Extraction and Studying The Effect of Ph And Temperature on Hemolysin Production by A Local Isolates of *Staphylococcus Aureus*

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Abstract

Background: *Staphylococcus aureus* is a ubiquitous bacterium that is generating increasingly bad press coverage due to its propensity to adopt a pathogenic lifestyle in hospital and community settings. *S. aureus* colonies are found in approximately 30% of the general population. It colonizes the skin readily and can lead to a wide range of pathological conditions from skin lesions to osteomyelitis, endocarditis, and septicemia. Many bacteria produce substances that are cytolysins i.e they dissolve red blood cells (hemolysins) or kill tissue cells or leukocytes (leukocidins). The β-toxin degrades sphingomyelin and therefore is toxic for many kinds of cells, including human red blood cells. Hemolysins were extracted and the optimum conditions for their production were extensively studied including optimum pH, temperature, incubation period, and various manipulations of the culture media.

Objectives

1-Extraction of hemolysin from a local isolate of *S. aureus*.

2-Studying the effects of pH and temperature on hemolysin production.

Methods: Bacterial samples were identified by subjecting them to the standard laboratory procedures while semi quantitative screening on blood agar (containing 5% human blood) revealed that all isolates were hemolysin producer but in different efficiencies. Determination of the optimal conditions for hemolysin production including the optimum pH and temperature were also performed.

Results: Bacterial samples were identified by subjecting them to the standard laboratory procedures and the results showed that forty isolates out of the total of 100 were identified as *Staphylococcus aureus*. Semi quantitative screening on blood agar (containing 5% human blood) revealed that all isolates were hemolysin producer but in different efficiencies. Depending on the semi-quantitative screening and hemolytic assays isolate SW-14 of *Staphylococcus aureus* was the higher hemolysin producing isolate. Determination of the optimal conditions for hemolysin production including the optimum pH and temperature were performed, the results demonstrated that the best hemolysin production was in the pH near neutrality (pH 7-7.5) and in temperature of 35-40^{o}C.

Conclusions

1.Conventional methods can be performed to extract hemolysins.

2.Hemolysin was maximally produced when the pH was near neutrality and incubation temperature was 37^{o}C and this conclusion indicates that hemolysin was produced when the conditions were similar to that of the host.

Key words: Hemolysin, semi-quatitative screening, pH, temperature

الخلاصه

موطنه: تعتبر بكتريا المكورات العنقوديه من انواع البكتريا الشائعة الانتشار وتحضي باهتمام متزايد وذلك لامراضيتها المعروفه في البيئه الاعتياديه وفي اجواء المستشفيات. توجد الهيملايسينات والسموم المحلله لانواع الخلايا في انواع

مختلفه من الكائنات الحيه. تمتاز هذه السموم (الانزيمات) وظيفيا بقابليتها على تحلل خلايا الدم الحمراء وارتباطها بخاصية الضراوه للعديد من الكائنات الحيه. يقوم الهيمولايسين من نوع بيتا بتحطيم السفنكومايلين لذلك يتعبر ساما للعديد من انواع الخلايا بضمنها خلايا الدم الحمراء استخلاص ودراسة تاثير الرقم الهيدروجيني ودرجة الحراره على انتاج الهيمولايسين من عزله محليه من بكتريا تنقية المكورات العنقوديه الذهبيه

الهدف :

1- استخلاص انزيم الهيمولايسين المنتج من عزله محليه من بكتريا المكورات العنقوديه الذهبيه.

2- دراسة تاثير الرقم الهيدروجيني ودرجة الحراره على انتاج الهيمولايسين.

طريقة العمل : جمعت 100 عينة من مناطق وآفات مختلفة من الجسم (إلتهابات المجاري البولية, الجروح, مسحات من الأذن...إلخ) من المرضى الراقدين والمترددين إلى مستشفى الكاظمية التعليمي في بغداد ومن كلا الجنسين للفترة من تشرين الثاني/2010 وحتى آذار/2011 لعزل وتشخيص بكتريا المكورات العنقودية الذهبية. أستخدمت طريقة الغربلة شبه الكمية على أكار الدم (الحاوي على 5% دم بشري) لتقييم قابلية العزلات على انتاج الهيمولايسين و تم تحديد الظروف المثلى لإنتاج الهيمولايسين والمتضمنة لكل من الرقم الهيدروجيني (pH) والحرارة المثلاوتين.

النتائج شخصت العينات البكتيرية بمعاملتها بالطرق المختبرية القياسية وقد بينت النتائج بأن 40 عزلة من مجموع 100 من العزلات قد شخصت بأنها بكتريا المكورات العنقودية الذهبية. أظهرت نتائج الغربلة شبه الكمية على أكار الدم (الحاوي على 5% دم بشري) بأن كل العزلات كانت منتجة للهيمولايسين ولكن بدرجات متفاوتة. إعتماداً على الغربلة شبه الكمية والفحوص التحللية كانت العزلة SW-14 هي الأكثر إنتاجاً للهيمولايسين ولذلك فقد أُنْتُخِبَتْ ورشحت لعزل وتنقية وتوصيف الهيمولايسين. أظهرت النتائج بأن أفضل إنتاج للهيمولايسين كان في درجة حموضة متعادلة تراوحت بين 7-7.5 ودرجة حرارة مابين 35-40 ^{0}م.

الاستنتاجات:

1-امكانية اسخلاص الهيمولايسين بالطرق التقليديه.

2- ان افضل الظروف لانتاج الهيمولايسين تكون عند الرقم الهيدروجيني المتعادل ودرجه حرارة الحضن الاعتياديه (37 مئويه) مما يدل على اهمية هذا الانزيم كاحد عوامل ضراوة البكتريا والذي يتم اعلى انتاج له في ضروف مماثله لما عليه الامر داخل المضيف.

Introduction

Staphylococcus aureus is a ubiquitous bacterium generating increasingly bad press coverage due to its propensity to adopt a pathogenic lifestyle in hospital and community settings [(1)].Hemolysins are cytolytic toxins found in a broad diversity of organisms. They are functionally defined by their ability to lyse erythrocytes and have often been associated with virulence for a variety of pathogenic microorganisms [(2)]. The β-toxin (β-hemolysin) degrades sphingomyelin and therefore is toxic for many kinds of cells, including human red blood cells [(3)].

Material and method

This study included a total of 100 samples which were collected from different body sites and lesions (UTI, wounds, and ear swabs...etc.) of in and out patients from both sexes who attended Al-Kadhumyia teaching Hospital in Baghdad during the period from November-2010 until March-2011. All specimens were immediately streaked onto Nutrient Agar (NA), MacConkey (MA) & Blood Agar (BA) and incubated at 37˚C for 24hours. The resulted growing colonies were Gram stained and were inoculated again onto Mannitol Salt Agar (MSA)this medium was used for the identification of pathogenic staphylococci, and the bacteria were identified according to the laboratory standard techniques relying on biochemical tests and identification was verified using API test. Semi-quantitative screening for Haemolysin production was performed by streaking blood agar medium with bacterial isolate and incubation at 37^{o}C for 24-48 hrs.

Hemolysin assay: Hemolysin was assayed by two methods, the first was performed according to the method applied by Al-Karkhi [(4)], and it was done by using micro-t iteration plates and the hemolytic Unit (HU)/ ml was defined as reciprocal of the highest titer giving hemolysis × 10. The second method used to Estimate hemolysin activity was that of Namdari & Bottone [(5)]

and hemolysin activity was reflected as the amount of hemoglobin produced and detected spectrophotometrically at 540 nm. The method of Biuret was applied to estimate the protein concentration [6,7].

Determination of the optimum pH and temperaturefor hemolysin production

The optimum pH for hemolysin production was determined by preparing 9 screw cup universals with 10 ml of brain heart infusion broth (BHIB) inside each, with different pH 5-9. The universals were inoculated from the chosen *Staphylococcus aureus* isolate (SW-14) and incubated at 37^{o}C for 24 hours. For the determination of the optimum temperature for hemolysin production, BHIBwas inoculated with the hemolysin producing isolate (SW-14) and was incubated with different degrees of temperature (25, 30, 35, 40, 45, and 50^{o}C) for 24 hours.

Results

The results showed that only 40 specimens were identified as *S. aureus*, reflecting a total percentage of 40%. Table 1 shows the ranking of *S. aureus* identified to be the causative agents of each type of the infections mentioned in the same table.

Table 1: Shows the ranking (frequency) of *Staphylococcus aureus* and the percentage of each infection from a total of 40 cases.

Specimens	Frequency	Percentage
wounds(Swabs)	15	37.5%
Ear swabs	5	12.5%
Sputum	8	20%
Urine samples	4	10%
Urethral discharge	2	5%
Vaginal swabs	1	2.5%
Blood samples	5	12.5%
Total (40)	40	100%

Identification was confirmed by API Staph identification kits and only 40 isolates were identified as *S. aureus* and selected for this study (table 2).

Table 2: The biochemical &API Staph system tests performed throughout the study.

Reaction	Result
Catalase	+
Haemolysis	+
Coagulase	+
Mannitol	+
Immune MASTASTAPH kit (slideagglutination test)	+
API Staph system	+

+ = Positive reaction

Estimating the ability of *Staphylococcus aureus* to produce hemolysin.

1. Semi-quantitative screening.

The results demonstrated the appearance of clear zones of hemolysis after the end of incubation period around the growing colonies with different diameters (table 3).

Table 3: Hemolytic zone diameters of hemolysin produced by the local isolates of *Staphylococcus aureus* grown on blood agar after 24 hours of incubation at 37^{o}C.

No. of isolate & its symbol	Diameters of hemolytic zones (mm)
SW-1	14
SW-2	10
SW-3	5
SW-4	9
SW-5	10
SW-6	13
SW-7	9
SW-8	11
SW-9	12
SW-10	10
SW-11	8
SW-12	10
SW-13	12
SW-14	15
SW-15	8
SW-16	10
SW-17	11.5
SW-18	7
SW-19	13
SW-20	10.5
SW-21	5
SW-22	3
SW-23	10
SW-24	9
SW-25	6
SW-26	11
SW-27	13
SW-28	12
SW-29	13
SW-30	10
SW-31	9
SW-32	7
SW-33	13
SW-34	11
SW-35	14
SW-36	10
SW-37	8
SW-38	12
SW-39	14
SW-40	12

2. Hemolysin assay.

Bacterial isolates were examined for their ability to produce hemolysin. The results showed that the highest titer of extract that caused hemolysis in HRBCs within the

microtiteration plate was 1/32, and the hemolytic unit for each ml of the extract was 320 U/ ml (figure 1).

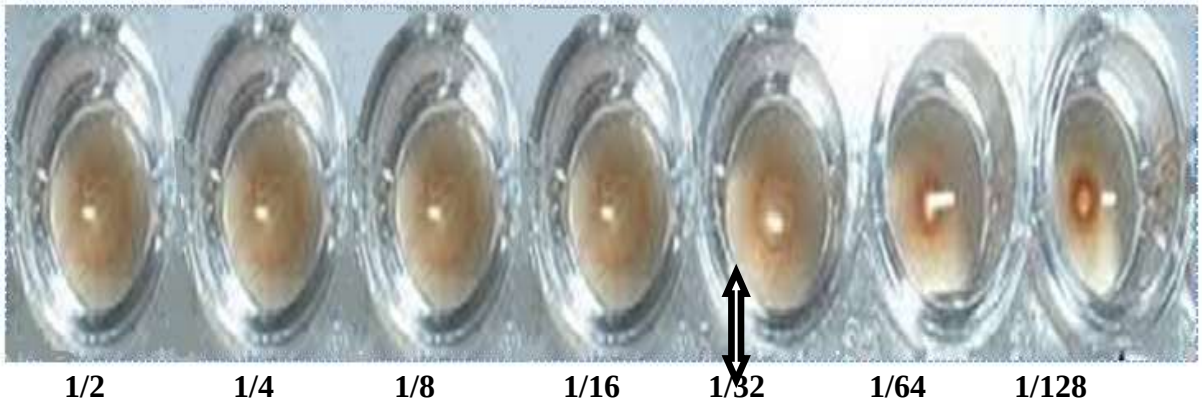

Figure 1: The titer of hemolysin on the microtiteration plate demonstrates the highest titer of the extract that cause hemolysis in HRBCs.

The activity of hemolysin produced by the local isolates enrolled in the current study was also determined as for the isolate No. 14 (table 4).

3. Selection of the highest hemolysin producing local isolate of *Staphylococcus aureus*.

Among the fourty identified pathogenic local isolates of *S. aureus* the highest hemolysin producing isolate was selected depending on the semi-quantitative screening (table 4) and hemolytic assay (table 4) which was the isolate SW-14 demonstrating the highest hemolysin activity (320 U/ml) as compared to the rest of the isolates.

Determining the optimum pH and temperature for hemolysin production.

1.Optimum pH for hemolysin production.

The results indicated that hemolysin production was maximal at pH near neutrality (pH 7) as indicated by an enzyme activity of 3905 U/ml and it was also clear that hemolysin production was significantly reduced as the pH of the medium shifted gradually towards acidity or alkalinity (figure2).

2. Optimum temperature for hemolysin production.

The results in figure 3 showed increasing hemolysin production when temperature of incubation ranged between 35^{o}C - 40^{o}C (3920 and 3732 respectively) which is the ordinary temperature of incubation suitable for almost all types of microorganisms while hemolysin activity decreased as far as the incubation temperature scheduled to change from normal degrees towards increased or decreased temperatures of incubation (figure3).

Discussion

Staphylococci remain among the most important pathogens isolated in the clinical microbiology laboratory [8]. *S. aureus*was superabundant and easy to collect from different body sites and lesions because of the following reasons: its a leading cause of hospital-acquired infections and it is the primary cause of lower respiratory tract infections and surgical site infections [9,10] and the second leading cause of nosocomial bacteremia [11] ; infections with *S. aureus*are especially difficult to treat because of evolved resistance to antimicrobial drugs [12].

Table 4: The activity of hemolysin produced by the local isolates of *Staphylococcus aureus* using the hemolysin assay on the micro-titration plate.

No. of isolate & its symbol	Activity (U/ml)
SW-1	320
SW-2	160
SW-3	40
SW-4	80
SW-5	160
SW-6	160
SW-7	80
SW-8	160
SW-9	160
SW-10	40
SW-11	40
SW-12	80
SW-13	160
SW-14	320
SW-15	160
SW-16	160
SW-17	160
SW-18	80
SW-19	160
SW-20	160
SW-21	40
SW-22	40
SW-23	80
SW-24	160
SW-25	40
SW-26	80
SW-27	160
SW-28	160
SW-29	160
SW-30	160
SW-31	160
SW-32	40
SW-33	160
SW-34	160
SW-35	160
SW-36	160
SW-37	80
SW-38	160
SW-39	320
SW-40	12

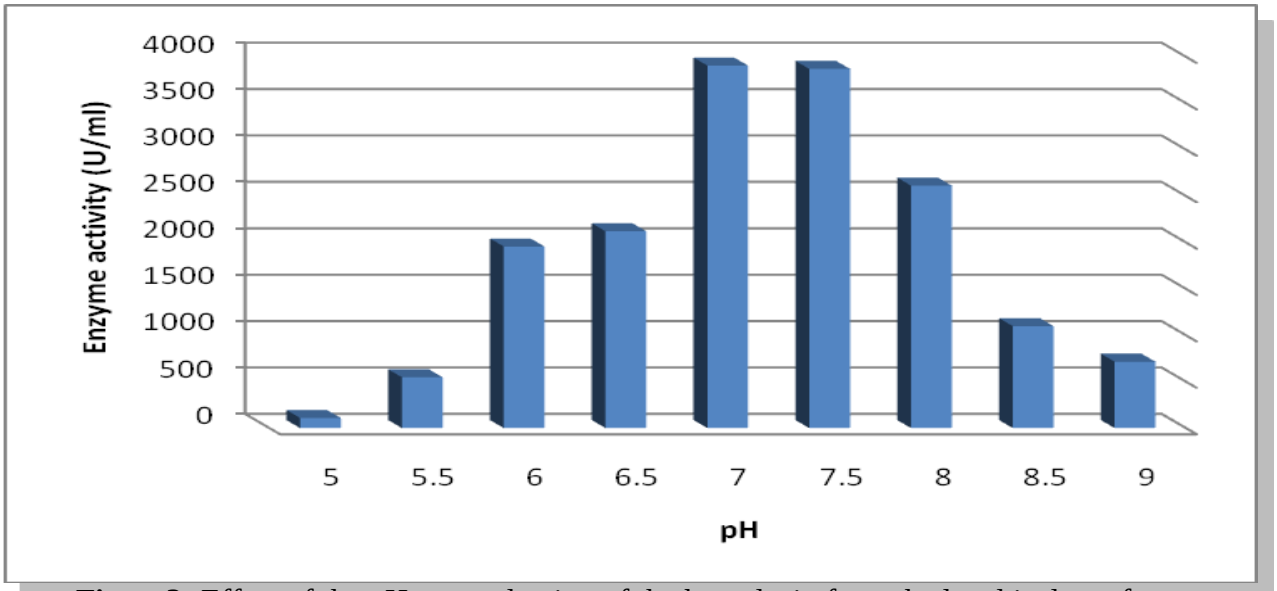

Figure2: Effect of the pH on production of the hemolysin from the local isolate of *Staphylococcus aureus* (SW-14).

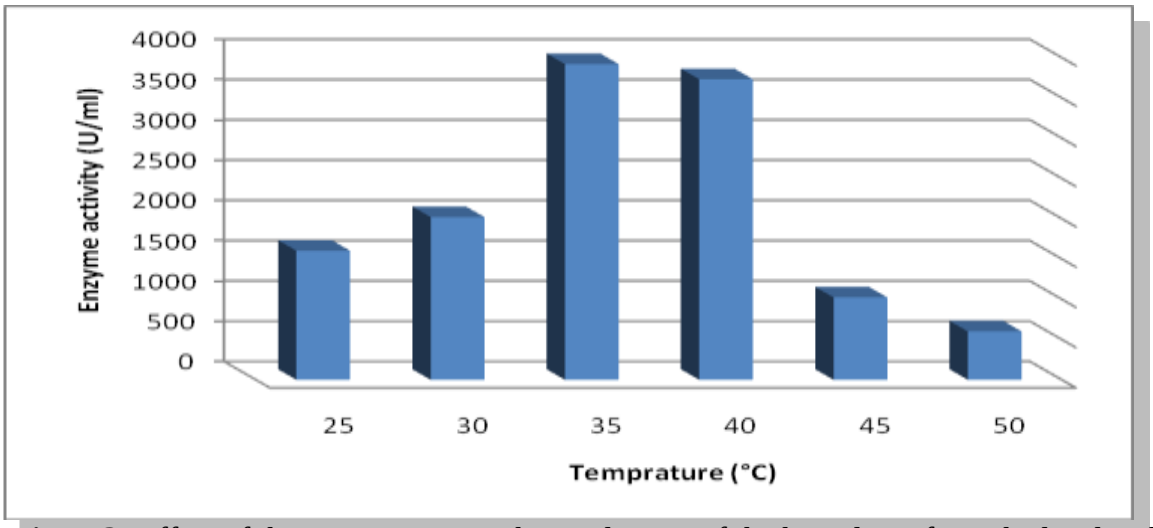

Figure3: Effect of the temperature on the production of the hemolysin from the local isolate of *Staphylococcus aureus* (SW-14).

In this study, data presented in table 1 showed that the percentage of *S. aureus* was 40%. Similar results revealed nearly close percentages of infection with *S. aureus* 27.6% to 67.3% as for that of the current study indicating that this bacterium was a dominant pathogen [13]. While the study of Chen *et al.*, [14] demonstrated a percentage of 58.5%. In the present study wound infections caused by *S. aureus* was the dominant and represented 37.5% and this result was very close to the result of Chen *et al.*, [14] who recorded 41.2%, whereas a clear disagreement to the results of the present study reported by Konvalinka *et al.*, [15]who demonstrated a percentage of 13.5%. In this study, *S. aureus*was isolated from 20%of sputum samples and this result is similar to the 21.8% found by Akortha *et al.*, [16] whereas a marked discording to the results of other researchers 3.8% [17, 18] and 83% [19] was observed.

Many studies had reported an increase in the incidence of hospital acquired *S. aureus* ear infections, and a percentage of 37.5% was reported by Kao & Shiao, [20] and 34.7% by Suh *et al.*, [21]

respectively, while Hwang *et al*., [22] gave 12.2% for ear infection caused by *S. aureus*. In our study we showed that the percentage of *S. aureus* ears infection was 12.5%, similar to many other studies and contradicting others, the results of the current study indicated an increasing problem of community acquired *S. aureus* infections during the period of the study. Urinary tract infection, in the present study, resembled a percentage of 10% of all infections caused by *S. aureus* which was close to the 8.7% found by Shrestha *et al*., [23] while it was little less than the 24.4% of UTIs caused by *S. aureus* reported by Imade *et al*., [24]. In the current study, urethral discharge revealed a percentage of 5% of infections caused by *S. aureus* and this result was approximately close to the 6% found by Weinberger *et al*., [25] while Oboho, [26] found a percentage of 53.7% and suggested that *S. aureus* may be a major cause of non-gonococcal urethritis A percentage of 2.5% of staphylococcal infections were reported, in the present study, when the vaginal swabs were considered revealing a great similarity to the results of Agbakoba *et al*., [27] who reported a percentage of 3.6% of Staphylococcal infections identified from the vaginal swabs while values of 17.1% and 38.7% were found by other researchers Chen *et al*., [28] and Momoh *et al*., [29], respectively. *S. aureus* was isolted from 12.5% of blood samples in the present study and this was approximately similar to 14.5% found by Jain *et al*., [30] and faraway from the 28.7% found by Soltani *et al*., [31]. These differences in the frequency of isolation could be attributed to factors such as the anatomical site of infection, type of lesion, age, and geographical area of isolation [16]. The results of the current study revealed that staphylococcal hemolysin produced a hemolytic zone of 15mm when semi quantitative method of screening for hemolysin was applied and the results also revealed that the hemolysin titer was 320 U/ml when the hemolytic assay was applied, these results were in marked discrepancy when compared to the results of Al-Makhzomi, [32] who reported 6 mm zone of hemolysis around bacterial colonies and a 640 U/ml hemolysin titer. The difference of the results reported by this study to the results reported by some other studies in regard to the hemolytic unit or the zone of hemolysis might be due to the fact that during the course of the purification of beta hemolysin, certain unexpected increases in the titer of the beta hemolysin preparations were noted because the addition polyethylene glycol to the diluent for the titration although it is not known whether the polyethylene glycol affected the erythrocytes or the beta hemolysin [33]. Another unexpected increase in the titer of beta hemolysin occurred during the lyophilization of the acetone-precipitated preparations of beta hemolysin. It is possible that this phenomenon is in some way related to the reported instability of the purified preparations of beta hemolysin [34] or beta hemolysin may have undergone fragmentation during lyophilization due either to freezing or to subsequent desiccation, or to both and the presence of an unknown inhibitor of beta hemolysin has also been suspected [35]. Beta hemolysin, is a "hot-cold" hemolysin; that is, hemolysis is significantly increased if incubation at 37 ^{o}C is followed by a period of holding at a lower temperature. Wiseman, (36) discussed the phenomenon, finding that rapid alteration of pH or NaCl concentration in suspensions of erythrocytes treated with hemolysin caused intensified hemolysis at 37^{o}C. He further suggested that a reduction in temperature may cause sudden contraction of the treated membrane which might break weak bonds and cause the structure to disintegrate Karthikayalu *et al*., [37] reported that the hemolytic activity of the crude extract was 6-mm diameter clear zone of lysis observed around the wells in human blood agar plates when 50 mg of

crude extract was added after incubation. The crude extract revealed 50% hemolysis at a concentration of nearly120 ng/ml by microtiteration plate assay; revealing obvious inconsistency with the results of the present study which might be due to the availability for different concentrations of divalent free cations that are known to have adverse effects on enzyme activity [38].The results of the present study indicated that hemolysin production was maximal when the pH of the medium was near neutrality (pH 7) and this result was different from that of Kreft *et al*., [39] who mentioned that the pH-optimal for hemolytic activity of the toxins produced by Listeria spp. was in the acidic range, this finding further supports the assumption that listeriolysin may play a key role in the escape ofvirulent Listeria from the acidic phagolysosomal environment.In another study conducted by Haider *et al*., [40], working with entero invasive *E. coli*, they showed that hemolysin expression at optimum pH appeared to be a strain specific feature and was found maximum inthe pH range 7.5-8.0 coinciding with the results of Muslim, [41] who reported an optimum pH of 7.5 for hemolysin production by *Aeromonas hydrophila* in TSB ,While another strain-specific study indicated that *B. pertussis* showed maximum activity at 7.5, whereas *B. parapertussis* and *B. bronchiseptica* showed activity at 7.5-8.0. Both studies revealed results that are nearly similar to the result of this study and the slight indifference might be attributed to the involvement of different determinant in host interaction and virulence of various bacterial species and strains within these species [42]. A wider range of temperature of incubation and an increased values of enzyme activity were adopted by Poole and Braun, [43] who claimed that Log-phase cells of *Serratamarcescens* cultured at 30°C were approximately 10-fold more hemolytic than those grown at 37°C and also by Donohue *et al*., [44] who mentioned that *Aspergillus niger* produced a proteinaceous hemolysin when incubated on sheep's blood agar (SBA) at both 23°C and 37°C, furthermore, Haemolysin production occurred at temperatures up to 42^{o}C, but was reduced at 18°Cand below [45]. Al-makhzomi, [32], In an identical results to that of the current study demonstrated that the optimum temperature for hemolysin production by *Vibrio cholerae* was 35-37^{o}C contradicting those of Yamamoto *et al*., [46] who mentioned that the optimum temperature for hemolysin production from other strain of cholera was 30^{o}C.

Study of temperature effect on hemolytic activity confirmed that it is a thermoregulatory phenomenon giving maximum activity at 37°C and decreasing with increases in temperature irrespective of the species. This can be attributed to the fact that virulence genes may be activated at this temperature and remain repressed at very low and high temperatures [47,48].

References

1.Russell, D. G. (2008). Staphylococcus and the Healing Power of Pus. *Cell Host & Microbe*. 3(3): 115-6.

2.Braun, V., and Focareta, T. (1991). Pore-forming bacterial proteinhemolysins (cytolysins). *Crit. Rev. Microbiol*. 18: 115-158.

3.Jawetz, Melnick & Adelberg's (2007). Medical Microbiology. Twenty-fourth edition. McGraw-Hill company, USA. 9: 156-226.

4.Al-Karkhi, Kifah Jasim Ahmed. (2005). Bacteriological and biochemical study of some virulence factors produced by *Vibrio cholera* isolated locally from cholera patients. PhD thesis. College of Science. Al-MustansereyUniversity.

5.Namdari, H., and Bottone, E. J. (1990). Microbiologic evidence supporting the role of Aeromonascaviae as a pediatric enteric pathogen. *J. Clin. Microbiol*. 28:837-840.

6.Koller, A., Kaplan, A., Pesce, A. J., *et al.*, (1984). Total serum protein. Clinical

chemistry. Pp: 1316-1324; 418. The C.V. Mosby Co. St Louis. Toronto. Princeton.

7. Burtis, C. A., and Ashwood, E. R. (1999). Tietz textbook of clinical chemistry. 3rd ed. WB Saunders, Philadelphia.

8. Kloos, W. E., and Bannerman, T. L. (1995). *Staphylococcus* and *Micrococcus*. In Manual of Cinical Microbiology, 6th edition, Pp. 282-298. Edited by Murray, P. R., Baron, E. J., Pfaller, M. A., Tenover, F. C., and Yolker, R. H. Washington, DC: American Society for Microbiology.

9. Richards, M. J., Edwards, J. R., Culver, D. H., and Gaynes, R. P. (1999a). Nosocomial infections in medical intensive care units in the United States. *Crit. Care Med.* 27: 887–92.

10. Richards, M. J., Edwards, J. R., Culver, D. H., and Gaynes, R. P. (1999b). Nosocomial infections in pediatric intensive care units in the United States. *Pediatrics.* 103: e39.

11. Wisplinghoff, H., Bischoff, T., Tallent, S. M., Seifert, H., Wenzel, R. P., and Edmond, M. B. (2004). Nosocomial bloodstream infections in US hospitals: analysis of 24,179 cases from a prospective nationwide surveillance study. *Clin. Infect. Dis.* 39: 309–17.

12. Lowy, F. D. (2003). Antimicrobial resistance: the example of *Staphylococcus aureus*. *J. Clin. Invest.* 111: 1265–73.

13. Dzwonkowska, J., Kurlenda, J., Baczkowski, B., Mazurkiewicz, S., Uzunov, I., Ziółkowski, W., and Markowicz, A. (2007). The effect of antibiotic therapy on the incidence of *Staphylococcus aureus* infections in orthopaedic patients. *Orthop. Traumatol. Rehabil*. 9(5): 532-47.

14. Chen, C.-J., Su, L.-H., Lin, T.-Y., and Huang, Y.-C. (2010). Molecular Analysis of Repeated Methicillin-Resistant *Staphylococcus aureus* Infections in Children. *Plos. One.* 5(12): e14431. doi: 10.1371.

15. Konvalinka A., Errett, L., and Fong, I. W. (2006). Impact of treating *Staphylococcus aureus* nasal carriers on wound infections in cardiac surgery. *Journal of Hospital Infection*. 64(2): 162-168.

16. Akortha, E. E., Aluyi, H. S. A. and Enerijiofi, K. E. (2011). Transfer of amoxicillin resistance gene among bacterial isolates from sputum of pneumonia patients attending the University of Benin Teaching Hospital, Benin city, Nigeria. *Journ. of Medicine and Medical Sciences*: 2(7): 1003–1009.

17. Moine, P., Vercken, J. B., Chevret, S., *et al*. (1994). Clinical investigation in critical care- Severe community acquired pneumonia: Etiology, epidemiology, and prognosis factors. *Chest*. 105:1487-95.

18. Nagalingam, N. A., Adesiyun, A. A., Swanston, W. H., and Bartholomew, M. (2005). A Cross-Sectional Study of Isolates from Sputum Samples from Bacterial Pneumonia Patients in Trinidad. *The Brazilian J. of Infectious Diseases*. 9(3): 231-240.

19. Sparham, P. D., Lobban, D. I., and Speller, D. C. E. (1978). Isolation of *Staphylococcus aureus* from sputum in cystic fibrosis. *J. of Clinical Pathology*. 31: 913-918.

20. Kao, J. C., and Shiao, A. S. (1998). Discharging ear with *Staphylococcus aureus* infection. *J. Taiwan Otolaryngol Head Neck Surg. (in Chinese)*. 33: 8-12.

21. Suh, H. K., Jeon, Y. H., Song, J. S., Hwang, S. J., and Cheong, H. J. (1998). A molecular epidemiologic study of MRSA infection in patients undergoing middle ear surgery. *Eur. Arch. Otorhinolaryngol*. 255: 347-351.

22. Hwang, J.-H., Tsai, H.-Y., Liu, T.-C. (2002). Community-acquired Methicillin-resistant *Staphylococcus aureus* Infections in Discharging Ears. *Tilor & Francis Health Sciences. Acta Otolaryngol*. 122: 827-830.

23. Shrestha, B., Pokhrel, B., and Mohapatra, T. (2009). Study of

nosocomial isolates of *Staphylococcus aureus* with special reference to MRSA in a tertiary care hospital in Nepal. *Nepal Med. Coll. J*. 11(2): 123-126.

24. Imade, P. E., Izekor, P. E., Eghafona, N. O., Enabulele, O. I., and Ophori, E. (2010). Asymptomatic bacteriuria among pregnant women. *Women North Am. J. Med. Sci*. 2: 263-266.
25. Weinberger, M., Cytron, S., Servadio, C., Block, C., Rosenfeld, J. B., and Pitlik, S. L. (1988). Prostatic abscess in the antibiotic era. *Rev. Inf. Dis.* 10(2): 239-249.
26. Oboho, K. O. (1984). Problems of Venereal Disease in Nigeria. 2. *Staphylococcus aureus* as a Possible Cause of Non-Gonococcal Urethritis. *Family Practice.* 1(4): 222-223.
27. Agbakoba, N. R., Adetosoye, A. I., Adewole, I. F. and Chukwuma, C. M. (2008). Isolation of Vaginal Pathogens along with Genital Mycoplasmas from Asymptomatic Gynaecology and Antenatal Clinic Attendees. *American-Eurasian J. of Scientific Research.* 3(2): 195-198.
28. Chen, K. T., Huard, R. C., Della-Latta, P., and Saiman, L. (2006). Prevalence of Methicillin-Sensitive and Methicillin-Resistant *Staphylococcus aureus* in Pregnant Women. *American College of Obstetricians and Gynecologists*. 108(3): 1, 482-487.
29. Momoh, A. R. M., Idonije, B. O., Nwoke, E. O., Osifo, U. C., Okhai, O., Omoroguiwa, A., and Momoh, A.A. (2011). Pathogenic bacteria-a probable cause of primary infertility among couples in Ekpoma. *J. Microbiol. Biotech. Res*. 1(3): 66-71.
30. Jain, A., Agarwal, A., Verma, R. K., Awasthi, S., and Singh, K. P. (2011). Intravenous device associated blood stream staphylococcal infection in paediatric patients. *Indian J. Med. Res.* 134(2): 193-199.
31. Soltani, R., Khalili, H., Rasoolinejad, M., Abdollahi, A., and Gholami, K. (2009). Antimicrobial susceptibility pattern of Staphylococcus aureus strains isolated from hospitalized patients in Tehran, Iran. *Iranian J. of Pharmaceutical Sciences*. 6(2): 125-132.
32. Al-makzomi, E. Y. (2010). Biochemical study of hemolysin enzymes produced from locally isolated bacteria *Vibrio cholerae*. Msc.thesis. Institute of Genetic Engineering and Biotechnology for Postgraduate Studies, Baghdad University.
33. Haque, R.-U., and Baldwin, J. N. (1969). Purification and Properties of Staphylococcal Beta Hemolysin. *J. of Bacteriology*: 100(2): 751-759.
34. Maheswaran, S. K., and Lindorfer, R. K. (1967). Staphylococcal β-hemolysin. II. Phospholipase C activity of purified β-hemolysin. *J. Bacteriol.* 94: 1313-1319.
35. Gow, J. A., and Robinson, J. (1969). Properties of purified staphylococcal β-hemolysin. *J. Bacteriol.* 97: 1026-1032.
36. Wiseman, G. M. (1970). In: Ajl, S. J. *et al*. (ed.), Microbial toxins, a comprehensive treatise, vol. 3, chapt. 6. Academic Press Inc., New York.
37. Karthikayalu, S., Rama, V., Kirubagaran, R., and Venkatesan, R. (2010). Characterization, purification and phylogenetic analysis of a cytolysin from the sea anemone *Heteractis magnifica* of the Indian Ocean. *J. Venom Anim. Toxins incl. Trop. Dis. Vol.* 16 (2): 223-240.
38. Chung, J. J., Ratnapala, L. A., Cooke, I. M., and Yanagihara, A. A. (2001). Partial purification and characterization of a hemolysin (CAH1) from Hawaiian box jellyfish (*Carybdea alata*) venom. *Toxicon.* 39: 981-990.
39. Kreft, J., Funke, D., Haas, A., Lottspeich, F., and Goebel, W. (1989). Production, purification and characterization of hemolysins from *Listeria ivanovii* and *Listeria monocytogenes* Sv4b. *FEMS Microbiology Letters.* 57: 197-202.
40. Haider, K., Albert, M. J., Hossain, A., and Nahar, S. (1991). Contact hemolysin

production by entero invasive *E. coli* and *shigella*. *J. Med. Microbiol.* 35: 330-336.

41. Muslem, S. N. (2005). Biochemical and genetic study of hemolysin produced by Aeromonas hydrophila isolated from diarrheal cases and superficial water. PhD Thesis. College of Science. Al-Mustansiriya.
42. Bodade, R. G., Khobragade, C. N., and Ahemad, N. (2009). Comparative study of hemolytic activity of *Bordetella* species. *Iranian J. of Microbiology:* 1(2): 26-31.
43. Poole, K., and Braun, V. (1988). Influence of Growth Temperature and Lipopolysaccharide on Hemolytic Activity of *Serratia marcescens. J. of Bacteriology*. 170(11): 5146-5152.
44. Donohue, M., Wei, W., Wu, J., Zawia, N. H., Hud, N., De Jesus, V., Schmechel, D., Hettick, J. M., Beezhold, D. H., and Vesper, S. (2006). Characterization of nigerlysin©, hemolysin produced by *Aspergillus niger*, and effect on mouse neuronal cells in vitro. *Toxicology.* 219: 150-155.
45. Lemcke, R. M., and Burrows, M. R. (1982). Studies on A Haemolysin Produced by *Treponema hyodysenteriae*. *Med. Microbiol.* 15: 205-214.
46. Yamamoto, K., Al-Omani, M., Honda, T., Takeda, Y., and Miwatani, T. (1984). Non-O1 *Vibrio cholerae* Hemolysin: Purification, Partial Characterization, and Immunological Relatedness to El Tor Hemolysin. *Infection and Immunity*. 45(1): 192-196.
47. Maurelli, A. T., Blackman, B., and Curtiss, R. (1984). Temperature dependant expression of virulence genes in *Shigela flexeneri*. *Infect. Immunol.* 43: 195-201.
48. Horomockyi, A. E., and Maurelli, A. T. (1989). Identification of shigella invasion gene by isolation of temperature regulated inv: lacz operon fusions. *Infect. Immunol.* 57: 2963-2970.