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RESEARCH ARTICLE

Studying Some Activities of Crude Alpha-Hemolysin Extracted from Staphylococcus aureus.

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Abstract

Crude alpha-hemolysin was produced by Staphylococcus aureus and it was extracted from the same bacteria which was isolated from clinical samples collected from skin tissue infection and sepsis.the hemolytic activity of crude alpha-hemolysin against rabbit erythrocytes was determined. The culture supernatant was treated with ammonium sulfate (75% saturation)and also hemolytic activity after addition of ammonium sulfate was measured. Skin test was determined against rabbit to detect its ability to induce delayed type hypersensitivity sensitivity and it gave positive results in compare with control and lethal toxin activity was tested in mice to reveal its ability to induce infection symptoms and mortility after 48 hour and 18 hour. so this study proved that crude alpha-hemolysin possess properties enable it to behavior as antigen and to be effective virulence factor in S. aureus.

Introduction:-

Staphylococcus aureusis a Gram-positive bacterium that often colonizes the human nares and the skin, in spite of being a commensal, S. aureusis cosidered as human pathogen thatresponsible for a variety of severe diseases(Lowy,1998). To set up a successful infection, S. aureus evolved an amazing variety of immune evasive strategies wiping out both innate and adaptive immune responses(do Vale et al.,2016).S. aureus is a dangerous pathogen that causes a variety of severe diseases because it possess large repertoire of virulence factors, among which secreted toxins play a preeminent role many of these toxins damage biological membranes, leading to cell deathespecially the potent hemolysins and leukotoxins (Otto,2014).Alpha-hemolysin is probably the best-known toxin of S. aureus(Berube and Bubeck Wardenburg,2013) and the first identified example of the beta-barrel forming toxins, which predominantly consist of beta sheets. It is lytic to red blood cells and a series of leukocytes, but not neutrophils (Valeva et al.,1997). It has 293 amino acids in length and forms a heptameric pore that leads to the efflux of monovalent and, at higher concentration, divalent ions. At higher concentrations, pore formation may be receptor-independent, but pore formation at lower concentrations has recently been shown to be dependent on the interaction with the ADAM10 receptor(Inoshima et al,2011). Alpha -hemolysin may play an important role in the pathogenesis of human disease, now primarily supported through two lines of evidence. First, carriers of *S. aureus* or individuals suffering from *S.* aureus disease develop serum antibody responses to the toxin consistent with toxin expression (Kolata et al.,2011; Adhikari et al.,2012; Fritz et al.,2013).

S. aureus produces two coagulases, staphylocoagulase and von Willebrand factor (vWF), which contribute to the formation of fibrin clots after binding to prothromb in (forming a complex called staphylothrombin) and several other plasma proteins, thereby triggering the conversion of fibrinogen to fibrin (Thomer et al.,2013). This leads to fibrin clots on the surface of S. aureus cells, inhibiting phagocytosis, causing abscess formation(Cheng et al.,2010). Toxin-deficient *S. aureus* strains display a virulence defect in a mouse model of lethal sepsis induced by intravenous inoculation of the pathogen; this correlates with toxin-mediated induction of endovascular injury and increased vascular permeability, as documented by dye extravasation studies (Powers et al.,2012).this study aims to extract the crued of Alpha -hemolysin from S. aureus in order to study some of its properties like hemolytic activity and

studying its ability to induce delayed type hypersensitivity sensitivity in immunized rabbits and also to prove its lethal activity in mice to corellate these results with S. aureus pathogenicity and to open the door to further studies in this field.

Materials and Methods:-

Bacterial isolatesamples were taken from 200 patients who were attending to Merjan Hospital , Babil,Iraq.they were sufferring from skin and sepsis infection ,during a period form March 2015-January 2016.only 30 sample gave Staphylococcus aureus bacteria positive results. Staphylococcus aureus was detected by morphological features of colony on mannitol salt agar and blood agar and doing all biochemical tests required for this bacteria (Collee et al., 1996).

only one isolate which given alpha- hemolysin and coagulase positive test was choosen to complete this study.bacterial ability to produce alpha -Hemolysin was detected on blood agar contain rabbit erythrocytes and bacterial ability to produce coagulase was detected by slide method(Bubeck Wardenburg et al.,2007).

Hemolysin production was detected by streaking bacteria on blood agar contain rabbit erythrocytes after 24 hour incubation aerobically the result revealed as clear zone around colony(Baron et al. ,1994).

Hemolytic activity against rabbit erythrocytes was determined by measurement of 50% hemolysis at 541 m in spectrophotometer. Equal volumes of 1 % red blood cell suspensions in phosphate buffer (pH 6.9) and doubling dilutions of toxin were incubated for 30 min at 37°C . and the optical density of 1 ml of the supernatant was determined. The hemolytic unit was defined as the reciprocal of the final diluition producing 50% hemolysis, when compared to a prepared standard (Lind et al.,1987) .

Preparation of crude toxin. Stock preparations of Staphylococcus aureus Cultivation was performed aerobically using a 4% inoculum from an exponentially growing proculture in 100 ml of medium (tryptic soya broth, Difco, 30 g/liter, pH 7.2) in 0.5liter indented Erlenmeyer flasks on a rotary shaker (120 rpm) at 37°C. At the times indicated growth was determined by measuring the absorbance of an appropriately diluted sample at 578 nm against the growth medium as a blank. The alpha-toxin content was assayed in the supernatant following centrifugation using the hemolytic titer. After 18 h the bacteria were harvested by centrifugation at 4°C (20 min at 16,000g). Solid ammonium sulfate was added (75%saturation) to the supernatant, which was kept for 2 h in a cold room. Theprecipitate formed was collected by centrifugation at 4°C (15 min at 16,000g) and storedfrozen as a stock of crude toxin (Lind et al.,1987).

Laboratory animal:-

Rabbits : In this study we use 9 local rabbits(*Oryctologus cuniculus*) males,their age between (8-9) months and weighing between (2-3.5) kg were kept in animal house of biology department,Babylon university .

Mice: White Swiss mice (6 weeks old) were used in thelethal activity test their wheight between (30-35) g were kept in animal house of biology department,Babylon university

Skin test

In this test 0.5 ml from crude toxin was injectedintradermally into each of three rabbit while animal control were injected with normal saline to see the result of this test(Tompkins *et al*.,1973) .

Lethal activity was measured by the intraperitoneal injection of 0.5 ml samples of doublingdilutions of toxin into groups of three mice. The end point was considered to be the final dilution which killed the entire group within 18 h. (Manohar et al.,1966).

Results:-

The value of Staphylococcus aureus was evaluated with the number of isolates contain coagulase and hemolysin in table(1)

Table(1)types of isolated microorganism.

samples	Number(%)	coagulase +	Hemolysin +
Staphylococcus aureus	**30(15)**	**20**	**18**
Other microorganism	**170(85)**	**-**	**-**
total	**200(100)**		

The hemolytic activity after crude toxin was detected and also after addition of solid ammonium sulfate was added with 75% saturation in table(2).

Table(2)crude toxin with its hemolytic activity

Purification steps	**Hemolytic activity(unit/ml)**
crude toxinsupernatant	320
crude toxinprecipitate	640

The effect of crude toxin was detected as seen in table (3) in three groups of rabbits by skin test which describe by erthyma ,induration diameter(cm) and necrosis.

Table (3) delayed type hypersensitivity sensitivity test in immunized rabbits.

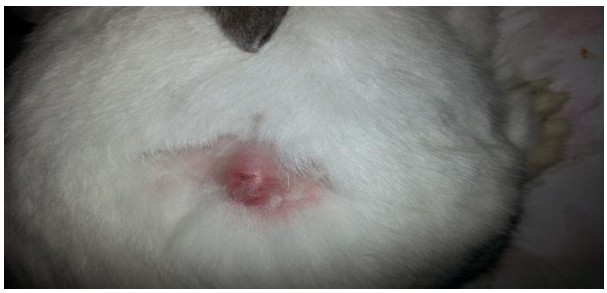
time	**4 h.**			**24 h.**			**48 h.**			**72 h.**		
	E	**ID**	**N**	**E**	**ID**	**N**	**E**	**ID**	**N**	**E**	**ID**	**N**
crude toxinsupernatant	+	_	_	+	**1.8**	_	+	**2.7**	+	_	**2.4**	_
	+	_	_	+	**1.9**	_	**--**	**3**	_	_	**3.1**	_
	+	_	_	+	**2**	_	+	**2**	+	_	**1.5**	+
crude toxinprecipitate	+	_	_	+	**1.9**	_	**--**	**2.5**	+	_	**2**	+
	+	_	_	+	**1.8**	_	+	**2.5**	+	_	**1.7**	+
	+	_	_	+	**1.9**	_	+	**3**	+	_	**2**	+
Control	_	_	_	_	_	_	_	_	_	_	_	_

E :Erthyma

ID:Induration diameter in(cm)

N:necrosis

Figure (1) below showed the effect of Crude alpha-hemolysin in rabbit's skin after 72 h.



Figuer (1) The effect of Crude alpha-hemolysin in rabbit's skin after 72 h.

Some changes could be seen in mice after injection with crude toxin to finshed with mice death table(4) revealed these results.

Table (4) The parameter that used to determine the infection.

Parameters	**Group1**	**Group2**	**Group3(control)**
dose	crude toxinsupernatant	crude toxinprecipitate	Normal saline
Volume/ ml	0.5	0.5	0.5
fever	-	-	-
inactivity	+	+	-
Loss of appetite	+	+	-
chill	-	+	-
Wheight loss	+	+	-
Mortality 100%	Occur after 48 h.	Occur after 18 h.	-

Discussion:-

Actual study referred to isolation of S. aureus bacteria from clinical samples these bacteria have two of important virulence factor .in this study we focused on alpha hemolysin to study some of its characters when alpha hemolysin is crued andprecipitated with ammonium sulfate. the crued of alpha hemolysin was extracted,hemolytic activity was measured to give 320(unit/ml)before addition of ammonium sulfate and 640(unit/ml) after addition other studies

also measured hemolytic activity in this steps and after purification(Kumar & Lindorfer,1962;Manohar et al.,1966; Lind et al.,1987) and to study its ability to induce delayed type hypersensitivity in rabbit skin and we can see that it gave positive results in comparesion with control this agree with (Berche *et al*.,1987) who found the role of exotoxin in the process of T cell activation wasstudied in vivo during the course of an experimental infection in the mouse. By usinghighly purified listeriolysin O, it was found that infection with viable, replicativebacteria induced in vivo the emergence of T cells specifically reacting against thisexotoxin, as demonstrated by eliciting the expression of delayed-type hypersensitivityto listeriolysin O in *Listeria*-immune mice .also thelethal activity was studyed in mice and revealed mortility in all mice this agree with other studies(Kumar & Lindorfer,1962;Manohar et al.,1966). so this lead us to conclude there is a role to alpha hemolysin in induce infection in bacterial isolate containing it.

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