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# Vancomycin Resistance Among Methicillin Resistant *Staphylococcus aureus* Isolates from Neonatal Sepsis Attending Intensive Care Unit in Shibin El-Kom Teaching Hospital, Egypt

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**Methicillin-resistant *Staphylococcus aureus* (MRSA) is a major cause of Nosocomial, community acquired infections and neonatal sepsis. The Glycopeptide vancomycin was the drug of choice for treating infections. Aim: Identifying the vancomycin- resistance phenotypically and genotypically among the MRSA isolates from Shibin El-Kom teaching Hospital. Materials and Methods: All samples were collected from Neonatal intensive care unit (NICU) of Shibin El-Kom teaching hospital in Minoufiya, Egypt and identified by conventional methods. S *aureus* and MRSA were isolated and identified from different clinical samples using conventional methods confirmed by antibiogram of the isolates and *mec A* gene detection. vancomycin MIC and Vancomycin screening agar were determined following CLSI guidelines. *Van A* was amplified by PCR using standard primers. Out of the 200 neonates included in this study, 85% were positive growth and 15% were negative growth. Among them, 25% isolates were staphylococci, 42 isolates had *nuc* gene. Out of 42 *S. aureus*, 80.95% had *mecA* gene and 19.05% had not *Mec A* gene. The VRSA isolates had not *van A* gene. Conclusions: Vancomycin was still the most effective drug against *S. aureus* infection. All MRSA in Shibin El-Kom Teaching Hospital had not vanA gene.**

**Keywords:** MRSA, VRSA, *Nuc* gene, Antibiotic resistance.

Over the past few decades, there has been an alarming increase in the prevalence of antibiotic-resistant pathogens and strains in serious infections. The occurrence of bacterial infection had decreased with the discovery of penicillin in 1940 until *Staphylococcus aureus* began producing B-lactamase, which destroys the penicillin B-lactam core ring (Khan, *et al*., 2013). This increase in resistance towards penicillin drove the development of methicillin drugs, which are virtually resistant against many genetic variations of the B-lactamase enzyme. Since then, MRSA has become endemic in hospitals and nursing homes worldwide (Noor & Munna, 2015). The treatment of the *S. aureus* infections has become problematic because of the increase of resistance to methicillin, vancomycin and other antibiotics (Mathews *et al*., 2010).

The glycopeptide vancomycin was considered to be the best alternative for the treatment of multi drug-resistant MRSA. However, there are increasing numbers of reports indicating the emergence of vancomycin-resistant S. aureus (VRSA) strains exhibiting two different resistance mechanisms. Initially, vancomycin-intermediate *S. aureus* (VISA) noted to be due to the thickened cell wall (Hiramatsu *et al.,* 1997), where many vancomycin molecules were trapped within the cell

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wall. The trapped molecules clog the peptidoglycan meshwork and finally form a physical barrier towards further incoming vancomycin molecules (Cui et al., 2006) The second was identical to the mechanism seen in vancomycin-resistant Enterococcus. Vancomycin-resistant Enterococcus faecium harbours the vanA operon, which contains five genes, VanS, -R, -H, -A and -X8. But Tiwari and Sen have reported a VRSA which is van gene-negative. (Tiwari and Sen 2006) Subsequent isolation of VISA and VRSA isolates from other countries has confirmed that the emergence of these strains is a global issue. Vancomycin was the drug of choice for treatment of infections caused by MRSA. High vancomycin MIC for MRSA which are susceptible to vancomycin may indicate the drug resistance to many antibiotics (Kshetry *et al*., 2016). The vancomycin has been a high molecular weight antibiotic, does not diffuse in concentration gradient manner while diffusing through the agar medium when the disc susceptibility test is employed. Moreover, it does not differentiate between vancomycin-susceptible isolates from vancomycin-intermediate isolates of *S. aureus* and hence disc diffusion testing is not used for detection of VRSA (CLSI 2012).

The aim of the present study was to identify the emergence of vancomycin-resistant MRSA among *S. aureus* isolates from patients with Neonatal sepsis attending the Intensive Care Unit in Shibin El-Kom Teaching Hospital.

## MATERIAL AND METHODS

Study Site and Population: The study was done at department of Microbiology, Shibin El-kom Teaching Hospital and Department of Microbial Biotechnology, Genetic Engineering and Biotechnology Research Institute, University of Sadat City. The present study was carried out among the Neonatal sepsis attending intensive care unit (ICU) at Shibin El-kom Teaching Hospital.

Isolation and Identification of *Staphylococcus aureus*: A total of 200 blood clinical samples were inoculated on blood culture and incubated aerobically at 37°C for 48 hours. *S. aureus* was identified by colony morphology, Gram stain, DNase, catalase and coagulase tests and fermentation of mannitol by conventional methods. (Oxoid, England) (De vos *et al.*, 2009). Staph isolates were confirmed by detection of the *nuc* gene.

Antibiotic susceptibility testing: The antibiotic resistance profile was determined by the disc agar diffusion (DAD) technique using different antimicrobial agents; Amikacin (30µg), Gentamicin (10µg), Streptomycin (10µg), Clarithromycin (15µg), Cefoxitin (30µg), Ampicillin (10µg), Ciprofloxacin (5µg), Erythromycin (15µg), Oxacillin (1µg), Vancomycin (30µg), Meropenem (10µg), Imepenem (10µg) and Cephardine (30µg) (Oxoid, England) according to the guidelines recommended by Clinical and Laboratory Standards Institute (CLSI, 2014).

Determination of MIC: MIC of vancomycin was determined by agar dilution method using CLSI guidelines (CLSI 2014). Briefly, gradient plates of Mueller-Hinton agar (Oxoid, England) were prepared with vancomycin (2-32 mg/l, Oxoid, England). 0.5 McFarland equivalent inoculum prepared using 18-24h; old culture was spotted on to gradient plates. Plates were incubated overnight at 35°C for 24h before assessing the visible growth.

Vancomycin screening plate: Brain heart infusion (BHI) was used for vancomycin (VA) screening plate (6µg/ml). All plates were inoculated with 10µl of an inoculum suspension prepared with growth from an overnight blood agar plate, with a turbidity equivalent to 0.5 McFarland standard. The plates were incubated for 48h, and growth was reported after both 24 and 48h (Bhateja *et al.,* 2005).

DNA Extraction: DNA of *S. aureus* isolated according to the Kit (Jena Bioscience. Germany) according to the manufacturer's recommendations. The eluted DNA was stored at –20°C until use

**PCR amplification for *Nuc, MecA and VanA* genes**

Amplification was carried out according to the following thermal and cycling condition for Nuc gene ( table 1) using Gene Amp (Applied Biosystems, USA) The primers and the PCR conditions were as described by (Zhang *et al*., 2004):and primeres are mentioned in table 1. Amplification condition consists of Initial denaturation at 94°C for 5 minutes. Thirty cycles of amplification consisting of Denaturation at 94°C for 60 seconds, annealing at 50°C for 60 seconds

and final extension at 72°C for 120 seconds. After the last cycle, a final extension of 10 minutes at 72 °C was done.

Amplification of *MecA* gene (Table 1) (Zhang *et al*., 2004):

a. Initial denaturation at 94°C for 5 minutes.

b. Thirty cycles of amplification consisting of:

- Denaturation at 94°C for 30 seconds.
- Annealing at 54°C for 30 seconds.
- Extension at 72°C for 30 seconds.

c. After the last cycle, a final extension of 5 minutes at 72 °C was done.

Amplification of *Van A* gene (table 1) (Biswajit *et al*., 2008):

a. Initial denaturation at 94°C for 5 minutes.

b. Thirty cycles of amplification consisting of:

- Denaturation at 94°C for 60 seconds.
- Annealing at 50°C for 60 seconds.
- Extension at 72°C for 120 seconds.

c. After the last cycle, a final extension of 10 minutes at 72 °C was done.

Amplified fragments were visualized on a 1% agarose gel electrophoresis stained with ethidium bromide. A DNA ladder (100–1500bp) was used to estimate allele sizes in base pairs (bp) for the gel.

## RESULTS

Out of the 200 neonates had neonatal sepsis, 85% were positive growth and 15% were negative growth. Out of 85% positive growth, 35% were Gram Positive, 37.5% were Gram-Negative and (12.5%) were Candida (Tab 2). Among 70 of Gram-Positive, 50 isolates were confirmed phenotypically as *S. aureus*. PCR amplification confirmed the presence of *nuc* gene in 42(84%) of the 50 isolates (table 4). The PCR product appeared a single DNA band with a size equal to 279 bp fragments (Fig 3).

**Table 1.** Primers used for amplification of *MecA* gene, *VanA* gene and *Nuc* gene

| Primer | Sequence(5'-3') | Reference | Size of Amplified Product (bp) |
|---|---|---|---|
| *Nuc* | 5'-GCGATTGATGGTGATACGGTT-3'<br>3'-AGCCAAGCCTTGACGAACTAAAGC-5' | Zhang et al., 2004) | 279 |
| *VanA* | 5'-ATGAATAGAATAAAAGTTGC-3'<br>3'-TCACCCCTTTAACGCTAATA-5' | (Biswajit *et al*., 2008) | 1030 |
| *MecA* | 5'-CCAATTCCACATTGTTTCGGTCATA-3'<br>3'-GTAGAAATGACTGAACGTCCGATAA-5' | Zhang *et al*., 2004 | 310 |

**Table 2.** Distribution of 200 Neonates with suspected sepsis according to Gram positive, Gram negative, Candida and No growth

| Type of growth | Gram positive | Gram negative | Candida | No growth |
|---|---|---|---|---|
| Number | No ( %)<br>70(35%) | No %<br>75 (37.5%) | No %<br>25 (12.5%) | No %<br>30 (15%) |

**Table 3.** Identification of 50 staphylococci according to biochemical test

| | *S. aureus* | CoNS |
|---|---|---|
| Coagulase Test | 42(84%) | 8(16%) |
| MSA | 45(90%) | 5(10%) |
| DNase | 42(84%) | 8(16%) |

**Table 4.** Detection of *Nuc* gene among 50 staphylococcal isolates

| | Positive No (%) | Negative No (%) |
|---|---|---|
| *Nuc* gene | 42 (84%) | 8(16%) |

Antibiotic susceptibility tests were evaluated by the disk diffusion method for all isolates of *S. aureus* (Table 5 & Fig 2). Isolates of *S. aureus* showed different level of resistance against some antibiotics (Fig 1).

Among the 50 clinical isolates of S. aureus, 50(100%) were identified as methicillin-resistant *S. aureus* (MRSA) by disc diffusion method. This was confirmed by detection of *mecA* gene. The PCR product appeared a single DNA band with a size equal to 310 bp fragment corresponding to the *mec A* gene for 34 (68%) isolates of *S. aureus* and 16(32%) had not *mec A* gene (Fig 4).

MIC of vancomycin was determined by agar dilution method using CLSI guidelines. MIC of Vancomycin for 50 staphylococci isolates were 48 isolates sensitive for vancomycin and two isolates resistant for vancomycin in concentration 2µg/mL while 49 isolates sensitive for vancomycin and one isolate resistant for vancomycin in concentration 4 and 8µg/mL while 50 isolates sensitive for vancomycin in concentration 16 and 32µg/mL (Tab 5). By Vancomycin Screening Agar for 50 staphylococci isolates were 49 isolates sensitive for vancomycin and one isolate resistant for vancomycin (Table 6).

The PCR amplification did not appear band corresponding to *van A* gene for *S. aureus* and 50 had not *van A* gene (Fig 5).

**Table 5.** Percentage of antibiotic resistant isolates of staphylococci (n=50)

| Antibiotic Name | Number of isolates % R | I | S |
|---|---|---|---|
| E | 39 (78%) | 7 (14%) | 4 (8%) |
| OX | 42 (84%) | — | 8(16%) |
| VA | 8(16%) | — | 42(84%) |
| S | 16(32%) | 3(6%) | 31(62%) |
| MEM | 37(74%) | 6(12%) | 7(14%) |
| CIP | 23(46%) | 8(16%) | 19(38%) |
| CE | 44(88%) | 4(8%) | 2(4%) |
| AK | 23(46%) | 10(20%) | 17(34%) |
| IPM | 20(40%) | 2(4%) | 28(56%) |
| CN | 30(60%) | 5(10%) | 15(30%) |
| CLR | 31(62%) | 6(12%) | 13(26%) |
| FOX* | 50(100%) | — | — |
| AM | 50(100%) | — | — |

R: Resistant, I: Intermediate, S: Sensitive
Amikacin (AK),Gentamicin (CN),Streptomycin (S), Clarithromycin (CLR), Cefoxitin (FOX), Ampicillin (AM), Ciprofloxacin (CIP), Erythromycin (E), Oxacillin (OX) (16%) & (0%) & (84%), Vancomycin (VA), Meropenem (MEM), Imepenem (IPM) and Cephardine (CE).

## DISCUSSION

The present study a total of 200 samples tested, 50 isolates were staphylococci. *S. aureus* was identified and classified by PCR using specific primers for housekeeping genes in these bacteria, such as the *nuc* gene. The PCR depending on a diagnostic protocol was used to detect the different genes (Mehrotra *et al*., 2000). *Nuc* gene is base line in identification and classification of *S. aureus* and in the present study, the nuc gene fragment was equal to 279 bp. Some reports indicated that the nuc gene was encoded to enzyme thermo nuclease

**Table 6.** Detection of *MecA* gene among 50 staphylococcal isolates

| | Positive No (%) | Negative No (%) |
|---|---|---|
| *MecA* gene | 34 (68%) | 16 (32%) |

**Table 7.** MIC of vancomycin for 50 isolates of *Staphylococci*

| MIC ( µg/mL) | No. of sensitive strains | Percentage |
|---|---|---|
| ≤ 2 | 48 | (96%) |
| 4-8 | 49 | (98%) |
| 16-32 | 50 | (100%) |

**Table 8.** Vancomycin Screening Agar for 50 staphylococci

| | Sensitive | Resistant |
|---|---|---|
| Vancomycin 6µg/ml | 49 | 1 |

**Table 9.** Detection of VanA gene among 50 staphylococcal isolates

| | Positive | Negative |
|---|---|---|
| VanA gene | 0 | 50 |

and the length fragment of *nuc* gene was equal to 279 bp (Brakstad *et al*., 1992). By applying PCR method, among the 126 clinical samples that were identified as *S. aureus* with phenotypic methods, 101 (80.2%) isolates were found to be *nuc* positive (Roxana *et al*., 2014), this agrees with our results.

Infections caused by methicillin-resistant *S. aureus* have been associated with high morbidity and mortality rates. MRSA is one of the common causes of hospital-acquired infections and 30 to 80 percent methicillin resistance in *S. aureus* based on antibiotic sensitivity tests has been reported from different hospitals Anupurba *et al.,* (2003).

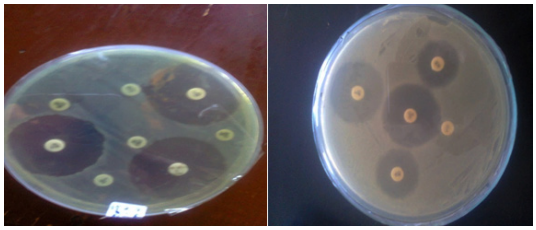

**Fig. 1.** Disc diffusion method with inhibition zone for some antibiotics against *S. aureus*

Antibiotic susceptibility testing has been found to be a good epidemiological marker for MRSA phenotyping. The present study, among MRSA isolates high degree of resistance was encountered for Ampicillin (100%), Cefoxitin (100%), Cephardine (88%), Oxacillin (84%), and Meropenem (74%). Our study similar to Mundhada *et al*., which also found a high level of resistance to Erythromycin (Mundhada *et al*., 2016). In our study, the isolates of *S. aureus* revealed increasing resistance against many antibiotics, such as Cefoxitin while they appeared sensitive to other antibiotics like Vancomycin. 84% isolates of *S. aureus* were Oxacillin-resistant,

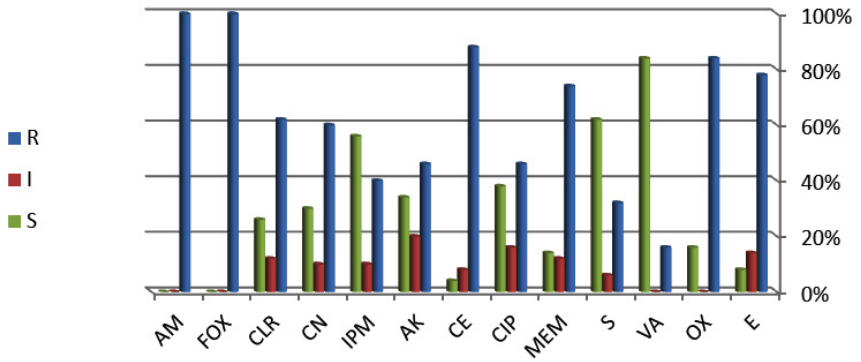


**Fig. 2.** Percentage of antibiotic resistant isolates of staphylococci

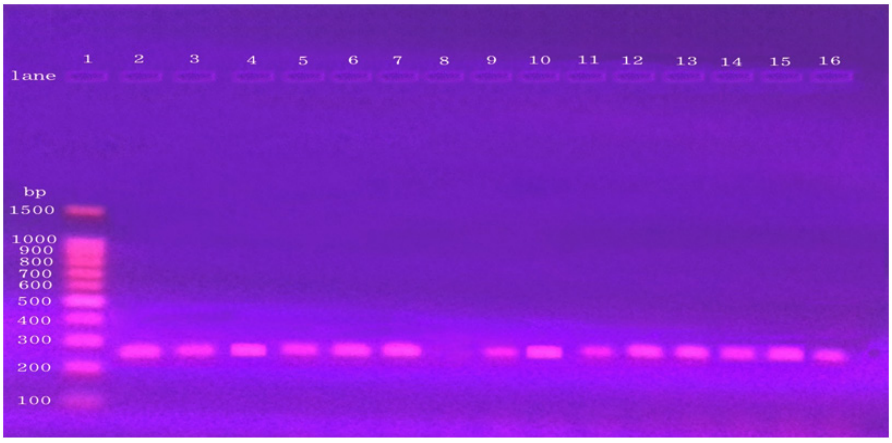


**Fig. 3.** Gel electrophoresis shows *Nuc* gene fragments

78% were Erythromycin-resistant and 16% were Vancomycin-resistant. In other reports, the percentage of Tetracycline-resistant *S. aureus* isolates was equal to 47.4% or 60.4% (Shittu *et al*., 2011) (Duran *et al.,* 2012). Our results did not agree with the reports of Duran *et al*., and Brakstad *et al*., which showed that all clinical samples of *S. aureus* were resistant to Vancomycin (Brakstad *et al*., 1992) (Duran *et al.,* 2012), and agree with Kruzel *et al*., Holland and Fowler showed that Vancomycin as a susceptible drug for treating *S. aureus* infection (Kruzel *et al*., 2011) (Holland & Fowler, 2011).

The cefoxitin disc diffusion method was more sensitivity and specificity than other routinely methods used for detection of MRSA. This method can be preferred in clinical microbiology laboratories, because it is easy to prepare (Mundhada *et al.,* 2016).

*S. aureus* is a major causes of community-acquired and nosocomial infections .They are also among the most frequently isolated bacteria in clinical microbiology laboratories (Gosbell *et al*., 2001). Simple and rapid identification and discrimination of *S. aureus* is detection of MET resistance is essential for prompt institution of effective antimicrobial chemotherapy and for limiting the unnecessary use of certain classes of antibiotics. Since MET resistance (encoded by *mec A*) are mediated by the expression of PBP2a, PCR detection of *mec A* is considered the "gold standard" for the detection of MET resistance (Zhang *et al*., 2004,

Our results confirmed the presence of *mec A* gene among 34 (68%) isolates of *S. aureus* and 16(32%) had not *mec A* gene.

Other investigators have shown that the presence of *mecA* gene correlates 100% with the detection of methicillin resistance in *S.aureus* when it is compared with the other methods (Thompson *et al*., 1982). From 126 *S.aureus* isolates, 87 (69%) isolates harbor the mec A gene and identified as methicillin-resistant *S.aureus* (MRSA) and the remaining 39 (31%) isolates were methicillin-susceptible (MSSA) (Roxana *et al*., 2014). Sixty seven (83.8%) of the 80 MRSA isolates and 26.8%

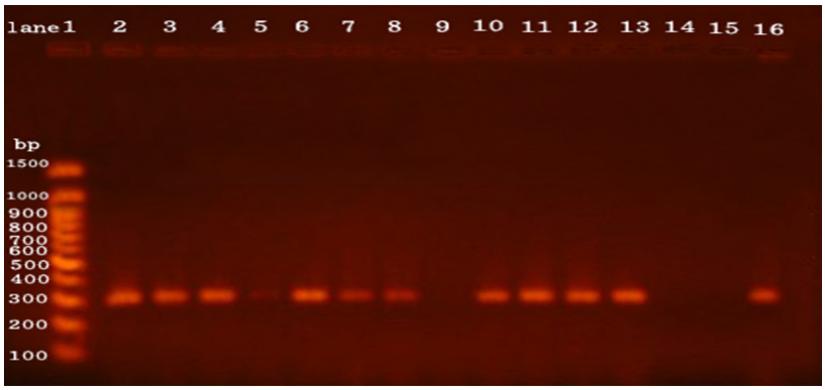


**Fig. 4.** Gel electrophoresis shows *MecA* gene fragments

**Fig. 5.** Gel electrophoresis shows *VanA* gene fragments

of the total 250 *Staphylococcus aureus* isolates which were tested, were found to be multidrug resistant MRSA (Rashedul *et al*., 2016).

The MIC value of vancomycin indicated that all isolates were sensitive to vancomycin. The genetic mechanism of vancomycin resistance in VRSA is not well understood. Several genes have been proposed as being involved in certain clinical VRSA strains (Jansen et al., 2007). The experimental transfer of the vanA gene cluster from *E. faecalis* to *S. aureus*has raised fears about the occurrence of such genetic transfer in clinical isolates of methicillin resistant *S. aureus*. In this study, all the phenotypically VRSA isolates contained *mec*A, but no isolates contained *van*A. Tiwari & Sen (2006) have also reported a *van* gene-negative VRSA.Results of Rana-Khara *et al*., showed that all strains sensitive for vancomycin (Rana-Khara *et al*., 2016), which agree with our result.

In conclusion, the results of this study showed the occurrence of *van*A gene-negative VRSA in Egypt. Though resistance to Vancomycin among *Staphylococcus aureus* isolates is not found in our study, the MRSA prevalence is higher and calls for measures to control its spread in hospital and infections due to it. Moreover regular testing to detect vancomycin resistance should be carried out so that the emergence of any resistant strain is detected, especially among the MRSA strains. A large proportion of these MRSA was found to be multidrug resistant, which takes attention to strict antibiotic policy should be enforced to curtail the irrational use of antibiotics.

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