Australian Journal of Basic and Applied Sciences, 5(5): 1281-1290, 2011
ISSN 1991-8178

Emergence of High-Level Vancomycin-Resistant *Staphylococcus aureus* in the Critical care patients Cairo University Hospitals

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Abstract: Vancomycin is a large hydrophilic glycopeptide, forming hydrogen bonds with the terminal D-alanyl-D alanine residues of the stem pentapeptide in the bacterial gram positive cell wall. From the screening profile of 200 patients enrolled in this study, the percent of methicillin resistance *Staphylococcus aureus* (MRSA) and vancomycin resistance *S. aureus* (VRSA) were represented by 19% and 14% of patients, respectively. Selection of the highly resistant strains was conducted according to MIC method. Using light cycler PCR in Detection of *Van A* (27%) and *Van B* (36%) genes responsible vancomycin resistance in

Key words:

INTRODUCTION

Vancomycin is a glycopeptide antibiotic first introduced by Eli Lilly company in the 1950s. Vancomycin resistance of *S. aureus was* first emerged in Japan (Hiramatsu *et al*. 1997) using the various clinical and laboratory standards institute (CLSI) criteria. National Morb. Mortal Wkly Rep. (1997) referred to these strains as vancomycin intermediate *S. aureus* or VISA, and are believed to be resistant to vancomycin due to a common phenotype including a thickened cell wall. Vancomycin resistance due to a second more well defined mechanism, involving the acquisition of *Van* genes, was first described by Morb. Mortal Wkly Rep. (2002 and 2004). Weigel *et al*. (2003) documented that the *Van* genes allow the bacterium to modify the drug target from D-ala-D-ala to D-ala-D-lac which is poorly bound by vancomycin as ensured by five additional strains of *S. aureus* carrying the *Van* genes. Strains containing *Van* operon are more highly resistant to vancomycin and are classified as vancomycin resistant *S. aureus*, or VRSA by CLSI standards. Both mechanisms of resistance will be discussed in more detail below. Vancomycin resistance in *S. aureus* has been received much attention, since the first report by Hiramatsu *et al* (1997). Furthermore, there is the equally alarming threat of the risk of transmission of these organisms between patients. The first case of *S. aureus* with reduce susceptibility to vancomycin was found in Japan in a 4-month- old infant following heart surgery (Hiramatsu *et al*. 1997). Also NMMWR (1997) reported that the first cause of VISA in the United States was described in a peritoneal dialysis patients following repeated courses of intravenous and intraperitoneal vancomycin to treat a recurrent of intravenous and intraperitoneal vancomycin to treat are current MRSA- peritonitis

The first documented cause of vancomycin resistance in *S. aureus* due to acquisition of the van genes occurred in Michigan in 2002 (Chang *et al*. 2003). The second case of VRSA was isolated from morbidly obese 70 year old male in Pennsylvania (Tenover *et al*. 2004). The third case of VRSA was isolated from the urine of along- term care facility patients (Morb. Mortal. Wkly Rep. 2004). The *Van* genes were first found in 1988 in an *Enterococcus faecium* isolated from France (Leclereq *et al*. 1988). Transfer of vancomycin resistance from enterococci to staphylococci was accomplished in vitro by Noble *et al*. (1992). Detection of genes responsible of vancomycin resistance van A and van B carried out by two different systems of light-cycler PCR (polymerase chain reaction). First, using light-cycler PCR instrument and using kit of VRE (vancomycin resistant enterococci) depending on the information which state that the van A vancomycin resistance gene could be transferred from vancomycin-resistant. *Enterococcus faecalis* to *S. aureus* (Noble *et al*. 1992). Also Celwell *et al*. (1974, 1985) demonstrated that *Enterococci* and *Staphylococci* are known to exchange genetic information, similar to transfer of the broad-host-range erythromycin-resistance plasmid and related element Horaud *et al*. (1985),Weaver *et al* (2002) and Janniere *et al*. (1993). Francia and Clewell, (2002) according to all this illustrated information detection of van A and van B genes in *Staph. aureus* by

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using kit of detection of van A and van B in *Enterococci* depending on genes exchange which take place between *S. aureus* and *Enterococci*. When the first proposal which state that it can detect the van A and van B by using kit used to detect the same genes van A/van B in *Enterococci* this proposal failed where all samples give negative result, so go to use light-cycler 480 high resolution melting master instrument by using dye enables detection of double strands DNA by fluorescence and this method give positive results illustrated later.

The present study was carried out to find out the presence of vancomycin intermediate *Staphylococcus aureus* (VISA) and vancomycin resistant *Staphylococcus aureus* (VRSA), by rapid and reliable detection by molecular genetics applications ,where genetic detection generally based on polymerase chain reaction (PCR) which give reliable guidance to clinicians concerning antimicrobial therapy and antimicrobial susceptibility patterns and causative pathogen and can be predicted early.

MATERIALS AND METHODS

1-Materials:

All clinical isolates of S. aureus (one per patient) from clinical samples of patients at critical care unit ,Cairo Hospital University, Mueller-Hinton agar supplemented with 6 mg/l oxacillin was purchased from Oxoid (England). Vancomycin was provided as a reference powder from Eli Lilly Sweden AB, Stockholm, Sweden. Light cycler VRE kit and Primer (Rouch)

2- Detection of MRSA according to (National Committee for Clinical Laboratory Standards, NCCLS, 2003)

A) Oxacillin Agar Screen Test:

It is the most reliable phenotypic screening test for the detection of the oxacillin resistance (Hackbarth & Chambers, 1989, Gerberding *et al*., 1994 and York *et al*., 1996). All MRSA isolates were spot inoculated on to Mueller-Hinton agar supplemented with 6 mg/L oxacillin and suspension (Kohner *et al*., 1999). The plates were incubated at 35°C for 24

b) Determination of minimum inhibitory concentration (MIC):

In a colorimetric MIC method, a redox indicator changes the color in response to bacterial growth, which enhances the detections of growth. Adding the indicator also helps to semi-automate the broth micro-dilution method through image processing. However, the indicators might affect bacterial growth, which in turn may change the interpretation of susceptibility, Tengerdy *et al*. (1967). In order to be able to select a suitable indicator we should compare more than one indicator for toxicity and suitability for computerized image analysis.

1. The redox indicator was 2, 3, 5 triphenyl-tetrazolium chloride (TTC) which enhance the estimate the bacterial growth.
2. Antimicrobial agents: prepare different dilutions of antibiotic (vancomycin) from 0.5 to 128mg/ml.
3. Preparation of microdilution plates of the antibiotics after prepare different antimicrobial dilutions put it in round bottomed micro plates were performed according to the NCCLS recommendations (2000), and then microplates were dried overnight with dry air at 25°C. all antimicrobial microdilution trays were stored in the cold room and used within 2 weeks after preparation.
4. Preparation of Bacterial strains where bacterial were cultured overnight on blood agar plates. Three colonies were transferred to 5ml of Mueller Hilton broth without blood and incubated at 37°C for 4h. to reach the exponential phase of growth from these cultures bacterial turbidity was adjusted to 0.5 on the McFarland turbidity standard as measured by absorbance (0.08 to 0.1 at 625 nm) in a spectrophotometer (Hitachi U-1100).

Corresponding to approximately 10^8 CFU/ml the adjusted bacterial suspensions were first diluted 1: 200 in Mueller Hilton broth and 100m was added to each well (5´ 10^4 CFU/ well), incubated for 16h. at 37°C. Then the indicator (TTC) was added to each well of the micro-plates then bacterial growth sucked up and down with the multi-pipette then incubated for 3h. longer, the micro-plates were scanned, by (ELISA Dialab DiaReader ELX 800G) red color indicate presence of bacterial growth however yellow color of well indicate No growth of bacterial inoculum.

***Ii- Bacterial DNA Extraction and Purification*:**

Bacterial DNA was isolated by automatic method by instrument of Rouche company called (Mag NA pure compact) , by using Mag NA pure compact Nucleic acid isolation kit contain plus its contents Bacteria lysis

buffer, where bacterial sample was 100ml suspension of cultured cells (containing no more than $1´ 10^6$ cells)

III- Using Light cycler VRE (vancomycin resistance enterococci) detection kit in detection of VanA/VanB in Staphylococcus aureus VRSA Strains:

Kit for the detection of vancomycin resistance genes (*Van A/ Van B*) was used during this study in research samples, using the light cycler instrument. The light cycler VRE detection kit provides primers and probes for amplification and sequence specific detection of Van A/ Van B genes. As well as kit specific internal control (IC) for the reliable interpretation of results. The components of the kit are designed for detection and differentiation of vancomycin resistance genotypes *Van A/ Van B* .

IV- Using Light cycler 480 high resolution melting master instrument in detection of VanA/VanB in Staphylococcus aureus VRSA Strains:

In principle, the light cycler 480 high resolution melting master can be used for species amplification of any DNA or cDNA target. However, you would need to adapt each amplification protocol to the reaction conditions of the light cycler 480 system and designed specific PCR primers for each target. The included light cycler 480 high resolution melting dye enables detection of double strands DNA by fluorescence, monitoring formation of amplicon during PCR cycling and melting curve analysis utilizing the high resolution data acquisition capabilities of the light cycler 480 instrument. And by using primer sequences for *Van A* and *Van B* used previously by Bell *et al* (1998) table(1).

Table 1: PCR primer sequences for vanA and vanB genes (Bell *et al*., 1998).

Primer	Sequence	Specificity
vanABF	GTAGGCTGCGATATTCAAAGC	*vanA*
vanAR	CGATTCAATTGCGTAGTCCAA	*vanA*
vanBR	GCCGAATCAAATCATCCTC	*vanB*

RESULTS AND DISCUSSION

I- Percent of patients have MRSA and VRSA:

Number of patients enrolled in this study 200 patients 19% from them have resistance to methicillin and from these 38 patients 28 have vancomycin resistance (14% of all patients) These results are also comparable to Haddadin *et al*. (2002) who state that patients who are elderly, immunosuppressed, have been exposed to antibiotics, prolonged ICU cure and MRSA carrier or infected patients are at risk of colonization and subsequent infection. Theaker *et al*. (2001) mentioned that between 30 and 60% of critically ill patients are probably because chronic illness and acute critical illness may allow for the formation of resistant organism on the skin or in the gastrointestinal tract.

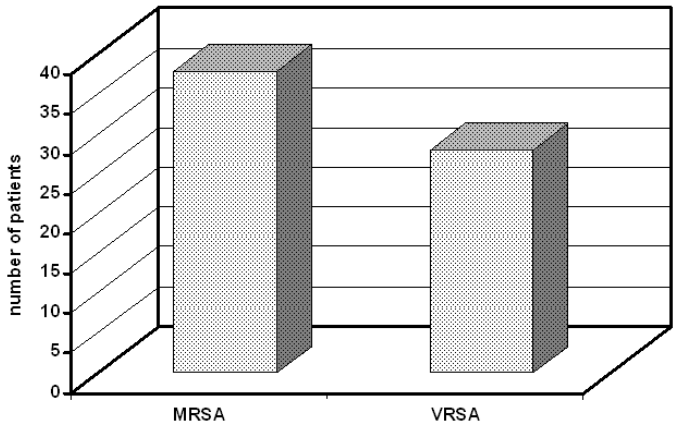


Fig. (1): Number of patients have MRSA and VRSA

Ii-evaluation of Oxacillin and Vancomycin MICS by Colorimetric Microdilution Method with Illustrated Different Degrees of Vancomycin Resistance from Patients at Admission:

Frequency of *Staphylococcus aureus* was 17 between all isolated microorganisms, show different MICs values to oxacillin and vancomycin as illustrated in Table (3). starting bacterial number of *S. aureus* in

Mueller-Hinton media was 10^{10} CFU/ml (O.D1.125) . Where 14 from these 17 isolates show isolates show resistance to oxacillin (O.D ^{3} 0.32 mm) with different values of CFU and 3 isolates show optical density less than 0.32 nm and have no growth. On the other hand, 11 isolates from 17 isolates show resistance to vancomycin and 6 sensitive to vancomycin, resistance to vancomycin in isolates which give O.D^{3} 0.36, where resistance show different type of resistance, hetero-resistance where value of O.D between 0.36 to 0.50 where this result confirmed by disc different method where these isolates cultured on brain-heart infusion agar plates containing vancomycin disc with concentration 30 mg/ml and these isolates when incubated to 24 h at 35°C show few small colonies around the vancomycin disc as illustrated in Table (2). and these hetero-resistance isolates were 4 from 11 isolates resistance to vancomycin. Measuring MIC by using this method which highly accuracy more than Agar dilution method where In Broth micro-dilation method, as with many susceptibility tests, problems might arise when determining the endpoints of growth, which may lead to false interpretations of susceptibility and to decreased reproducibility (Cunha, 1997), Since turbidity is measured visually, the end point of bacterial growth may be under estimated. This problem has been partly solved in some laboratories by using indicator in the conventional broth microdilution method, a procedure refereed to as colorimetric MIC method (Baker *et al*. 1994, Baker and Tenover 1996 and Johnson *et al*. 1985).

III-Van A and Van B detections by real time light cycler PCR (Rouch company). By using VRE detection kit:

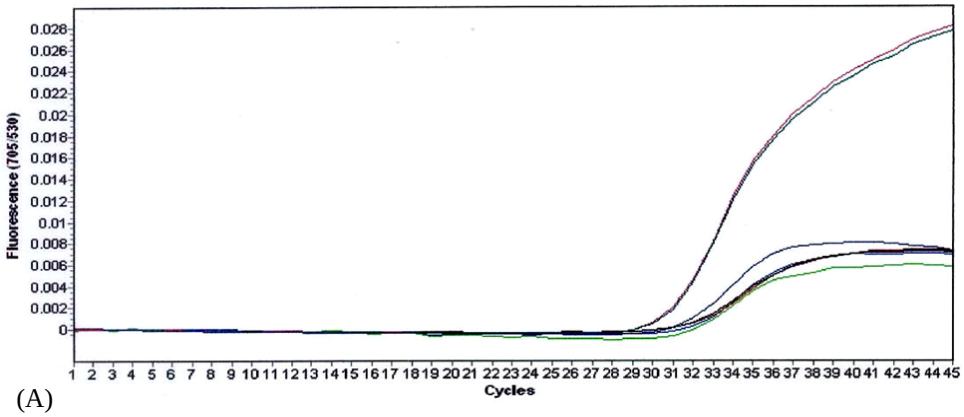
By this realtime PCR technique, the detection of *vanA* and *vanB* genes carried out by using light cycler VRE (vancomycin resistance enterococci) detection kit for detection *vanA/vanB* enterococci, to detect the *vanA* and vanB in *S. aureus*, where Fosheim *et al*. (2007) reported that the VRE EVI gene detection assay has been evaluated previously for its ability to detect *vanA* and vanB genes in *VRE* also state that in his study he can detect the *vanA* gene in *Staph. aureus* by using this kite, when depend on this previous information and carried out this technique to detect *vanA* and *vanB* in isolated VRSA strains in this thesis, all strain give negative result, on the other hand, with this negative results for *vanA/vanB* detection in *Staph. aureus* strains, we rerun confirmation test anther time to confirm that this strains give positive result to vancomycin resistant and try with anther technique to detect *vanA* and vanB in *Staph. aureus*, there are several formats available for real time amplification and instrument used in this thesis, the light cycler PCR instrument is different from the conventional thermal cyclers since the heating and cooling is controlled by air instead of a heating block, which allows rapid temperature exchange rates. In addition, the PCR reactions are performed in glass capillaries with a high ratio of surface area to volume for efficient heat transfer. Figure (3).

IV-Detection of van A and van B by light cycler 480 high resolution melting master kit:

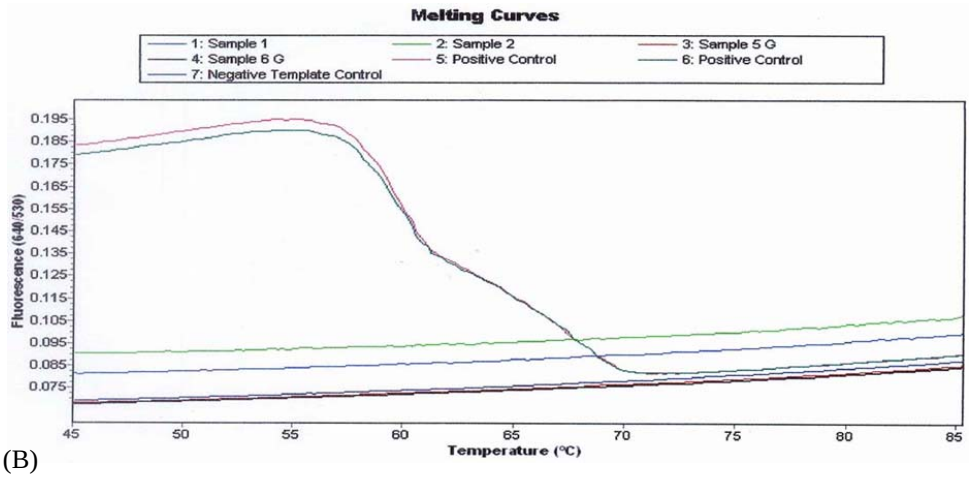
By using light cycler 480 high resolution melting master kit with another primer different from which found in VRE kit, and sequence of this primer showed in chapter of materials and methods and by using this high new technology of light cycler techniques and using of Sybr green 1 stain for dsDNA and detection of van B in 11 isolates which highly resistant to vancomycin, only 4 isolates from 11 isolates give positive result to van B occurrence, this isolates number 1, 7, 9, 11 and the rest of the samples 3, 3, 4, 5, 6, 8, 10 give negative results, as following in table and Fig. (4). where melting temperature were B7 (Tm=80.9), B9 (Tm=80.9), B10 (Tm=77.8).

On the other hand three isolates give positive result from 11 isolates for van A detection as follow number 3, 4 and 5 give positive result, from illustrated data show that no isolate give positive result for detection of *Van A* and *Van B* together, but confirm that the presence of *Van A* and *Van B* in resistance isolates to vancomycin and on the other hand the percent of occurrence of *Van B* in the isolates 36.3% and van A 27.2% from all 11 isolates as illustrated in the following figure (5). Although all researcher reports which confirm the transfer of *Van A* or *Van B* from enterococci to Staph. aureus, but in our study I cannot confirm this informations because, if vanA or *VanB* genes transfer as plasmid from enterococci to *Staph. aureus*, it can give positive result for *VanA*, and *Van B* detection in *Staph. aureus* strains by using kit used to detect *VanA* and *VanB* in enterococci, but give negative result for detection in all strains, if plasmid carrying *VanA* and *VanB* transfer from enterococci to *S. aureus*, the kit can detect it easily in *Staph. aureus* because it still as separate unite and not incorporate into *S. aureus* DNA, so this thesis not agree the using of this kit in detection of *VanA* and *VanB* in *S. aureus*.On the other point of view explain the phenomenon of vancomycin resistance in *Staph. aureus* due to thickening of the cell wall with re-organization of cell wall metabolism (Cui *et al*., 2003). Also Hiramatsu *et al* (1998) and Sieradzki *et al*., (1999) demonstrated that the thickening of cell wall is closely associated with the mechanism of vancomycin resistance in the VRSA strains. As we and other researchers proposed previously, trapping of vancomycin molecules in the cell wall peptidoglycan would be

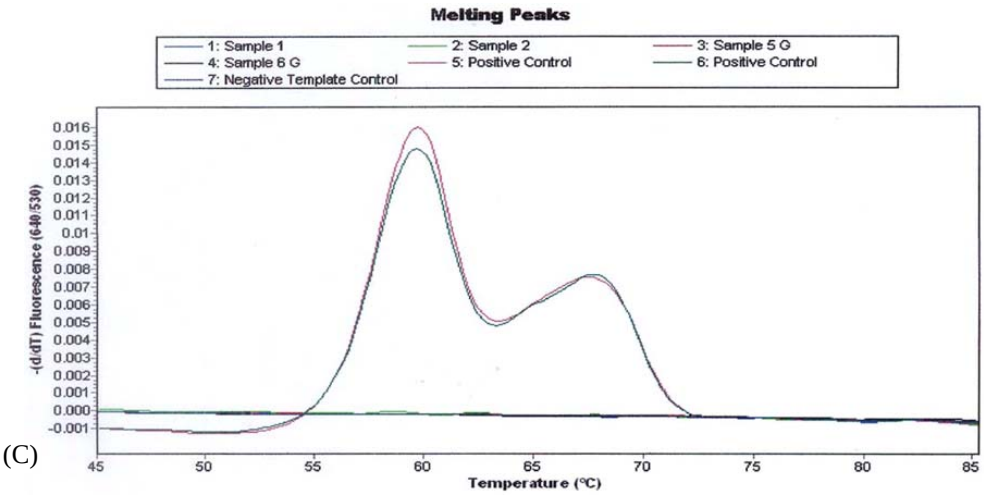
the essential contributor. The thicker the cell wall, the more vancomycin molecules would be trapped within the cell wall, thus allowing decreased number of vancomycin molecules to reach the Cytoplasmic membrane where the real functional targets of vancomycin are present, where vancomycin binds to the stem peptide of the membrane-anchored murein monomer (lipid II) at its lys-D-Ala-D-Ala residue and thus prevents the murein monomer from being incorporated into the nascent peptidoglycan.



(A)

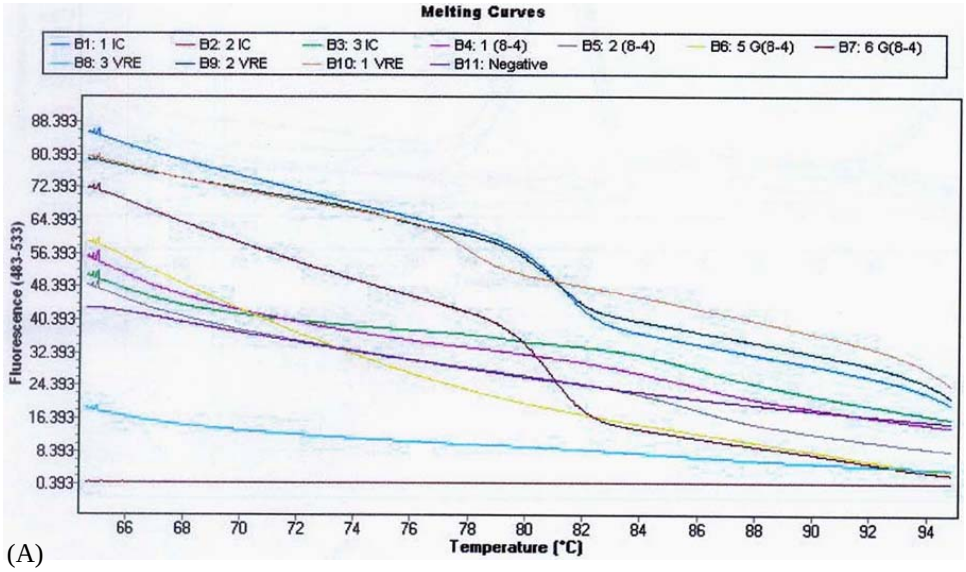


(B)

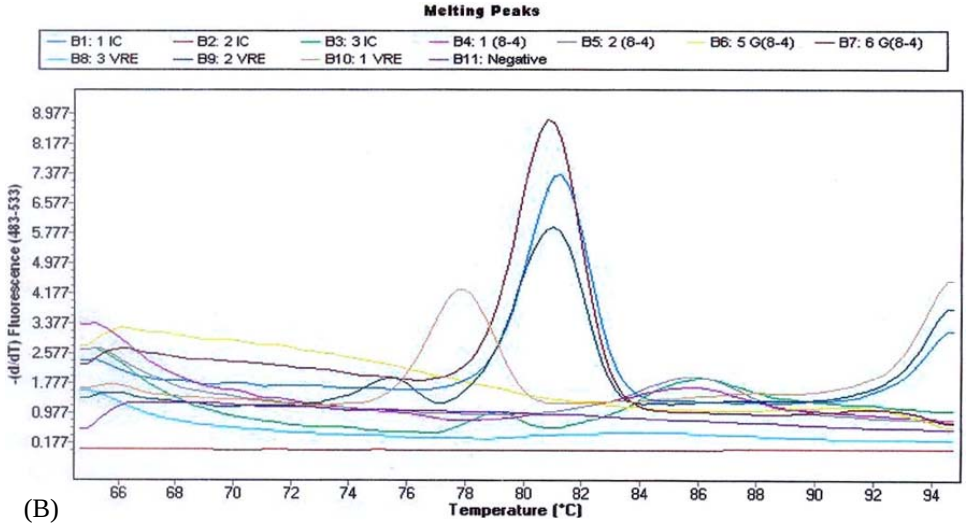


(C)

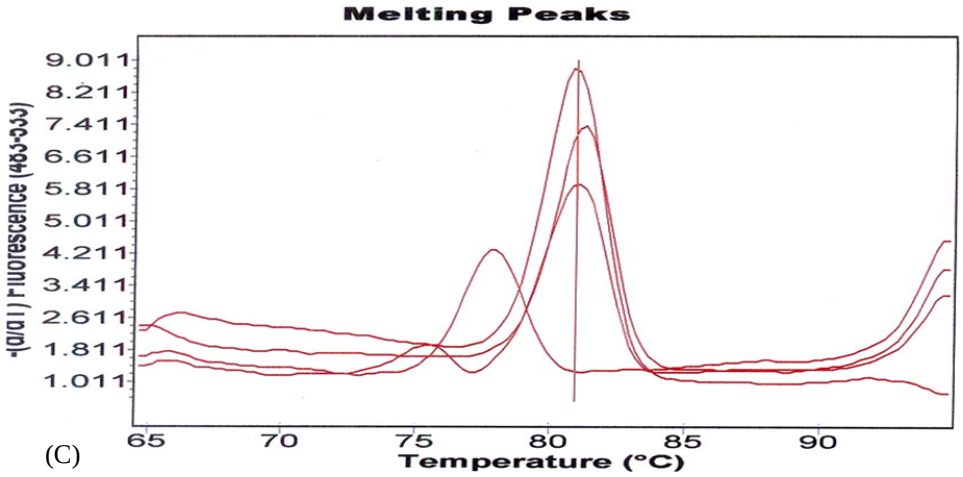
Fig. 2: Show the negative result of VanA and AVanB detection by VRE kit (A)number of cycles and(B, C) Thermal melting points



(A)

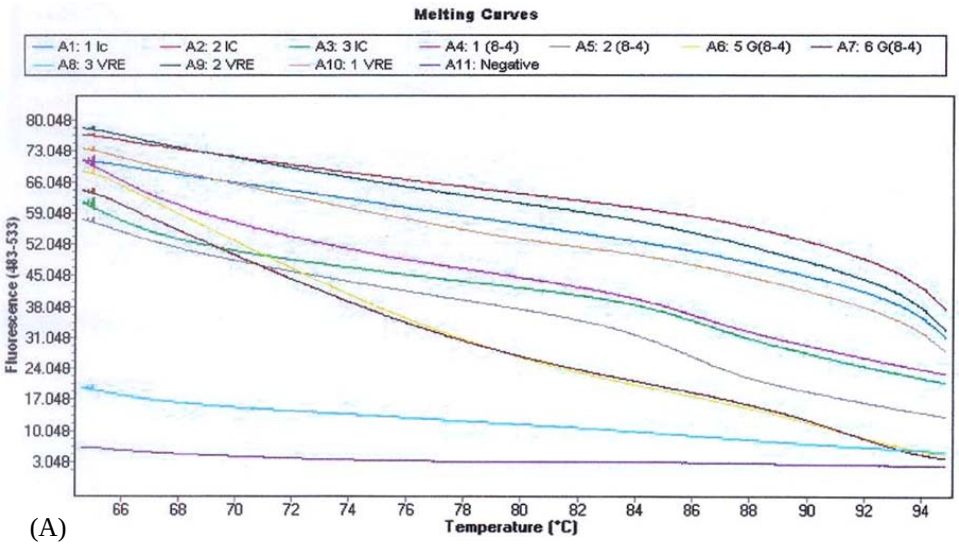


(B)

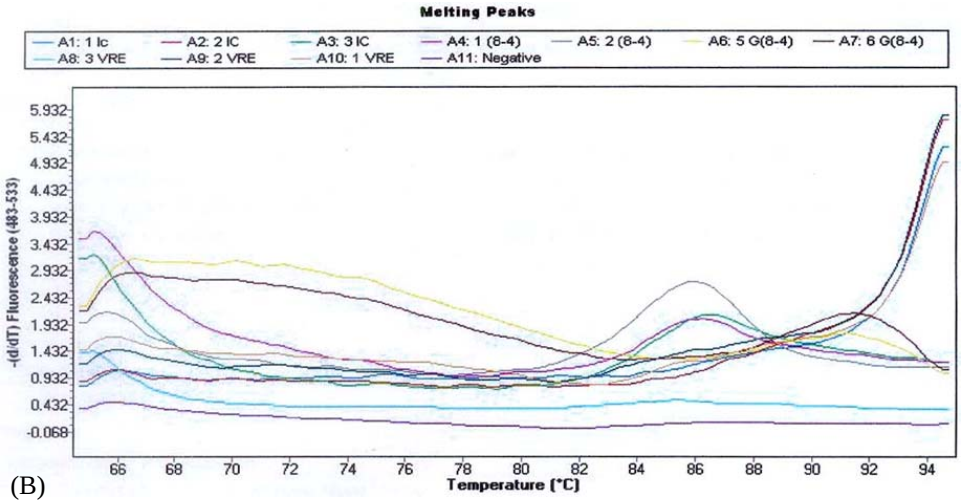


(C)

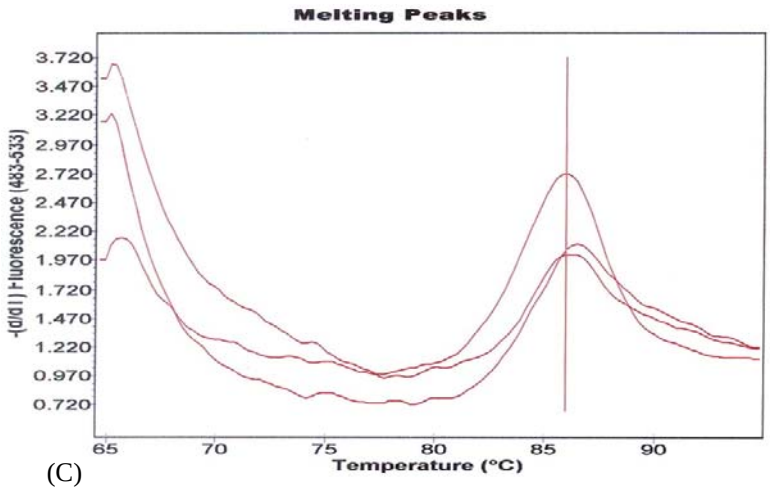
Fig. 3: Detection of van B by light cycler 480 high resolution melting master kit:



(A)



(B)



(C)

Fig. 4: Detection of van A by light cycler 480 high resolution melting master kit

In this current thesis some strains of isolated VRSA strains were negative for *VanA* and *VanB* gene (36%) by PCR, therefore the absence of *VanA/B* genes in this strains does not role out that these strains are not VRSA. Hanaki *et al*. (1998) and Cui *et al*. (2000), hypothesis that cell wall thickening is responsible for development of vancomycin resistance, the mechanism of vancomycin has been extensively studied with the first clinical VRSA strain, where biochemical and transmission electron microscopy (TEM) examination of the VRSA strains, suggested that it produces increased amounts of peptidoglycan. More murein monomers and more layers (probably 30-40 layer as judged by cell-wall thickness observed with TEM) of peptidoglycan are considered to be present in the cell wall. As a result, more vancomycin molecules are trapped in the peptidoglycan synthesis occurs. Moreover, a higher concentration of vancomycin would be required to saturate all the murein monomers that are supplied at an increased rate in VRSA strains.
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Table 2: Evaluation of oxacillin and vancomycin MICs by colorimetric microdilution method.

No. of isolates	Oxacillin MIC optical density (OD)	(CFU/ml)	Interpretation of oxacillin (OD)	Vancomycin MIC optical density (OD)	(CFU/ml)	Interpretation of vancomycin (OD)
1	0.730	$5x10^2$	(+)(R)	0.811	$5x10^3$	(+)(R)
2	0.890	$5x10^3$	(+)(R)	0.547	$5x10^2$	(+)(R)
3	0.626	$5x10^2$	(+)(R)	0.456	5x10	(+)(h)
4	0.803	$5x10^3$	(+)(R)	0.664	$5x10^2$	(+)(R)
5	0.810	$5x10^3$	(+)(R)	0.715	$5x10^3$	(+)(R)
6	0.239	0.0	(-)(S)	0.294	00	(-)(S)
7	0.570	5x10	(+)(R)	0.735	$5x10^3$	(+)(R)
8	0.674	$5x10^2$	(+)(R)	0.397	5	(+)(h)
9	0.638	$5x10^2$	(+)(R)	0.173	00	(-)(S)
10	0.811	$5x10^3$	(+)(R)	0.912	$5x10^4$	(+)(R)
11	0.954	$5x10^4$	(+)(R)	0.427	5x10	(+)(h)
12	0.260	0.0	(-)(S)	0.013	00	(-)(S)
13	1.096	$5x10^4$	(+)(R)	0.263	00	(-)(S)
14	0.212	0.0	(-)(S)	0.118	00	(-)(S)
15	0.618	$5x10^2$	(+)(R)	0.471	5x10	(+)(h)
16	1.059	$5x10^4$	(+)(R)	0.859	$5x10^3$	(+)(R)
17	0.642	$5x10^2$	(+)(R)	0.211	00	(-)(S)

+ : Positive to resistance
- : Negative to resistance
R : Resistant
S : Sensitive
h : Hetero-resistance
* Oxacillin concentration in plate wells was 30 mg/ml
*Vancomycin concentration in plate wells was 30 mg/ml.
*CFU colony forming unit

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