Molecular characterization of *Staphylococcus aureus* isolated from hospital acquired sepsis in pediatrics, relation to antibiotics, resistance and virulence genes

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Abstract

Introduction **The objective of this study was to determine the prevalence of antibiotic resistance genes *mecA, vanA, B, C* and virulence genes Panton-Valentine Leucocidin (PVL) and fibronectin-binding protein (*fnBPA*) among *S. aureus* isolates from hospital-acquired sepsis from pediatric intensive care units.**

Methods **The study was a retrospective cross-sectional study, including 250 unique isolates of *S. aureus* obtained from pediatric patients with hospital-acquired sepsis. The isolates were subjected to study of antibiotic susceptibility by disc diffusion method and molecular analysis of antibiotic resistance genes and certain virulence genes (PVL and *fnBPA* genes).**

Results **Methicillin resistant *S. aureus* represented 178 (71%) of the isolated *S. aureus* and reduced susceptibility to vancomycin was detected by minimum inhibitory concentration in 39 (22%) isolates. It was found that there was a strong association between the MRSA strains and resistance to some antibiotics, devices association ($p<0.001$) and patient outcomes ($p=0.003$). There was a significant association between reduced vancomycin susceptibility ($p=0.010$), the presence of a central line catheter ($p=0.000$) and *fnBPA* gene ($p<0.001$) and mortality rate.**

Conclusions **The present study highlights that major *S. aureus* strains isolated from sepsis in pediatric patients were methicillin resistant with a substantial proportion of reduced susceptibility to vancomycin. Although none of the isolates had *van* genes responsible for vancomycin resistance, this finding warrants a considerable attention for study as it was a risk factor for mortality in those patients. The virulence genes fibronectin-binding protein and Panton-Valentine Leucocidin were not uncommon in *S. aureus*.**

Keywords ***S. aureus, mecA, van, fnBPA*, PVL.**

Introduction

Staphylococcus aureus (*S. aureus*) is a widespread pathogen associated with multiple infections in both healthcare facilities as well as community-acquired infections. The pathogen is responsible for multiple infections ranging from infections of the soft tissues to serious invasive infections such as sepsis and pneumonia. Its pathogenicity is due to many factors such as the resistance to antibiotics, production of enzymes and toxins. Methicillin resistant *S. aureus* (*MRSA*) has been a significant concern in healthcare infections worldwide.[1]

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Methicillin resistant *S. aureus* reflects the antibiotic resistance pattern of this species which simply reflects the resistance to betalactam antibiotics and limits the options available for antibiotic therapy. Methicillin resistance is due to the presence of *mecA* gene. *MecA* gene codes for the penicillin-binding protein 2a (PBP2a) which reduces the binding affinity for the betalactam antibiotics, even the penicillinase-resistant penicillin. The *mecA* gene, known as the staphylococcal cassette chromosome *mec* (*SCCmec*) is present on a mobile genetic element. The *mecA* gene complex has plasmids and transposons insertion sites which are associated with the development of resistance genes to other antibiotics.[2]

Many studies since 2002 until now reported the emergence of *S. aureus* strains with reduced susceptibility to vancomycin, a glycopeptide antibiotic that is reserved as an alternative treatment of MRSA.[3] Two different resistance mechanisms were present, intermediate resistance to *S. aureus* that occurs as a result of thickening in the cell wall, where many vancomycin molecules were trapped within the cell wall. The trapped molecules block the meshwork of peptidoglycan and eventually create a physical barrier to more incoming molecules of vancomycin;[4] these isolates are called vancomycin-intermediate *S. aureus* (VISA) with MIC of 4–8 μg/mL.[5] The second mechanism of resistance to vancomycin is due to the presence of *vanA* gene or other *van* resistance determinants, these isolates are called vancomycin-resistant *S. aureus* (VRSA) with MIC ≥ 16 μg/mL.[5]

Among the virulence factors expressed by *S. aureus* are fibronectin-binding protein A (fnBPA) and fibronectin-binding protein B (fnBPs), which are important virulence factors that mediate its action via *S. aureus* adhesion to the fibronectin, fibrinogen and elastin.[6] Additionally, fnBPA is known to be an important coating component for *S. aureus* in its internalization by nonprofessional phagocytic cells to be protected against immune response and antibiotic treatment, the process that is associated with serious infections and septic death.[7]

Another factor of virulence expressed by *S. aureus* is the Panton-Valentine Leucocidin (PVL), which is a leucotoxin that mediates the destruction of leukocytes and tissue necrosis, it is encoded by two genes, *lukS-PV* and *lukF-PV*.[8]

The aim of this study was to investigate the prevalence of *mecA*, *vanA*, *B*, C antibiotic resistance genes and virulence genes Panton-Valentine Leucocidin (PVL) and fibronectin-binding protein (fnBPA) among *S. aureus* isolates from hospital-acquired sepsis of intensive-care units in pediatric patients.

Methods

Study design

The study was a retrospective cross-sectional study that included 250 unique isolates of *S. aureus* collected from pediatric patients from intensive care units, with hospital-acquired sepsis, from Mansoura University Children Hospital, Egypt, from January 2015 to March 2018. Hospital-acquired sepsis was diagnosed according to the center of disease control criteria.[9] Their ages ranged between 20-65 months. The study was approved by the Mansoura ethical committee and approval to use isolates in future genetic studies of the microbes was received from each patient's parent.

Data collection

The resulting demographic and clinical data were obtained from the recorded electronic data system for each patient.

Bacterial isolates

Bacterial isolates were collected from each sample by standard microbiological techniques. *S. aureus* was identified by Gram stain, coagulase, catalase tests and mannitol fermentation.

Antimicrobial susceptibility test

Antibiotic disc diffusion method was used to detect antibiotic susceptibility according to Clinical and Laboratory Standards Institute guidelines (CLSI).[10] The antibiotic discs used were cefoxitin, ciprofloxacin, clindamycin, erythromycin, gentamicin, amikacin, oxacillin, rifampin, tetracycline, sulfamethoxazole plus

trimethoprim (Oxoid, Basingstoke, UK). Cefoxitin disc (30 µg) and oxacillin disc (1 µg) were used for the detection of methicillin-resistant isolates. Decreased susceptibility of the isolates to vancomycin was determined by observing the minimum inhibitory concentration (MIC) by agar dilution according to the CLSI guidelines.[10]

Following the definition of the Clinical and Laboratory Standards Institute, *S. aureus* isolates with vancomycin MIC 4–8 µg/mL were classified as vancomycin intermediate *S. aureus*, and those with MIC ≥16 µg/mL were classified as vancomycin resistant *S. aureus*, and *S. aureus* with reduced susceptibility to vancomycin with MIC from 2 µg/mL to 4 µg/mL.[10]

Detection of *mecA, vanA, B, C, fnBPA,* and PVL genes by PCR

DNA extraction

S. aureus was grown at 37°C for 18 hours on blood-agar plates. DNA was extracted by the use of DNeasy by Blood & Tissue Kit according to the manual procedures. Extracted DNA was kept frozen at -20°C before amplification procedures. The sequences of the primers used for all genes have been summarized in Table 1.

PCR detection of mecA

Amplification was achieved by using Qiagen ready to use mixture for amplification. A 1 µL volume of prepared DNA (0.5 µg) was applied to 25 µL PCR mixture with 0.7 µL of 0.8 µmol/L of each primer. The PCR thermal cycling protocol included first denaturation of 95°C for 3 min, followed by amplification for 33 cycles of 94°C for 1 min, 53°C for 30 s and 72°C for 1 min, with a final extension at 72°C for 6 min. Electrophoresis visualized the amplified products by staining with ethidium bromide and seen using UV transilluminator.[11]

PCR detection of vanA-C genes

The amplification was carried out using Qiagen amplification mixture. A 1 µL volume of prepared DNA (0.5 µg) was applied to 25 µL PCR mixture with 0.7 µL of 0.8 µmol/L of each primer. The PCR program consisted of initial denaturation step at 94°C for 3 min; followed by denaturation at 94°C for 30 seconds, annealing at 82°C, 59°C, and 58°C for *vanA*, *vanB* and *vanC* respectively for 2 min, and DNA extension at 72°C for 2 min. The reaction was terminated for 6 min after the last cycle by incubation at 72°C and the products were deposited at 4°C. PCR products (5.0 µL) were analyzed with electrophoresis after staining with ethidium bromide and seen using UV transilluminator.[12]

PCR detection of PVL gene

The PCR program consisted of initial denaturation step at 94°C for 1 min; this was followed by denaturation at 94°C for 30 s, primers annealing at 50 for 1 min, and DNA extension at 72°C for 2 min. After the last cycle, the reaction was terminated by incubation at 72°C for 6 min. PCR products (5.0 µL) were analyzed by 1% agarose gel electrophoresis and made visible by ethidium bromide staining and UV transillumination.[13]

PCR detection of fnBPA gene

After amplification for 30 cycles (30 s of denaturation at 94°C, 30 s of annealing at 57°C, and 1 min of extension at 72°C. PCR products were analysed by electrophoresis through 0.8% agarose gel stained by ethidium bromide staining and UV transillumination.[13]

Control strains

The control strains were used for the laboratory tests. For biochemical identification and antibiotics test susceptibility by disc diffusion method, *S. aureus* ATCC 29213 was used. For PCR for PVL gene, *S. aureus* ATCC 49775 was used. For detection of *fnBPA* gene, *S. aureus* ATCC was used. For *mecA* gene detection by PCR, *S. aureus* ATCC 33591 was used.[14]

Statistical analysis

Table 1. The sequences used for the studied genes and the base pair (bp) of the amplified products

Gene	Sequences of the primers	bp	Reference
mecA	F:5′-AAAATCGATGGTAAAGGTTGGC-3\ R 5′-AGTTCTGGAGTACCGGATTTGC-3\	533	11
vanA	F5′-ATGAATAGAATAAAAGTTGCAATAC R 5′-CCCCTTTAACGCTAATACGAT	1029	12
vanB	F: 5′-CCCGAATTTCAAATGATTGAAAA R 5′-CGCCATCCTCCTGCAAAA	457	12
vanC	F5′-GCTGAAATATGAAGTAATGACCA R 5′-CGGCATGGTGTTGATTTCGTT	811	12
PVL	Luks-PV: 5′AGTGAACTTATCTTTCTATTGAAAAACACTC-3\ Luks-PV: 5′-GCATCAASTGTATTGGATAGCAAAAGC-3\	433	13
fnBPA	F: 5′-CACAACCAGCAAATATAG-3\ R: 5′-CTGTGTGGTAATCAATGTC-3\	1362	13

The data were analyzed by the use of Statistical Package for Social Science (SPPS v24), (IBM, Armonk, NY, USA). The qualitative data were expressed as numbers and percentages and compared with the use of Chi-square test and p values were considered significant if <0.05. The quantitative data were analyzed as mean and standard deviation (SD). The risk factors assessment was measured by binary logistic regression and p values were considered significant if <0.05.

Results

The study included 250 children with mean age 39.9±13.1 months complaining of healthcare-acquired sepsis due to S. *aureus*.

The sepsis was diagnosed as primary sepsis with no obvious primary infection in 57% of the patients. The most frequent underlying disease was mainly renal diseases and diabetes mellitus (23% and 22% respectively), the mortality rate was 16% – Table 2.

Methicillin resistant S. *aureus* represented 178 (71%) of the isolated S. *aureus* and reduced sensitivity to vancomycin was detected by MIC in 39 (22%) isolates, data not shown.

The study of antibiotic resistance genes *mecA* and *van* genes revealed that *mecA* was detected in 71% of the isolated S. *aureus* while none of the isolated S. *aureus* had *van* genes. The prevalence of virulence genes was 24% for *fnBPA* and 4% for PVL.

The antibiotic resistance was more frequent among MRSA than non-MRSA strains and statistically significant increases were found in resistance to gentamicin (p=0.008) and clindamycin (p=0.013). There was a statistically significant increase in PVL (p=0.036) and statistically non-significant increase in *fnBPA* in MRSA than non-MRSA strains, (p=0.450). The most frequent devices associated with MRSA were urinary catheters and ventilators (p <0.001).

	Total patients (n=250)	
	No.	%
Gender		
Male	123	49%
Female	127	51%
Age, months (mean±SD)	39.9 ± 13.1	
Underlying etiology		
Diabetes mellitus	54	22%
Renal diseases	57	23%
Respiratory infections	46	18%
Hepatic disorders	33	13%
Meningitis	17	7%
Malignancy	43	17%
Type of sepsis		
Primary	141	56%
Secondary	109	44%
Type of device		
Central venous line	57	23%
Ventilator	51	20%
Urinary catheter	142	57%
Patient outcome		
Discharge	211	84%
Death	39	16%

Table 2. Demographic and clinical data of pediatric patients

Table 3. Comparison between MRSA and non-MRSA regarding antibiotics resistance, device association and patient's outcome

	MRSA (n=178)		Non-MRSA (n=72)		Chi-square value	df value	95% confidence interval		P value
	No	%	No	%			Lower bound	Upper bound	
Oxacillin	178	100	3	4	235.612	1	0.005	0.051	p<0.001
Gentamicin	42	24	7	10	6.261	1	1.222	6.729	0.008
Amikacin	40	22	19	26	0.436	1	0.430	1.520	0.307
Clindamycin	34	19	8	11	5.373	1	1.132	5.735	0.013
Vancomycin	39	22	10	14	2.093	1	0.816	3.707	0.100
Tetracycline	25	14	7	10	0.858	1	0.625	3.683	0.240
Erythromycin	35	20	11	15	0.657	1	0.647	2.847	0.268
Rifampicin	63	35	21	29	0.891	1	0.735	2.409	0.214
Trimethoprim/sulfamethoxazole	81	45	25	35	2.441	1	0.890	2.770	0.077
Ciprofloxacin	34	19	7	10	3.289	1	0.924	5.205	0.048
Cefoxitin	178	100	0	0	250.000	1			<0.001
fnBPA	44	46	19	26	0.076	1	0.490	1.711	0.450
PVL	4	2	6	8	4.945	1	0.069	0.925	0.036
Gender (male)	82	46	41	57	2.427	1	0.372	1.121	0.078
Device					19.269	2			
CLABSI	28	16	29	40					<0.001
Ventilator	36	20	15	20					
Urinary catheter	114	64	28	38					
Outcome									
Death	20	11	19	26					0.003
Discharge	158	89	53	73					

CLABSI – central line-associated bloodstream infection.

Table 4. Study of demographic, clinical and microbiological risk factors associated with mortality

Parameters	Died (n=39)					
	No.	%	Odds ratio	95% confidence interval		P value
				Lower bound	Upper bound	
Gender						
Male	24	62%	2.1	1.1	3.9	0.032
Reduced vancomycin sensitivity	16	41%	2.6	1.4	4.4	0.010
Device associated with sepsis						
Central venous catheter versus other	25	64%	4.3	2.5	7.6	<0.001
mecA	30	77%	2.02	0.9	4.6	0.064
fnBPA	23	59%	4.3	2.4	7.5	<0.001
PVL	4	10%	2.7	1.2	6.2	0.058

Moreover, a significant statistical association with patient outcomes among MRSA strains (p=0.003) was seen – Table 3.

There was a significant association between male gender (p=0.032), reduced vancomycin susceptibility (p=0.010), the presence of a central line catheter (p<0.001) and *fnBPA* gene (p<0.001) and mortality – Table 4.

Discussion

S. aureus has emerged as a major pathogen in invasive infections affecting hospitalized patients. There is unceasing rise of MRSA strains among these patients, thus, influencing proper empiric antibiotic choice and requiring longitudinal control.[15]

In the present study, strains resistant to methicillin represented 71% of the isolated *S. aureus*. Previous analysis about MRSA frequency among *S. aureus* isolates denoted that the rates ranged from 13 up to 74%.[16] These wide variations could be justified by the difference in geographical area from one country to another, different risk factors and the degree compliance with guidelines of infection control within the health system.[17]

The current findings indicate that there is a higher frequency of resistance to commonly used antibiotics for *S. aureus* treatment. A statistically significant association was reported between MRSA strains and resistance to gentamicin (p=0.008) and clindamycin (p=0.013). These findings are in line with previous data claiming that MRSA strains have emerged with concomitant resistance to many commonly used antibiotics from groups like aminoglycosides, macrolides, fluoroquinolones, chloramphenicol, and tetracycline.[18] Once a *S. aureus* isolate is characterized as an MRSA, it is instantly classified as multiple drug resistant infection as it will be non-susceptible to all categories of β-lactam such as all categories of penicillins, cephalosporins, β-lactamase inhibitors, and carbapenems. [19]

The best antibiotic of choice for treatment of MRSA is vancomycin. However, there are reports about the emergence of intermediate resistance to vancomycin.[4] The standard method for determination of vancomycin susceptibility is by MIC as accepted by Clinical and Laboratory Standard Institute.[10]

Using MIC to assess *S. aureus* susceptibility to vancomycin showed reduced susceptibility in 39 (22%) isolates. This was consistent with a previous study; it reported that 21.2% of isolated *S. aureus* had intermediate resistance to vancomycin.[20] Inappropriate long-term vancomycin usage leads to gene mutation which results in changes in the thickness of the cell wall of *S. aureus*, that may be associated with reduced sensitivity to vancomycin.[21] Nevertheless; the present data showed that none of the isolates had *van* genes. This result was in agreement with a previous study that evaluated the rare existence of *van* genes among the clinical isolates of *S. aureus*.[22]

Moreover, the frequency of virulence genes in the present study was found to be 24% for *fnBPA* and 4% for PVL. These findings are different from a previous study done in Saudi Arabia on 50 clinical isolates, it reported lack of PVL gene and lower prevalence of *fnBPA* gene (8%).[14] This may be owed to the fact that PVL is a common gene in *S. aureus* isolated from community-acquired infections, with a lower prevalence in *S. aureus* isolated from hospital-acquired infections.[22] It was found that its presence is statistically significant especially with MRSA strains (p=0.036). This finding came in agreement with other studies that focused on the spread of PVL positive MRSA strains in hospital-acquired infections.[23]

Furthermore, the invasive process of infection associated with *S. aureus* needs the presence of fibronectin-binding proteins that act as *S. aureus* invasins and the deletion of the gene encoding *fnBPA* in invasive laboratory strains, leading to a decrease in the invasive ability of these strains.[24]

The current results showed that the mortality rate reached 16% of the enrolled cases. This was comparable to an earlier study among children suffering from sepsis, which found the rate to reach 13%.[25] Generally, the mortality rate from sepsis may vary according to different factors such as the gender, the development of septic shock and multiple organ dysfunctions and the etiological pathogen of the disease (especially antibiotic-resistant bacteria) and the presence of comorbidities affect the prognosis and explain the differences from one place to another.[26]

Male gender was one of the risk factors for mortality in the present study showing statistical significance (p=0.032). There was previous assumption regarding the sexual dimorphism in the immune responses to the infection that may

have an impact on the mortality as the androgens may have immunosuppressive effects.[27] Whether this hypothesis is applicable in children or not, needs further studies.

The other risk factors for grave outcomes were the association with central venous catheter (p<0.001). This is a well-known risk factor for invasive *S. aureus* infection and bacteremia.[28] Moreover, there was an evident association between mortality and reduced sensitivity to vancomycin (p=0.010). This agreed with a previous study that demonstrated this association of reduced sensitivity to vancomycin in *S. aureus* and severe complications of sepsis.[29] Consistently, in the present study, the presence of *fnBPA* gene was found to be associated with mortality (p<0.001). Further studies are required to validate these findings.

Conclusions

The present study highlights that major *S. aureus* strains isolated from sepsis in pediatric patients were methicillin resistant with a considerable proportion of these isolates with reduced sensitivity to vancomycin. Although none of the isolates had *van* genes responsible for vancomycin resistance, this finding warrants a considerable attention for study as it was a risk factor for mortality in those patients. The virulence genes fibronectin-binding protein and Panton-Valentine Leucocidin were not uncommon in *S. aureus*. This finding can be placed under more investigation to be used as a useful strategy in developing new treatment or vaccine for *S. aureus* infections. But also, prospective studies and continuous surveillance are needed to support these finding.

Authors' contributions statement: MZ designed the research plan, organized the study and participated in the main role of editing and revising the manuscript. SG, ARE, RAL carried out all laboratory tests and coordinated the data analysis. AM collected and supervised all clinical issues of patients. The corresponding author is DA and had a major contribution in writing of the manuscript and had a role in follow up of all steps of the study. All authors read and approved the final version of the manuscript.

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