EXTRACTION AND PURIFICATION OF ALPHA- HEMOLYSIN WHICH IS PRODUCING BY *STAPHYLOCOCCUS AUREUS* ISOLATED FROM DIFFERENT CLINICAL SAMPLES[+]

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Abstract :

Four hundered seventy six clinical samples were collected from patients since June 2003 to May 2004 .The number of *Staphylococcus aureus* isolated from the samples was 163 isolates as follows : urine 52 isolates , wound swab 38 isolates , ear swab 21 isolates , tonsil 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 was lost after treatment with 30 mg/ml for30 min. too.

المستخلص :

جمعت أربعمائة وست وسبعون عينة مرضية للفترة من الأول من حزيران ٢٠٠٣ إلى نهاية مايس ٢٠٠٤ وجد إن ١٦٣ حالة مرضية كانت بسبب المكورات العنقودية الذهبية وكانت كالأتي : الإدرار ٥٢ عزلة ، مسحات الجروح ٣٨ عزلة، مسحات الإذن ٢١ عزلة، مسحات اللوزتين ١٧ عزلة ، مسحات الخراج ١١ عزلة بالإضافة إلى ٢٤ عزلة قد تم جمعها من مسحات الأنف.

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تم التحري عن قابلية عزلات المكورات العنقودية الذهبية على إنتاج الهيمولايسين بواسطة مقايسة الحالة الدموية ووجد إن ٧٠ عزلة شكلت نسبة ٤٢% كانت منتجة للهيمولايسين واستخلص الهيمولايسين من المزروع السائل للعزلة المنتجة المختارة بالنبذ المركزي المبرد وتم تنقيته بالترسيب بكبريتات الامونيوم والترشيح بالهلام كما حددت فعالية الهيمولايسين إذ كانت فعاليته ٥٢٦٠ وحدة تحلل/مل . تم تحدي درجة حرارة الحضن المثلى وتأثيرها على فعالية الهيمولايسين ووجد إن الهيمولايسين ذو فعالية في مدى حراري يتراوح بين ٢٠-٤٠ م. أما فترة الحضانة المثلى فقد كالاتى ١٤ ساعة والأس الهيدروجيني الأمثل لفعالية الهيمولايسين هو ٧. حددت سمية الهيمولايسين المنتج من قبل المكورات العنقودية الذهبية على خلايا الدم البيضاء للإنسان إذ وجد إن هنالك تناقص في عيوشية هذه الخلايا إلى ٣٨% عند معاملتها بتركيز ١٥ ملغم/مل بعد مرور ٣٠ دقيقه فيما انعدمت عند تركيز ٣٠ ملغم /مل بعد مرور ٣٠ دقيقة أيضا.

Introduction:

Staphylococcus aureus have been reported as the causative agents of many diseases, it causes a variety of supportive (pus-forming) infections and toxins in humans,it causes 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 endocarditic. *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* casuses 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 play a significant role in its pathogenesis.[4].Among theses virulence factors is production of hemolysin and the best is characrized and most potent membrane damaging toxin of *Staphylococcus aureus* is alpha toxin.[4].It is expressed as a monomer that binds the membrane of susceptible cells subunits then oligomerize to form heptametrical rings which a central pore through which cellular contents leak.[5] In humans, platelets and monocytes are particularly sensitive to alpha–toxin .Susceptible cells 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 somatic lyses.[6,7,8].This study was undertaken to producte of hemolysin from local of Staphylococcus aureus isolates and investigate some of the characteristics of alpha- hemolysin .

Materials and methods

Source of *staphylococcus aureus:*

This study deals 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, and 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 for 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 Kumar & lindorfer**,** [10] and after incubating of isolates on brain heart infusion broth at 37C for 18 hrs.The supernatants were collected in sterile test tube by cooling centrifuge 7000\ min. for 15 min. then hemolysin assays were done for each sample to detect the producer isolates by using rabbit blood .Protein concentration for each sample was measured according to Damon & Wuepper, [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 took place at three different temperatures (30, 37, 40) C to 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 (2,4,6,8,10,12)was 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 verified 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 *Staphyloccus aureus* isolates:

Staphyloccus aureus isolates were collected from different clinical samples obtaind from 476 patients and found 163 cases were caused by *Staphyloccus aureus* as follows UTI 52 isolates; wound swab 38 isolates ; ear swab 21 isolates ; tonsil swab 17 isolates ; boil swab 11 isolates; and nasal swab 24 isolates . Table (1).

Table (1): No. of *Staph. 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 *Staphyloccus 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 Staph. 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 was extracted and purified by cooling centrifugation, precipitation by using ammonium sulphate and gil –filtration (sephadex G 100) . Table (3) shows 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 was 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) shows the relationship between incubation temperature on hemolysin activity of alpha hemolysin, our results 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).

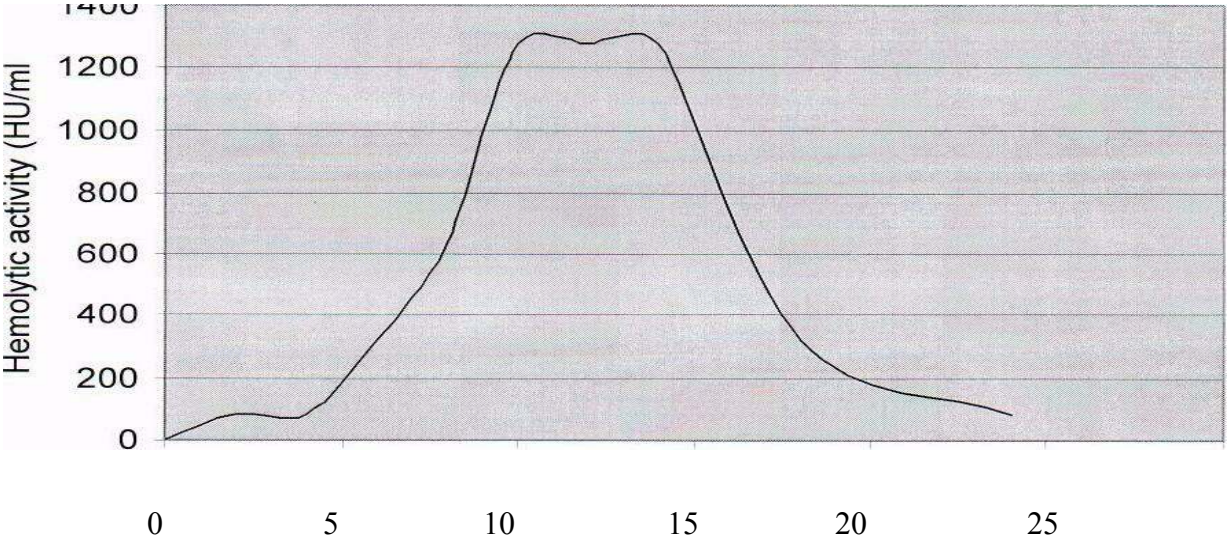

Fig.(1) : Relationship between incubation time and hemolytic activity.

Effect of pH values on hemolysin activity:

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

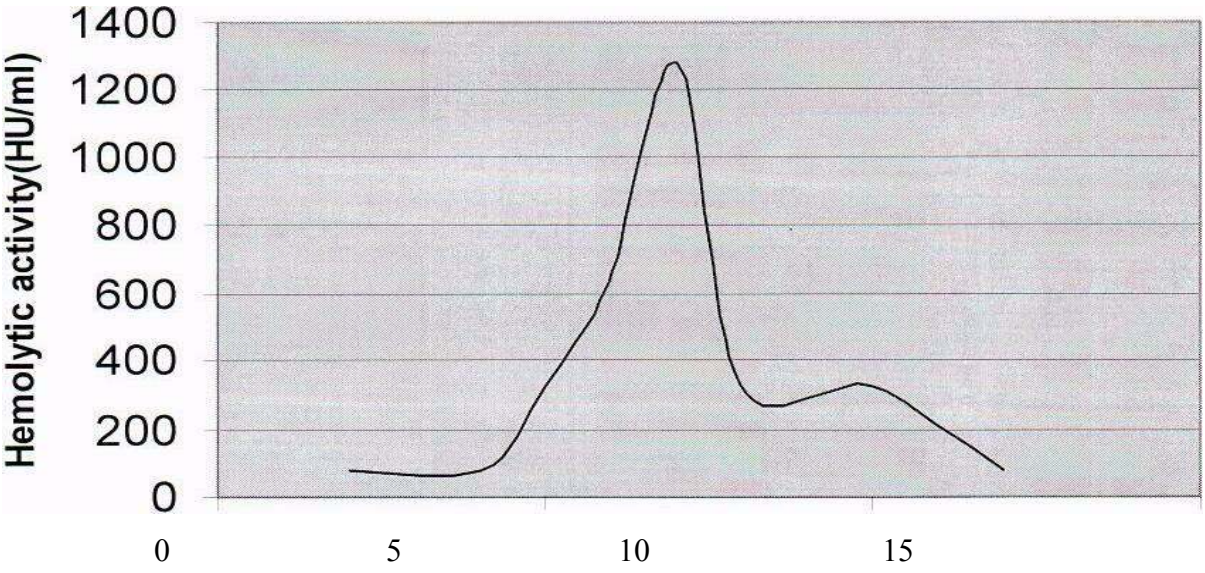

Fig.(2): Effect of pH values on hemolysin activity

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 it was 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 W B Cs 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 under 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 shows this bacterium is responsible for many diseases such as UTI , wound infections otitis media etc. details are summarized in table (1). This may be because to it has many virulence factors ,in additionally the bacterium has become a particularly important pathogen in hospital environments as up to 90% of health care workers carry *Staphylococcus aureus* and contact patients susceptible to infection:elderly or immuno-compromized patients or these with an opon wound or catheter entering their body [15] .

The exotoxin .alpha- hemolysin is an important virulence factor for the *Staphylococcus aureus* and many strains 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 AL.Ibadi, [9]. This may because to it has many virulence factors.The role of alpha hemolysin in disease is mentioned in many reports [17, 18 ,19] they mention 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 researchers also suggest 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 goes with AL.Ibadi,s [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 produced a large amounts of alpha-hemolysin and high activities of purified hemolysin when compare with crude hemolysin. [20]. Our results shows hemolytic activity and specific activity of purified hemolysin is in the last step (gel-filtration by sephadex G 100) 2560 and 8258 respectively, this result is in agreement with AL.Ibadi,s [9].

When some factors affecting activity of alpha-hemolysin were studied results showed the optimum temperature was 20-40 C , this result go with Bernheimer and LiL results [21], Fig. (1) shows the relationship between incubation time and hemolytic activity of alpha-hemolysin, and it is found the optimum incubation period is 12 hrs. Measured by higher hemolytic activity (1280 HU/ml), this result goes with Duncan and Cho,s[16]. because they mention the toxin production begins at the end of logarithmic growth phase. The optimum pH for hemolysin activity is 7 when the purified hemolysin is exposed to different pH values for one hour and our results are in agreement with other reports [22,23].

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

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