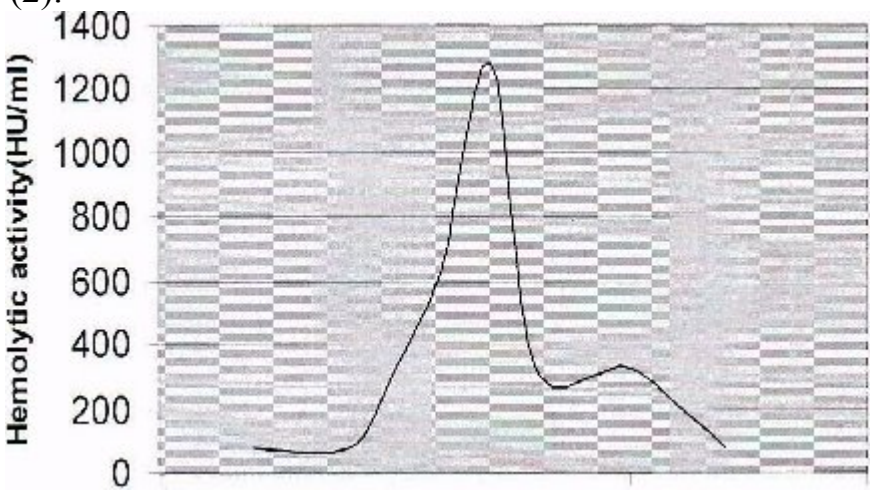


Fig.(1) Relationship between incubation time and hemolytic activity.

Effect of pH values on hemolysin activity

The purified hemolysin was exposed to different pH values for 1 hr. and found the optimum pH for hemolysin activity was 7.0 .Fig (2).



0 5 10 15

Fig. (2):Effect of pH values on hemolysin activity

Effect of alpha – hemolysin on human WBCs

Table (5) reveals inverse relationship between concentration of hemolysin and percentage viability of human WBC upon incubation for various periods of time, and found time required for 50% killing varied inversely with hemolysin concentration.

Table (5): Cytotoxic effect of purified hemolysin on human WBCs

Hemolysin concentration	Percentage of viable WBCs after			
	30 min.	60 min.	90 min.	120 min.
0	90	90	90	90
5	50	44	33	27
10	47	38	30	21
15	38	29	23	17
20	8	-	-	-
25	-	-	-	-
30	-	-	-	-

(-): No viable cells

Discussion

Approximately 30% of the human population is colonized by *Staphylococcus aureus* and certain conditions , this bacterium is capable of causing a range of diseases from superficial skin infections such as boils to serious disorders like endocarditis[1,15]. Our results shown this bacterium was responsible for many diseases such as UTI , wound infections otitis media etc. details were summarized in table (1). This may due to it has many virulence factors ,in additionally the bacterium has become a particularly important pathogen in hospital environment as up to 90% of health care worker carry *Staphylococcus aureus* and contact patients susceptible to infection- elderly or immunocompromized patients or these with an upon wound or catheter entering their body [15] .The exotoxin .alpha- hemolysin is an important virulence factor for the *Staphylococcus aureus* and many strain of this bacterium produce alpha-

hemolysin, the toxin production begins at the end of logarithmic growth but 80% of toxin is produced during stationary phase [16].

The percentage of the local hemolytic *Staphylococcus aureus* isolates (42.9%) in the present work is slightly higher than that reported by [9]. This may due to it has many virulence factors the role of alpha hemolysin in disease was mentioned in many reports [17, 18 ,19] they mentioned the organism is capable of infecting virtually every type of human tissue or organ and the toxicity of alpha-hemolysin is dependent upon the site of infection in localized lesions, the toxin will cause tissue death while in septicemic infections , lethal amounts of the toxin are produced while other research also suggests the Staphylococcal virulence factors work synergistically : alpha-hemolysin can permeabilize the membrane and allow entry of other enzymes that destroy normal host cell metabolism[19] . this result go with [9]. *Staphylococcus aureus* was originally cultured in meat infusion broth; this did not produce large amounts of alpha-hemolysin [19]. Extraction and purification of *Staphylococcus aureus* alpha-hemolysin was done by cooling centrifuge, precipitation by ammonium sulphate in (20- 40) % saturation and gel-filtration by sephadex G100 this produce a large amounts of alpha-hemolysin and high activities of purified hemolysin when compare with crude hemolysin. [20]. Our results shown hemolytic activity and specific activity of purified hemolysin was in the last step (gel-filtration by sephadex G 100) 2560 and 8258 respectively, this result in agreement with [9].When some factors affecting activity of alpha-hemolysin were studied results showed the optimum temperature was 20-40 C , this result go with [21], Fig. (1) Shown the Relationship between incubation time and hemolytic activity of alpha-hemolysin, and found the optimum incubation period were 12 hrs. Measured by higher hemolytic activity (1280 HU/ml), this result go with [16] .because he mentioned the toxin production begins at the end of logarithmic growth phase. The optimum pH for hemolysin activity was 7 when the purified hemolysin exposed to different pH values for one hour and our results in agreement with other reports [22, 23]. Cytotoxicity of alpha-

hemolysin had also been done for human peripheral leukocytes and found our result in agreement with [12, 21].When compare the results of the present work and those of others, we found that the cytotoxic activity of alpha-hemolysin various with the source of isolates and source of cells.

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