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GENETIC STUDY OF METHICILLIN RESISTANT *S. AUREUS* (MRSA) ISOLATED FROM NEONATAL INFECTIONS

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ABSTRACT

This study aimed to detect of methicillin resistant *S. aureus* (MRSA) which caused neonatal infections and to detect the genes being responsible of antibiotic resistance in these bacteria by molecular technique (PCR). Antibiotic sensitivity test results demonstrated that, *S. aureus* isolates were highly resistant against most antibiotics, 100% for each AMP, AMC, CTX, CTR, CAZ, KF and K. whereas it's resistance ≥70% for most other antibiotics. While 0% resistance (100% sensitivity) was shown in each IPM, MRP. The results of minimum inhibitory concentration MIC by using HiComb test showed that *S. aureus* has 100% resistance for both MET and OX with MIC values ranged between (5-240µg/ml and 32-256µg/ml respectively). While 0% resistance reported for VA (0.024-0.512µg/ml). PCR results demonstrated that, among 35 isolates of *S. aurus* were determined previously as MRSA by phenotypic methods, 91.4% have *mecA* gene, 37% have *nuc gene* and 2.9% have *vanA*.

KEYWORDS: Neonates, Infections, Methicillin Resistant *S. aureus*

INTRODUCTION

Resistance to commonly used antibiotics is emerging and constitutes an important problem worldwide. In addition, the preading of antibiotics resistant bacteria in hospitals is a recognized problem, although neonates admitted from the community may also carry resistant pathogens. The wide availability of over the counter antibiotics and the inappropriate use of broad spectrum antibiotics in the community may explain this[1]. Reports of multi-drugs resistant bacteria causing neonatal infection in developing countries are increasing, particularly in neonatal intensive care unit(NICU). This study focuses on Methicillin Resistant *S. aureus*(MRSA) due to most studies indicate that this bacteria are the most common resistant[2,3]. Introduction of methicillin into medical practice in the early 1960s quickly resulted in methicillin-resistant *S. aureus* (MRSA). MRSA have resistance to the action of methicillin, and related beta-lactam antibiotics e.g. penicillins and cephalosporins as well as to several classes of antibiotics. Some MRSA are resistant to all but one or two antibiotics, notably Vancomycin-Resistant *S. aureus* (VRSA)[4]. Molecular techniques(e.g. Polymerase chain reaction(PCR) offer rapid and sensitive methods to detect the presence of resistance genes and play a critical role in the elucidation of resistance mechanisms[5].

MATERIALS AND METHODS

During a period of March (2012) to February (2013), a total of 666 samples were collected from neonates, mothers and environment in Babylon Hospital for Pediatric and Gynecology in Hilla\Iraq. Those samples included blood, urine, CSF and swabs (oral cavity, umbilical cord, skin, eye, respiratory secretions, nose and surgical site) from neonates.

While samples from mothers included amniotic fluid from mothers in delivery room, umbilical cord blood from recent neonate (cord blood) and HVS from pregnant women. Finally, samples from environment included swabs from mask of mechanical ventilator, cannula, nursery, curtage unit, disinfectant, fluid sucker, catheter, floor and stage of delivery room. Bacterial isolates were investigated for identification according to their characteristics and compared with referential references[6, 7, 8]. The characteristics being investigated for diagnosis are: colonial and cellular morphology, grame stain, selective media, biochemical testes, and API identification kits were also used to confirm the diagnosis.

Antimicrobial Susceptibility

Disc Diffusion Test was performed according to Bauer method[9] on by using (27) antibiotic discs. The antibiotic discs were placed on an inoculated Muller-Hinton agar plate and incubation at 37ºC for 18-24 hrs. Then, inhibition zone was measured and compared to standard criteria in CLSI[10].

Minimum Inhibitory Concentration (MIC) by HiComb Test: the HiComb strip was applied to an inoculated Muller-Hinton agar plate and was incubated at 37ºC for 18-24 hrs. After incubation an ellipse will appear, that intersects the MIC value scale (in µg/ml) the lowest concentration that will inhibit the growth of a test organism as determined visually by the lack of turbidity over a defined interval related to an organism's growth rate, most commonly after 18 to 24 hrs[11,12] .

Moleculler Assays: Polymerase Chain Reaction (PCR) were performed for detection the responsible genes of antibiotic resistance which included *mec* A, *nuc* and *van* A in MRSA by using the primers and PCR conditions which detailed in table 1.

Table 1: Primers Sequences and PCR Conditions for Methicillin Resistant *S. Aureus*(MRSA)

Gene	Primer Sequence (5' 3')	Product Size Bp	References	PCR Cycle Program
mec A	F: 5'- AAA ATC GAT GGT AAA GGT TGG C - 3 R: 5'- AGT TCT GCA GTA CCG GAT TTG C – 3	533	Torimiro and Torimiro (2012)	94ºC 5min 1x ---------- 94ºC 30sec 55ºC 30sec 40x 72ºC 30sec ---------- 72ºC **5min 1x**
nuc	F: 5'- GCG ATT GAT GGT GAT ACG GTT-3' R: 5'-AGC CAA GCC TTG ACG AAC TAA AGC-3'	280	Akinkunmi and Lamikanra(2010	
van A	F: 5' - GGG AAA ACG ACA ATT GC – 3' R: 5' - GTA CAA TGC GGC CGT TA –3'	732	Florence *et al*.(2004)	

RESULTS AND DISCUSSIONS

Among atotal of 510 bacterial isolates were identified from different samples revealed positive results for bacterial culture *S. aureus* accounted 50(9.80%).

Antimicrobial Susceptibility

Disc Diffusion Test: The percentages of antibiotic resistance in *S. aureus* are shown in table 2. Based on these

data, fully sensitivety (100%) was found against each of IPM, MRP which can be attributed to the fact that Carbapenems are broad-spectrum antibiotics, and it's ß-lactam rings are resistant to hydrolysis by most ß-lactamases. The activity of meropenem against most clinical isolates was comparable with imipenem[13].

S. aureus isolates were highly resistant to most antibiotics as following: 100% for each AMP, AMC, CTX, CTR, CAZ, KF and K. whereas, 94% for CPM, 82%(TOB), 80%(OX), 72%(GEN) and 70%(ME), table 2. The resistance to β-lactams is mostly due to either production of β-lactamases or lack of penicillins receptors on cell wall and/or alteration in their permeability to β-lactam antibiotics preventing the uptaking of them[14]. Level of resistance in *S. aureus* against OX and MET were accounted 80% and 70% respectively, table 2. These percentages were higher than those obtained by other studies[15,16,1713]. This variation can be assign to the popular administration of antibiotics without physician's consulting and its availability in the hospitals and pharmacies and due to differences in source of samples. The percentages of resistance to OX and ME in the present study manifest the fact that not necessarily that all ORSA isolates will be MRSA. This finding was in agreement with astudy in Egypt[18].

Among 50 isolates of *S. aureus*, 10% were vancomycine resistant(VRSA), table 2. This finding was in contrast with other studies in Iraq were reported no VRSA isolates, where found high sensitivity rate (100%) to vancomycin in Nassirya, Najaf and Babylon, respectively[19,17,20]. This variation of VRSA percentage between our result and others may be attributed to the disk diffusion procedure can not differentiate isolates with reduced susceptibility to vancomycin from susceptible isolates even when incubated for 24 hrs. Additionally, vancomycin resistant *S. aureus* (VRSA) strains may produce only subtle growth around a vancomycin disk[21,10]. On the other hand, the widespread use of vancomycin makes resistance to the drug a significant worry, Vancomycin resistance evolved in more common pathogenic organisms during the 1990s and 2000s, including vancomycin-intermediate *S. aureus* (VISA) and vancomycin-resistant *Staphylococcus aureus* (VRSA)[22].

Table 2: Percentages of Antibiotic Resistance in S. *Aureus* Isolates in This Study

Antibiotic	**AMP**	**OX**	**MET**	**AMC**	**KF**	**CPM**	**CTR**	**CTX**	**CAZ**	**IMP**	**MRP**	**GEN**	**AK**	**TOB**	**TE**	**CIP**	**NX**	**VA**	**K**	**DA**	**E**
Resistance%	**100**	**80**	**70**	**100**	**100**	**94**	**100**	**100**	**100**	**0.0**	**0.0**	**72**	**20**	**82**	**24**	**22**	**20**	**10**	**100**	**20**	**70**

AMP=Ampicillin;OX=Oxacillin;MET=Methicillin;AMC=Amoxiclave;KF=Cephalothin;CPM=Cefepime;CTR=Ceftriaxone;CTX=Cefotaxime;CAZ=Ceftazidime;IMP=Imepenem;MRP=Meropenem;GEN=Gentamycin;AK=Amikacin;TOB=Tobramycin;TE=Tetracycline;CIP=Ciprofloxacin;NX=Norfloxacin;VA=Vncomycin;K=Kanamycin;DA=Clindomycin;E=Erythromycin.

Prevents growth of a given organism the present study used MIC HiComb test and MIC values were based on break point recommended by CLSI[10] for estimation of the response. Eight antibiotics included CAZ, AK, CIP, CPM, AMC, MET, OX, and VA were tested for (MIC) against 10 isolates of *S. aureus*, MIC values for the studied antibiotics were detailed in table 3.

Table 3: The Values of Minimum Inhibitory Concentration (MIC) for Some Antibiotics against *S. aureus* Isolated in this Study

Antibiotics	CAZ 0.016 - 256	AK 0.001-256	CIP 0.001-240	CPM 0.001-240	MET 0.001-240	OX 0.016-256	VA 0.001-240	AMC 0.001-240
Standard values ≤S I R≥	8 16 32	16 32 64	1 2 4	8 16 32	8 _ 16	2 _ 4	2 4-8 16	4 _ 8
MIC ranges µg/ml	16-128	0.032-64	0.25-240	4-64	5-240	32-256	0.024_0.512	4-240
Percentage of resistant bacteria	70%	30%	10%	90%	100%	100%	0%	90%

As detailed in table 3, the percentages of resistant *S. aureus* isolates were 70%(CAZ), 30%(AK), 10%(CIP), 90%(CPM), 100%(MET), 100%(OX), 90%(AMC) and 0%(VA)(VRSA), (table 3). This findings were in agreement with a recent study in Iraq/Al-Diwanyia where reported that all *S. aureus* isolates have 100% resistant for each MET(30-240) µg/ml and OX(16-256) µg/ml[23]. While different from another report in Babylon province where the result was revealed that 75% of MRSA isolates were fully susceptible to vancomycin(VSSA) and 25% of them were VISA[20]. This variasion may be attributed to that the researchers depend on breakpoints recommended by various international committees which recommended variable values of breakpoints for vancomycin resistance[20]. As compared with disk diffusion method, the MIC seems to be more reliable and more acceptable, some published studies proposed that the MIC method is a well-standardized and reliable reference method that is useful for research purposes which provides accurate and precise results[24].

Molecular Assays

S. aurus isolates revealed positive amplification for three genes. It is found that among 35 S. *aurus* isolates were determined previously as MRSA by phenotypic methods, 32(91.4%) have *mecA* gene, 13(37.1%) have *nuc gene* and 1(2.9%) have *vanA* gene. The distribution of these genes varied in respect to the isolation source (table 4). The molecular detection of *mecA* gene, which responsible of methicillin resistance, revealed positive amplification with product size accounted for 532 bp, as shown in figure 1 *mecA* gene was distributed as detailed in table 4. The highest spread of it was found among S. *aurus* isolated from blood samples 12(37.5%). This finding was higher than many other studies who reported that incidence of MRSA which have *mec*A gene are 45.5% and less than 5% respectively[23, 25]. This results were in contrast with other reports where reported that all isolates (100%) were *mec*A positive[20, 19]. *mec*A gene carried on the Staphylococcal Cassette Chromosome *mec* (SCC*mec*). SCC*mec* is inserted into the S. *aureus* chromosome near the origin of replication[26]. Most of antibiotic resistance is transferred by plasmids, while this high prevalence of *mec*A gene in may be due to horizontal gene transfer from MRSA to MSSA isolates by transduction[27].

In the light of these results that 35 isolates were detected as MRSA depending on antibiotic susceptibility test (disk diffusion test), these isolates were selected for genetic study. As a result of PCR, only 32(91.4%) isolates revealed positive amplification for *mecA* gene. This contrast may be

Table 4: Distribution and Percentages of Responsible Genes of Antibiotic Rasistance in S. *AUREUS*

Gene / Source	*mec*A	*nuc*	*van*A
Blood	12(37.5%)	8(61.5%)	1(2.9%)
Oral Cavity swab	5(15.6%)	1(7.7%)	---
Urin	1(3.1%)	---	---
Umbilical Cord swab	3(9.5%)	2(15.4%)	---
Skin swab	5(15.6%)	---	---
Nosal swab	1(3.1%)	---	---
Surgical Sites swab	1(3.1%)	1(7.7%)	---
HVS	2(6.3%)	1(7.7%)	---
Caesarian Section	1(3.1%)	---	----
Fluid Sucker	1(3.1%)	---	---
Total	**32(91.4%)**	**13(37.1%)**	**1(2.9%)**
Total number of isolates	35	35	35

explaine by the fact that some hyper producers of penicillinase give no zone, and will therefore be falsely reported as MRSA. As well as, expression of methicillin resistance in *S. aureus* depends on environmental factors such as temperature and osmolarity[28].

Figure 1: Gel Electrophoresis of PCR Product of Meca Gene with Product Size= 532bp. The Isolates Numbered (1, 2, 3, 5, 6, 7, 8) Were Positive for *Meca* Gene, Whereas Isolates Numbered (4, 9) Were Negative

Figure 2: Gel Electrophoresis of PCR Product of Nuc Gene with Product Size= 280bp. The Isolates Numbered (1, 2, 4, 5, 6, 7, 8) Were Positive for *Nuc* Gene, Whereas Isolate Numbered (3) Was Negative

The molecular detection of *nuc* gene, which responsible of oxacillin resistance, revealed positive amplification with product size accounted for 280 bp, as shown in figure 2 Among 35 isolates, only 13(37.1%) revealed positive results and the highest freqiunce of *nuc* gene was recorded among isolates from blood 8(61.5%) , (table 4). This finding was lower than result by another studies in Iraq[23] and in Egypt[18] who found that 36.5% and 45.4% isolates had *nuc* gene, respsctively. On the other hand, The molecular detection of *van*A gene, which responsible of vancomycin, revealed positive amplification with product size accounted for 732 bp, as shown in figure 3. Only one isolate from blood sample revealed

positive amplification for *van*A gene 1(2.9%), table 4. This finding was lower than those reported in Egypt[18] where vancomysine resistance was 54.1%. On the other hand, it was in contrast with other studies in Iraq[20] were reported no VRSA isolate.

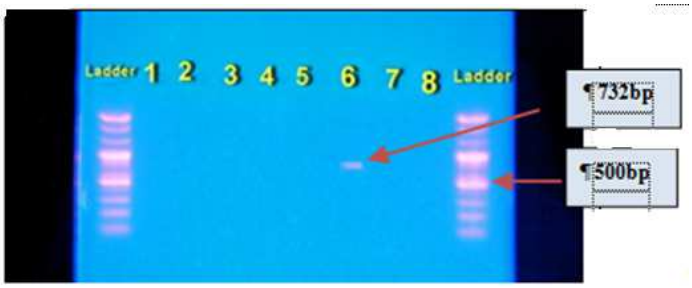

Figure 3: Electrophoresis of PCR Product of Vana Gene with Productsize= 732bp. The Isolate Numbered (6) Was Positive For *Vana* Gene, Whereas Isolates Numbered (1, 2, 3, 4, 5, 7, 8) Were Negative

VRSA refers to strains of *S. aureus* that have become resistant to the glycopeptide antibiotic vancomycin. Although high-level vancomycin resistance in *S. aureus* has been rarely reported, the increase of staphylococcal resistance to vancomycin (or another glycopeptide antibiotic, teicoplanin) which is often a treatment of choice in infections with methicillin-resistant *S. aureus* (MRSA) represents a dangeriouse healthy problem[29]. *In vitro* and *in vivo* experiments reported in 1992 demonstrated that vancomycin resistance genes from *E. faecalis* could be transferred by horizontal gene transfer to *S. aureus*, conferring high-level vancomycin resistance to *S. aureus*[30]. Until 2002 such a genetic transfer was not reported for wild *S. aureus* strains. In 2002, a VRSA strain was isolated from the catheter tip of a diabetic, renal dialysis patient in Michigan[31].

As detailed in table 4, according to the PCR results, among 35 isolates of *S. aureus* , 13 isolates were ORSA and MRSA in the same time, there is no ORSA withuot MRSA. this finding differs from the results of antibiotic susceptibility by disk diffusion test in the present study, table 2, where 10 isolates were ORSA not MRSA. On the other hand, This finding was in disagreenent with a study in Egypt, where obtained that among Out of 51 isolates of *S. aureus*, 26 had single resistance, oxacillin resistance(ORSA only)[18], This finding may indicated that losing of *mecA* gene which was in agreement with a study by[34] who clearly demonstrated that *mecA* gene was lost in 36 (14.4%) of 250 methicillin-resistant *S. aureus* isolates after 2 year of storage and the older strains could have contained a larger percentage of *mecA* negative cells to begin with, due to their longer storage period, than did the more recently isolated strains.

CONCLUSIONS

Among bacterial isolates isolated from neonatal infections, methicillin resistant *S. aureus* (MRSA) have high levels resistance against many types of antibiotics. In addition, this bacteria harbores the genes which render the organism to resist antibiotic in high rate.

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