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Autolysis Activity of Vancomycin Resistance *Staphylococcus epidermidis*

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Abstract: Out of one hundred clinical samples were taken from different sources which include burns, blood cultures, wounds and nasal swabs infections ; 90 isolates developed growth on mannitol salt agar. Among these, 40 (44.4%) were Coagulase positive (*Staphylococcus aureus*) isolates, 50 (55.5%) belong to coagulase negative staphylococci in which *Staphylococcus epidermidis* isolates were 30(60%).The effect of vancomycin resistant on cell autolysis activity of *S. epidermidis* was detected by whole cell autolytic assay . Three isolates of *S. epidermidis* ,vancomycin sensitive (VSSE),vancomycin resistance (VRSE) and vancomycin intermediate (VISE) were tested. The results revealed that was significant difference among three isolates , the VSSE isolate (*S.epidermidis* NO. 22) have the highest autolytic activity in the presence of antibiotic , followed by the VRSE isolate (*S. epidermidis* NO.1) and the VISE isolate (*S. epidermidis* NO.14) which was the lowest autolytic activity with the presence of antibiotic. The result of transmission electron microscope (TEM) showed that the VRSE isolates (*S.epidermidis* NO. 1) have thicker cell wall followed by VISE (*S.epidermidis* NO. 14) isolates .However , the VSSE (*S.epidermidis* NO. 22) didn't showed any cell wall thickening.

Keywords: *S. epidermidis*, vancomycin resistant, autolysin,VRSE ,VISE, VSSE.

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Introduction:

The first reports of vancomycin resistance in coagulase-negative staphylococci were in 1979 and 1983 (1-2). Though a cause for concern, these reports did not generate a great deal of attention as coagulase-negative staphylococci are generally considered to be relatively a virulent organisms(3).

Some reports have pointed out reduced susceptibility of glycopeptides within *S. epidermidis* in various European countries, suggesting that such strains might be highly disseminated in the community and in hospitals(4-6).

Alterations of autolytic activities are always associated with vancomycin resistance in Staphylococci. A slight increased whole cell autolytic activity was observed for the presence of vancomycin in the assay buffer. Reduction in autolytic activity may allow the cell to tolerate vancomycin that might otherwise cause lysis-inducing effects. This might be an important step in the development of vancomycin resistance in *S. aureus* (7-8). A key feature of the resistance mechanism in this mutant appears to be some alternation of cell wall structure such that it allows capture of the glycopeptide molecules at the periphery of the cells distant from sites of cell wall biosynthesis, which thus become protected from the antibiotic(9). This study aims to Detect the presence of Autolysin gene (*aae*) in clinical isolates Vancomycin resistant *S. epidermidis*

from patients in Baghdad by PCR technique and Studying the effect of Vancomycin on bacterial autolysis activity.

Materials and Methods:

Bacterial isolates:

Thirty *S. epidermidis* isolates were isolated from clinical specimens which include burns, blood cultures, wounds and nasal swabs infections from patients attending Baghdad teaching Hospital, Laboratory teaching of Baghdad medical city and AL-kindy teaching Hospital for the period from August 2013 to December 2013. The isolates were identified depending on the morphological features on culture media and biochemical tests according to Bergey's Manual. *S. epidermidis* was identified according to the morphological features on culture medium and biochemical test with the use of API Staph system(10-11). Minimum inhibitory concentrations of vancomycin to thirty *S. epidermidis* isolates were determined. This test was achieved according to Morello *et al.*(12).

DNA Extraction from *S.epidermidis* isolates by using Wizard® Genomic DNA Purification Kit:

1. All *S.epidermidis* isolates were cultured on mannitol salt agar, after incubation for 24 hr. at 37ºC. The bacterial isolates were inoculated in tubes contained 5 ml of sterile brain heart infusion broth and incubated overnight 18 hr. at 37 ºC.
2. One and half ml of bacterial growth was transferred to a 2ml micro centrifuge tubes and centrifuged at 14000g for 2 min, discard the supernatant.
3. Resuspend the pellet with 480µl of 50mM EDTA, then 120 µl of lysozyme was added to the resuspended cells and gently the pipetting to mix, after incubation at 37ºC for 2hr. in water bath, the micro centrifuge tubes were centrifuged for 2 minutes at 14000g and the supernatant was removed.
4. Six hundred ml of Nuclei Lysis Solution was added to the pellet cells and pipetting gently to mix then incubate in water bath at 80ºC for 10 min, allow to cool at room temperature.
5. Three ml of RNase solution was added to the cell lysate in microfuge tubes were inverted gently to mix, incubated in water bath for 60 min. at 37ºC.
6. Two hundred of Protein Precipitation Solution was added to the RNase treated cell lysate, Vortex vigorously at high speed for 20 sec. incubate the microfuge tubes in ice bath for 10 min. then centrifugation at 14000, for 3 min
7. Transfer the supernatant to new clean 1.5 microfuge tubes containing 600 µl of room temperature isopropanol, gently mix by inversion until the thread-like strands of DNA form a visible mass, centrifuged the tubes at 14000g for 2 min.
8. The supernatant carefully poured off and the tubes drained on clean absorbent paper, Room temperature 70% ethanol was added to wash the DNA pellet, centrifuged the tubes at 14000g for 2 min. and the ethanol was aspirated.
9. DNA Rehydration Solution 100µl was added to the DNA in the microfuge tubes and rehydrate through incubation at room temperature overnight.
10. The DNA was stored in -20ºC.

The DNA concentration and purity:

The DNA concentration and purity were determined by using Nano drop instrument from ACT gene (China). The quality of the extracted DNA was checked by 0.8 % agarose gel electrophoresis.

Polymerase Chain Reaction (PCR) Technique:

Primers used in present study listed in (Table 1), these primers were provided in a lyophilized form, dissolved in sterile distilled water to give a final concentration of 10 pmol / µL and stored in deep freezer until used in PCR amplification.

Table (1): The primers and their sequences used in conventional PCR for detection of *S. epidermidis*.

Gene	Primer Name	Sequence 5' ⟶ 3'	Size bp	References
aae	**F**	GAG GAG GAT TTT AAA GTG C	858	Heilmann *et al*.(2003) Gazzola and Cocconcelli (2008**)**
	R	AAC ATG ACC ATA GTA ACC-	858	

PCR Amplification:

The extracted DNA, primers and PCR premix (Bionner), were thawed at 4°C, vortex and centrifuged briefly to bring the contents to the bottom of the tubes.PCR mixture was set up in a total volume of 20 µL included 5µL of PCR premix ,2 µL of each primer, 5 µL of template DNA have been used and 1.5 µL DMSO. The rest volume was completed with sterile de-ionized distilled water, then vortexed and finally 5 µL of template DNA was added(13). Negative control contained all material except template DNA, so instead that distilled water was added.

PCR reaction tubes were centrifuged briefly to mix and bring the contents to the bottom of the tubes, and placed into thermocycler PCR instrument where DNA was amplified as indicating in the (Table 2).

Table (2): PCR amplification Program for *aae* gene.

Stage	Temperature	Time	Cycle	References
Initial denaturation	95 ºC	1 min	30	Gazzola and Cocconcelli (2007).
Denaturation	94 ºC	30sec		
Annealing	52 ºC	1 min		
Extension	72 ºC	1min		
Final Extension	72 ºC	10min		

The PCR product were analyzed by PCR and the results confirmed by using 2 % agarose gel electrophoresis(14).

Cell autolysis assays:

Autolysis assays for *S. epidermidis* strains were performed as described by Qin *et al*. (15) ,cell samples (50 ml) were collected from exponential-phase cultures growing in TSB medium (OD_{580}=0.7) containing 1 M NaCl, and cells were pelleted by centrifugation. The cells were washed twice with 50 ml ice-cold water and resuspended in 50 ml 0.05 M Tris/HCl (pH 7.2) containing 0.05% (v/v) Triton X-100. The cells were then incubated at 30°C with shaking, and OD_{580} was measured at 30 min intervals.

Transmission electron microscope:

Transmission electron microscope was made according to Paul *et al.*(16) by using negative stain in College of Medicine, AL-Nahrain University.

Statistical Analysis:

In this study, the Statistical Analysis System- SAS(17) was used to effect of different factors in study parameters. Least significant difference –LSD test was used to significant compare between means.

Results and Discussion:

Vancomycin susceptibility was determined by the minimum inhibitory concentration (MIC) for 30 *S.epidermidis* isolates, according to Clinical and Laboratory Standards Institute(18), the results showed that 12 isolates were resistant to Vancomycin (VRSE), 4 isolates were intermediate resistant (VISE)and 14 isolates were sensitive(VSSE).

***Aae* gene amplification by monoplex PCR technique:**

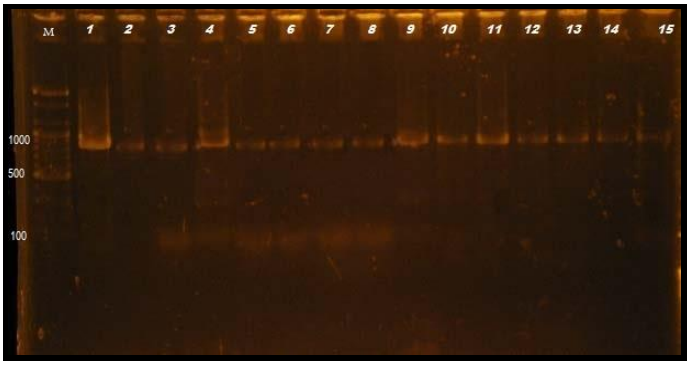
To detect the virulence factors (autolysin,adhesin), staphyloccal surface protein, *aae* gene in *S.epidermidis* was used, thirty isolates *S.epidermidis* were subjected to PCR technique in a monoplex pattern .All *S.epidermidis* isolates gave positive result for the presence of *aae* gene as shown in Figure (1a,b). The result demonstrated that the 30 (100%) of *S.epidermidis* isolates were obtained from clinical samples found to has the *aae* gene with molecular weight 858 bp.

The positive result of *aae* gene was confirmed by 2% agarose gel electrophoresis stained with ethidium bromide, electrophoresed in 70 volt for 1.5 hrs. and photographed under ultraviolet (UV) trans illuminator.

The result agree with Heilmann *et al*. (13). When reported *aae*, a novel autolysin/ adhesion from *S. epidermidis* having both bacteriolytic and adhesive properties also,the same result agree with Widerström (19) in the amplification reaction revealed a PCR product of 858 bp.

The function role of Atl /*Aae* was bind to fibrinogen, fibronectin and vitronectin(19). The bind vitronectin, indicating *atl* plays a role in binding of the cell not only to naked polystyrene surface during early stage of adherence but also to plasma protein –coated polymer surface during later stage of adherence. *Atl* has a role in mediating the attachment of bacterial cells to polymer surface, representing the prerequisite for biofilm formation(20).

In conclusion, *aae* is a surface-associated protein with bacteriolytic and adhesive properties representing a new member of the staphylococcal autolysin/adhesins potentially involved in colonization. the expression of the *atl* gene increased in the vancomycin resistance- induced *S. haemolyticus*, but the expression of the other autolysis-related genes – *lrgAB*, *lytS* and *sarA* – did not change the correlation of expression of *atl* and autolytic activity has been investigated in vancomycin resistant *S. aureus* strains and some showed increased *atl* gene expression or lower autolysis activity than that of parent isolates(21) and the *S. epidermidis* contained the gene *atlA* coding for the *atlA* autolysin, which has an adhesive function that is involved in the first phase of biofilm formation, as described by Helimann *et al*.(13).



Figure(1 a): Gel electrophoresis of amplified PCR product of *aae* gene in monoplex pattern, all strains well positive to it, agarose (2%), TBE buffer (1x), 70 volt for 1.5 hrs. stained with ethidium bromide. M: DNA ladder (100 bp),line from 1-15 indicate *aae* gene bands from *S.epidermidis* isolates(NO.1-15).

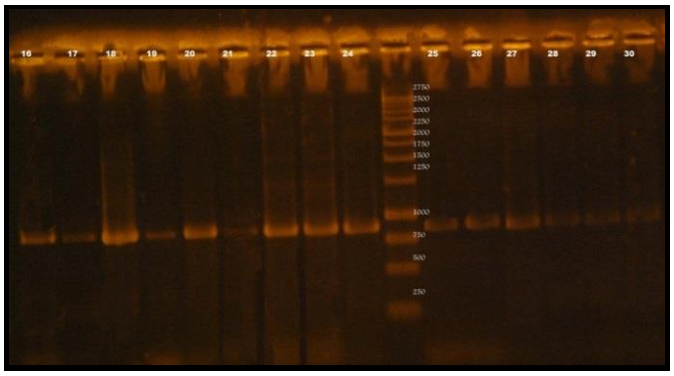


Figure (1 b): Gel electrophoresis of amplified PCR product of *aae* gene in monoplex pattern all strains well positive to it. Agarose (2%), TBE buffer (1x), 70 volt for 1.5 hrs. stained with ethidium bromide. M: DNA ladder (250 bp) ,line from 16-30 indicate *aae* gene bands from *S.epidermidis* isolates (NO. 16-30).

Autolysis assay:

Whole cell autolysis:

To examine the effects of Vancomycin on whole cell autolytic properties of laboratory-derived (VRSE, VISE, VSSE) isolates,the drug at a concentration of one half of MIC was included in the assay buffer before the OD580 was taken. The whole cell autolytic activities of *S.epidermidis* 1(VRSA) isolate was shown in (Figure 2).

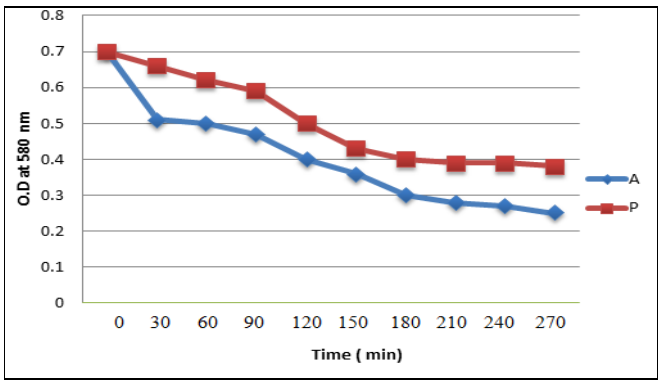


Figure (2): Whole cell autolytic activity profile of VRSE isolate. Profile of *S.epidermidis* 1. (P) present of Vancomycin;(A) Absence of Vancomycin.

The results showed that VRSE isolates (*S.epidermidis* 1) had low autolytic activity in presence of antibiotic compare to its in absence of antibiotic. There is a significant difference in OD between present and absent of vancomycin (*S.epidermidis* 1).

In presence of vancomycin, the autolytic activity increased inVISE isolates (*S.epidermidis* 14) compare to its in absence of antibiotic. As shown in (Figure 3). There was significant difference in OD between present and absence of Vancomycin.

In presence of vancomycin the autolytic activity increased in VSSE (*S.epidermidis* 22). As showed in (Figure 4). There was significant difference in OD between present and absence of Vancomycin.

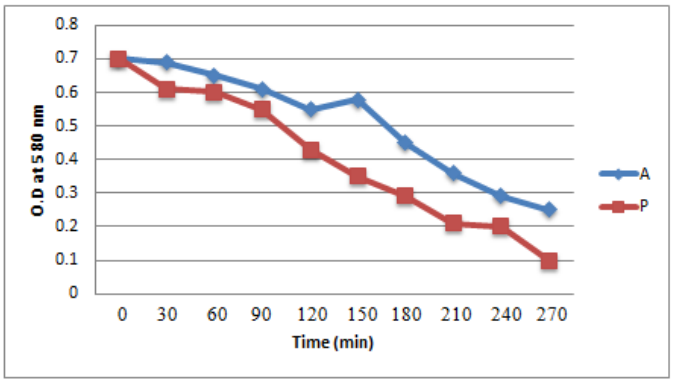


Figure (3): Whole cell autolytic activity profile of laboratory-drvied VISE isolate.Profile of *S.epidermidis* 14. (P) present of Vancomycin;(A) Absence of Vancomycin.

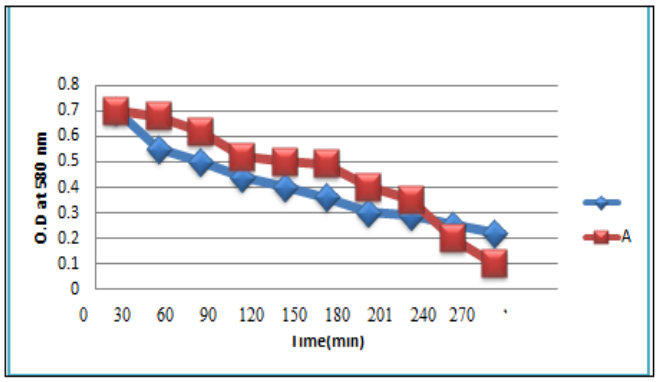


Figure (4): Whole cell autolytic activity profile of VSSE isolate.Profile of *S.epidermidis* 22. (P) present of Vancomycin; (A) Absence of Vancomycin

Finally, The results of comparison between autolysis activities of three *S.epidermidis* isolates revealed that there was significant difference among these isolates (VRSE,VISE and VSSE) in presence and absence of antibiotic (Vancomycin), as shown in (Table 3), the VSSE isolate (*S.epidermidis* 22) have the highest autolysis activity in the presence of antibiotic , followed by the VRSE isolate (*S. epidermidis* 1) and the VISE isolate (*S. epidermidis*14) which was the lowest autolysis activity with the presence of antibiotic. Calculations of autolysis rate were calculated according to Kim *et al*. (22) as in formula.

Percent lysis at time t = [(OD0-ODt)/OD0]×100.

The vancomycin resistant S. *epidermidis* isolate (VRSE MIC= 256), exhibited autolysis rates was (58%) , VSSE and VISE which were (70%), (64%) respectively in absence of vancomycin similar to result of Kim *et al.* (2012). The autolysis rates increased in the presence of vancomycin in each of *S.epidermidis* isolate (VSSE 22 and VISSE 14) were (85%),(57%), and decreased in VRSE was (44%) same as Kim *et al*. (22), also came with study of Gazzola and Cocconcelli[5].

Autolysis is linked to the process of cell division, and is therefore related to the growth of the cell and the expression of autolysins, which hydrolyses cell wall components. The autolysis rates of *S.epidermidis* with induced vancomycin resistance were less than those of the sensitive isolate . This suggests that resistance to autolysis indicates reduction of cell wall turnover(7,23).

Table (3): Comparision between autolytic activities of VSSE isolates (*S.epidermidis*22) in the presence and absence .

Time (min.)	O.D		LSD value
	Absence	Present	
0	0.70	0.70	0.00 NS
30	0.55	0.68	0.058 *
50	0.50	0.62	0.066 *
90	0.44	0.52	0.054 *
120	0.40	0.5	0.061 *
150	0.36	0.49	0.072 *
180	0.30	0.4	0.068 *
210	0.29	0.35	0.062 *
240	0.25	0.2	0.075 *
270	0.22	0.1	0.062 *
LSD value	0.126 *	0.137 *	---
* (P<0.05).			

Finally, there was significant difference in autolytic activity between three *S.epidermidis* isolates (VRSE, VISE and VSSE) in presence and absence of Vancomycin, as shown in (Table 4).

Table 4: Comparison between autolytic activities of 3 *S.epidermidis* isolates.

NO. of isolates	OD (mean ± SE)		T-test value
	Absence	Present	
NO.1 VRSE	0.507 ± 0.039	0.404 ± 0.045	0.094 *
NO.14 VISE	0.513 ± 0.052	0.404 ± 0.064	0.091 *
NO.22 VSSE	0.401 ± 0.047	0.555 ± 0.027	0.102 *
LSD value	0.078 *	0.094 *	---
* (P<0.05).			

Transmission electron microscopy:

TEM was performed on *S.epidermidis* isolates (VRSE MIC=256, VISE MIC=16, VSSE MICN=4) to assess cell wall thickness. The result of this study showed that the VRSE contain thicker cell wall as shown in (Figure 5), than VISE and VSSE as shown in (Figure 6A &B) by staining cells by negative stain. Cell wall thickening is a common feature of vancomycin resistant staphylococci. TEM revealed that cell wall thickness

of vancomycin sensitive *S. hemolyticus*, VSSH were 21.48 nm, increase in thickness of VRSH ,VISH 28% and 89 %, respectively by Kim *et al*.(22) The results demonstrated a characteristic change in cell wall thickness similar to that reported in previous studies on with induced vancomycin resistance (5, 22-24). In the study of Kim *et al* . (22), the cell wall thickness of the isolate with induced vancomycin resistance was almost 1.9-fold higher than that of its sensetive isolate . In *S. aureus*, the *atl* gene plays a fundamental role in cell division and separation. Decreased *atl* gene expression in vancomycin-resistant *S. aureus* may produce a build-up of peptidoglycan layers contributing to a thickened cell wall. However, only *atl* gene expression was upregulated in the vancomycin-resistance-induced *S. epidermidis* isolates, while cell wall thickness was much greater than that in the parent strain(7). Vancomycin-resistance-induced *S.epidermidis* exhibited a typically thick cell wall, decreased cell growth phenotype, decreased autolysis activity(23). Because S. *epidermidis* is prevalent in hospitals and is regarded as an important nosocomial pathogen with a tendency to develop multiple resistance, further understanding of the mechanisms underlying vancomycin resistance should be acquired through future detailed studies.

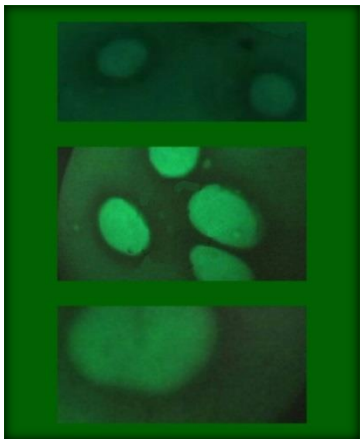
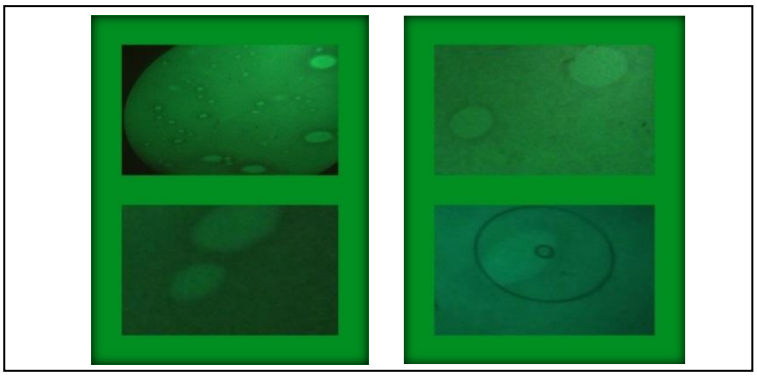


Figure (5): VRSE isolate with MIC= 256 µg\ml, under transmission of electron microscope 13000x(up), 34000x(mid) and 64000 x(down)



A **B**

Figure (6): S. *epidermidis* under transmission of electron microscope 34000x(up) and 64000x(down).
A- VISE isolate with MIC= 16 µg\ml
B- VSSE isolate with MIC= 4 µg\ml

In this work the results showed that there were significant differences in autolytic activity between three *S.epidermidis* isolates(VRSE,VISE and VSSE) in presence and absence of vancomycin, the VSSE isolate (*S.epidermidis* 22) have the highest autolysis activity in the presence of antibiotic , followed by the VRSE isolate (*S. epidermidis* 1) and the VISE isolate (*S. epidermidis*14) which was the lowest autolysis activity with the presence of antibiotic. vancomycin has effect on autolytic activity but don't have any effect on presence of *aae* genes as showed in PCR. The cell wall of VRSE was thicker in compared with VSSE as showed in TSM.

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