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Original article

Phenotypic and genotypic detection of antimicrobial resistance and virulence factors among *Staphylococcus aureus* clinical isolates

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ABSTRACT

Background: *Staphylococcus aureus (S. aureus)* is a world-wide nosocomial and community-acquired infectious agent. This study aimed to determine the prevalence rate of *S. aureus* infections with assessment of their antibiotic susceptibility patterns and virulence profiles using available phenotypic and genotypic methods. **Methodology:** *Staphylococcus aureus* isolates were collected and identified by conventional methods. Antimicrobial susceptibility testing was done and interpreted according to Clinical and Laboratory Standards Institute guidelines (2022) followed by macrolide lincosamide streptogramin B (MLS_B) phenotyping by D test. Detection of staphylococcal virulence (hla and etb) and antibiotic resistance (ermB and msrA) genes were also done. **Results:** Out of 152 *S. aureus* isolates, 84 (55.3%) and 68 (44.7%) were methicillin resistant (MRSA) and methicillin susceptible *S. aureus* (MSSA) respectively. Almost all MRSA isolates were beta hemolytic and susceptible to linezolid, streptogramins (100% for each) vancomycin (95.2%) and ceftaroline (90.5%). About 84.5% and 45.2% of MRSA compared to 36.8% and 27.9% of MSSA were resistant to erythromycin and clindamycin respectively. Regarding MLS_B phenotyping 44%, 9.5% and 31% of MRSA and 28.1%, 1.5% and 10.3% of MSSA were constitutive $cMLS_B$, inducible $iMLS_B$ and MS_B phenotypes respectively. hla, erm B and msrA genes were detected in 91.7%, 10.7% and 1.2% of MRSA isolates respectively. While etb gene was not detected at all among them. **Conclusion:** Methicillin and MLS_B resistance among *S. aureus* are concerning. Therefore, great efforts should be made for their accurate detection in hospital settings. Proper antibiotic stewardship program is strongly recommended to keep the benefit of antibiotics with acceptable susceptibility pattern.

Introduction

Staphylococcus aureus (S. aureus) is one of the leading causes of morbidity and mortality in both hospital and community-acquired infections especially those with high antimicrobial resistance rates [1]. About 500,000 *S. aureus*-related infections occur annually in the United States [2]. In Egypt, methicillin resistant *S. aureus* (MRSA) contributes to about 40-80% of *S. aureus* healthcare-associated infections [3].

Many reports described MRSA general prevalence rate of ≥ 20% among hospitalized patients in different Arabian countries. *Staphylococcus aureus* has the ability to survive for days or even weeks on environmental surfaces in healthcare facilities. It can withstand a wide range of temperatures, humidity and exposure to sunlight. These characteristics enable *S. aureus* to contaminate a wide range of hospital items with high infectivity [4,5].

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The World Health Organization (WHO) has considered MRSA as important antibiotic resistant bacteria and put them on their priority list. All organisms on that list require novel therapeutic approaches and substantiate an urgent need for new antibiotics options [6].

Vancomycin is the drug of choice for treating severe MRSA infections. However, its use has several limitations like: poor tissue penetration, narrow therapeutic index, slow activity potential nephrotoxicity and ototoxicity [7]. Few alternatives are available for treatment of MRSA infections. Ceftaroline (CPT) is a fifth-generation cephalosporin approved by the Food and Drug Administration for the treatment of complicated staphylococcal infections with increased affinity to penicillin binding proteins [8].

Streptogramins (Dalfopristin / Quinupristin) represent one of the few potential protein interrupting anti-staphylococcal agents that interfere with protein synthesis by binding to the 50S subunit of the bacterial ribosome like macrolides and lincosamides explaining the cross resistance to macrolide, lincosamide and streptogramins (MLS) antibiotics mediated by many resistance genes including; *erm* & *msrA* genes [9].

Different *S. aureus* strains carry different virulence factors with varying pathogenic outcomes [10]. Haemolysins and exfoliative toxins are characterized virulence factors in *S. aureus*. Haemolysins make holes in the host cell membrane, facilitating toxins entry and cell damage [8]. Also, exfoliative toxins enhance host colonization and invasion of skin and injured mucosa as they are proteases which can recognize and hydrolyze skin proteins [11].

The alarming high levels of virulent MRSA in Egyptian hospitals made awareness of MRSA control measures among the medical staff at different health centers a must; emphasizing the need for well-organized antibiotic stewardship programs with proper hand hygiene and strict disinfection measures to keep the prevalence of MRSA carriage and infections as low as possible [7]. The aim of this work is to determine the prevalence rate of *S. aureus* infections in Menoufia University Hospitals and to assess their virulence profiles & antibiotic susceptibility patterns (with the evaluation of alternative treatment options) using available phenotypic and genotypic methods focusing on MLSB phenotypes.

Methods

This cross-sectional study was performed in Medical Microbiology and Immunology Department and Clinical Pathology Department, Faculty of Medicine, Menoufia University during the period from February 2021 to May 2022. Written consents and full patient history (name, age, sex, duration of hospital stay, presence of associated co-morbidities and exposure to invasive procedures) were obtained from all patients who were admitted at different departments and ICUs of Menoufia University Hospitals. This study protocol was approved by the local Ethics Committee of the Menoufia University.

Processing of the samples:

Different clinical samples (blood, pus, urine, sputum plus surgical wound swabs) were collected from patients. These collected specimens were immediately delivered to Microbiology Laboratory within two hours to be processed and examined. Each sample was inoculated on nutrient, sheep blood and mannitol salt agar. All were incubated aerobically at 37°C for 24-48h [12].

Identification of Staphylococcus aureus:

Staphylococcus aureus isolates were identified according to conventional methods by colonial appearance (golden yellow colonies on nutrient agar, white to yellow, creamy opaque beta haemolytic colonies on sheep blood agar, mannitol fermenting colonies on mannitol salt agar); Gram's staining (Gram positive cocci arranged in in grape like clusters) and biochemical reactions (catalase positive & tube coagulase positive) [12].

Antimicrobial susceptibility tests:

Susceptibility screening tests were done for all *S.aureus* isolates by disk diffusion method and interpreted according to the Clinical and Laboratory Standards Institute guidelines (CLSI) 2022 [13]. The used antibiotic disks (Oxoid) were: penicillin (10 µg), cefoxitin (30 µg), ceftaroline (30 µg), linezolid (30 µg), macrolides; erythromycin (15 µg), lincosamides; clindamycin (2µg), streptogramins; quinupristin / dafloprístin (15 µg), tetracycline (30 µg), doxycycline (30 µg), nitrofurantoin (300 µg), trimethoprim-sulfamethoxazole (1.25 / 23.75 µg), and ciprofloxacin (5 µg). *S. aureus* ATCC 25923 was used as quality control strain.

Detection of methicillin resistance

Detection of methicillin resistance was done using cefoxitin disc (30 µg); the diameter of inhibition zone was measured, categorized as resistant if zone

diameter ≤ 21mm after 16-18 incubation hours at 34 ºC considered as MRSA [13].

Detection of vancomycin susceptibility

Vancomycin minimal inhibitory concentration (MIC) for all S. aureus isolates was determined using broth dilution method (home prepared). Vancomycin MICs ≤2 µg/mL, from 4-8 µg/mL and ≥16 µg/ mL were interpreted as vancomycin susceptible *S.aureus* (VSSA), vancomycin intermediate *S.aureus* (VISA) and vancomycin resistant *S.aureus* (VRSA) respectively according to CLSI guidelines [13].

Macrolide lincosamide streptogramin B (MLS_B) phenotyping

Staphylococcus aureus isolates were investigated for macrolide lincosamide streptogramin B (MLS_B) phenotyping by erythromycin-clindamycin double disk diffusion test (D-Zone test). The test results were interpreted as different phenotypes as follows [13,14]:

- Constitutive cMLS phenotype: if isolate was resistant to both erythromycin and clindamycin.
- Inducible $iMLS_B$ phenotype: if isolate was resistant to erythromycin and susceptible to clindamycin with positive D-zone test (flattening of clindamycin growth inhibition zone adjacent to erythromycin disc or even hazy growth).
- MS_B phenotype: if isolate was resistant to erythromycin and susceptible to clindamycin with negative D test.
- L phenotype: if isolate was resistant to clindamycin and susceptible to erythromycin.
- S phenotype: if isolate was susceptible to both erythromycin and clindamycin .

Molecular detection of S.aureus virulence genes (hla & etb) and resistance genes (ermB & msrA)

DNA extraction was carried out using the Pure Link Genomic DNA Mini Kit (Invitrogen by Thermo Fisher Scientific, USA) applying manufacturer instructions. The concentrations of DNA were assessed using the Nano-Drop™ 2000 system (Thermo Scientific, USA). The DNA extracts were kept at -20 °C until use as a template for polymerase chain reaction (PCR) amplification. PCR assay was used to detect virulence genes (*hla & etb*) and resistance genes (*msrA* & *ermB)* using PCR thermocycler (Biometra, Germany). The used primers are demonstrated in **table (1).** The PCR mixtures were subjected to thermal cycling (4 min at 95ºC for primary denaturation then 35 cycles of 30 s at 94 ºC for denaturation, followed by annealing at 54º C for 60 s, extension at 72ºC for 45 s and final extension at 72ºC for 7 min) [11,15]. PCR products were separated by agarose gel electrophoresis (1.5%) and DNA bands were visualized with UV transilluminator.

Table 1. Primers used in the study.

Gene	Primer sequence	Size BP	Reference
hla	5`- ATGAAAACACGTATAGTCAGCTCAGTAACAA-3` 5`- TTAATTTGTCATTTCTTCTTTTTCCCAATCGA- 3`	960	2
etb	5-ACAAGCAAAAGAATACAGCG-3` 5-` GTTTTTGGCTGCTTCTCTTG- 3`	226	11
msrA	5`- TCC AAT CAT TGC ACA AAA TC-3` 5`- CAA TTC CCT CTA TTT GGT GGT- 3`	162	15
ermB	5´-CCG TTT ACG AAA TTG GAA CAG GTA AAG -3´ 5´-GAA TCG AGA CTT GAG TGT GC-3	360	9

Statistical analysis

The collected data were tabulated and analyzed by SPSS (statistical package for the social science; SPSS Inc., Chicago, IL, USA) version 15 for Microsoft Windows. Descriptive statistics were used and expressed as number and approximated percentages**.**

Results

Among 405 non-repetitive Gram-positive cocci clinical isolates revealed from 842 patients

admitted at Menoufia University Hospitals during the period from February 2021 to May 2022, 152 (37.5%) were *S. aureus* (20.7% were MRSA and 16.8% were MSSA) and 191 (47.2%) were Coagulase negative *Staphylococci* (CoNS) as shown in **figure (1).**

Staphylococcus aureus were isolated from 82 male (54%) and 70 female (46%) patients from all age groups with (mean age=33.6± 12) and were most commonly isolated from surgical wound (66/152- 43.4%) followed by urine (24/152 - 15.8%), sputum (22/152- 14.5%), blood and pus samples (20/152 - 13.15% for each).

According to methicillin susceptibility, *S. aureus* isolates were classified into methicillin resistant *S. aureus* (MRSA) (84/152-55.3%) and methicillin susceptible *S.aureus* (MSSA) (68/152-44.7%). There was no statistically significant difference between MRSA and MSSA regarding type of sample but there was statistically significant difference between them regarding patient gender (53.6% of MRSA were isolated from female patients) as shown in **table (2)**. Also, there was statistically significant difference regarding age, duration of hospital stay, associated co-morbidities and exposure to invasive procedures as approximately 52.4%, 73.8%, 41.7% and 36.9% of MRSA strains were isolated from patients aged more than 50 years, stayed for more than 7 days in hospitals, with associated co-morbidities and invasive procedures respectively.

Antimicrobial susceptibility patterns of *S. aureus* isolates obtained by disk diffusion method are illustrated in **table (3)**. MRSA isolates were highly susceptible to linezolid, streptogramins (100% for each), vancomycin (95.2%) and ceftaroline (90.5%). There was a high statistically significant difference between MRSA and MSSA regarding penicillin, erythromycin and ciprofloxacin susceptibility and a statistically significant difference regarding ceftaroline, clindamycin, nitrofurantoin and trimethoprim-sulfamethoxazole susceptibility.

According to macrolide lincosamide streptogramin B (MLS_B) phenotyping by D test**,** statistically significant difference was shown between MRSA and MSSA regarding $cMLS_B$, $iMLS_B$, MS_B and S phenotypes but without statistically significant difference regarding L phenotypes as demonstrated in **table (4)**.

Regarding vancomycin susceptibility by broth dilution method, 95.2% of MRSA strains were VSSA and 4.8% were VISA. While all MSSA were VSSA as shown in **table (5).**

On blood agar, 113/152 *S.aureus* isolates (74.3%) showed beta hemolysis (positive haemolysin) including all MRSA strains (100%) and 29 isolates (42.6%) of MSSA strains with high statistic significant difference between MRSA and MSSA.

By multiplex PCR, haemolysin *hla* gene was detected in 77 MRSA isolates (91.7%). Regarding antibiotic resistance genes, *erm B* resistance gene was detected in 9 (10.7%) MRSA isolates. While *msrA* resistance gene was detected only in one MRSA isolate (1.2%). On the other hand, *etb* gene was not detected in any isolate under previously recommended PCR thermos-cycling conditions. Antimicrobial susceptibility profile regarding *erm* B gene presence in MRSA isolates was illustrated in **table (6)**.

Table 2. Samples source of *S.aureus* isolates.

Sample	**Methicillin resistant *S.aureus* MRSA (n=84)**		**Methicillin sensitive *S.aureus* (MSSA) (n=68)**		**Chi square**	***p* value**
	Males	**Females**	**Males**	**Females**		
	No (%)	**No (%)**	**No (%)**	**No (%)**		
Blood (n=20)	5 (12.8%)	5 (11.1)	7 (16.3)	3 (12)		
Wound (n=66)	18 (46.2%)	19 (42.2%)	20 (46.5%)	9 (36%)	**4.27**	**>0.05**
Pus (n=20)	4 (10.3%)	6 (13.3%)	6 (13.95%)	4 (16%)		
Sputum (n=22)	5 (12.8%)	7 (15.6%)	6(13.95%)	4 (16%)		
Urine (n=24)	7 (17.9%)	8 (17.8%)	4 (9.3%)	5 (20%)		
Total (152)	**39 (46.4%)**	**45 (53.6%)**	**43 (63.2%)**	**25(36.8%)**	**3.276**	**<0.05**

Table 3. Antimicrobial susceptibility patterns of *S.aureus* isolates.

Antimicrobial agent	MRSA(n=84)		MSSA(n=68)		Chi square	*p* value
	Susceptible No (%)	Non-susceptible No (%)	Susceptible No (%)	Non-susceptible No (%)		
Penicillin	0 (0%)	84(100%)	45(66.2%)	23(33.8%)	25.99	**<0.001**
Cefoxitin	0 (0%)	84(100%)	68(100%)	0(0%)	144	**<0.001**
Ceftaroline	76(90.5%)	8(9.5%)	68(100%)	0(0%)	4.3	**<0.05**
Ciprofloxacin	30(35.7%)	54(64.3%)	51(75%)	17(25%)	23	**<0.001**
Erythromycin	13(15.5%)	71(84.5%)	43(63.2%)	25(36.8%)	36.8	**<0.001**
Clindamycin	46(54.8%)	38(45.2%)	49(72.1%)	19(27.9%)	4.797	**<0.05**
Tetracycline	29(34.5%)	55(65.5%)	31(45.6%)	37(54.4%)	1.9	**>0.05**
Doxycycline	29(34.5%)	55(65.5%)	33(48.5%)	35(51.5%)	3.05	**>0.05**
Nitrofurantoin	24(28.6%)	60(71.4%)	32(47.1%)	36(52.9%)	5.5	**<0.05**
Trimethoprim-sulfamethoxazole	21(25%)	63(75%)	29(42.6%)	39(57.4%)	5.3	**<0.05**
Linezolid	84(100%)	0(0%)	68(100%)	0(0%)	0.02	**>0.05**
Streptogramins	84(100%)	0(0%)	68(100%)	0(0%)	0.02	**>0.05**

Table 4. MLS_B antibiotics phenotypes in *S. aureus* isolates**.**

Phenotypes	Erythromycin susceptibility result	Clindamycin susceptibility result	MRSA (n=84)	MSSA (n=68)	Chi square	*p* value
			No (%)	No (%)		
[a]cMLS$_B$	Resistant	Resistant	37 (44%)	17 (25%)	5.952	<0.05
[b]iMLS$_B$	Resistant	Susceptible and D test positive	8 (9.5%)	1 (1.5%)	4.38	<0.05
[c]MS	Resistant	Susceptible and D test negative	26 (31%)	7 (10.3%)	10.193	<0.05
[d]L	Susceptible	Resistant	1 (1.2%)	2 (2.9%)	0.595	> 0.05
[e]S	Susceptible	Susceptible	12 (14.3%)	41 (60.3%)	35.026	<0.05

MLS_B: macrolide lincosamide-streptogramin B family of antibiotics

a- $cMLS_B$: constitutive resistance to MLS_B antibiotics
b- $iMLS_B$: inducible resistance to MLS_B antibiotics
c- MS_B: macrolide-streptogramin $_B$ phenotype (resistance only to erythromycin)
d- L: resistance only to clindamycin
e- S: susceptible to both erythromycin and clindamycin.

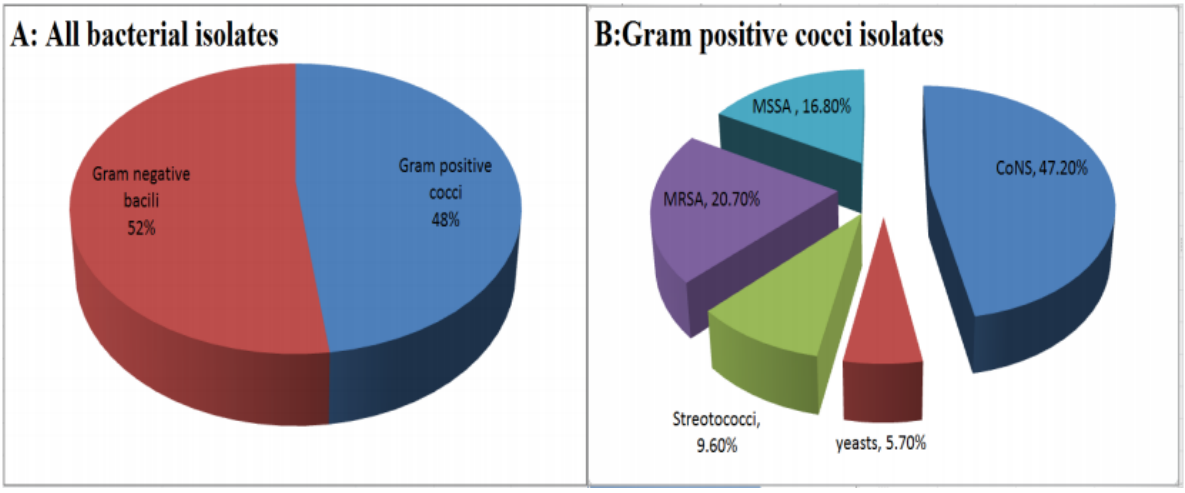
Table 5. Vancomycin susceptibility pattern of *S.aureus* isolates.

Vancomycin susceptibility	VSSA (MIC ≤2 µg /mL) No (%)	VISA (MIC 4-8 µg /mL) No (%)	VRSA (MIC ≥16 µg /mL) No (%)
MRSA (N=84)	80 (95.2%)	4 (4.8%)	0 (0%)
MSSA (n=68)	68 (100%)	0 (0%)	0 (0%)

Table 6. Antimicrobial susceptibility profile of MRSA regarding *ermB* gene **.**

Antimicrobial agent	*ermB* positive (n=9)		*ermB* negative (n=75)	
	Susceptible		Susceptible	
	No.	%	No.	%
Penicillin	0	0	0	0
Cefoxitin	0	0	0	0
Ceftaroline	6	66.7	70	93.3
Ciprofloxacin	0	0	28	37.3
Erythromycin	0	0	13	17.3
Clindamycin	1	11.1	45	60
Tetracycline	0	0	35	46.7
Doxycycline	0	0	35	46.7
Nitrofurantoin	0	0	27	36
Sulfa-trimethoprim	0	0	25	33.3
Linezolid	9	100	75	100
Streptogramins	7	77.8	75	100

Figure 1. Distribution of isolated bacteria.



Discussion

The center for disease prevention & control (CDC) has labeled MRSA as a serious threat to human health. It is implicated in numerous diseases ranging from superficial skin infections to sepsis**.** Accurate numbers describing the prevalence and characteristics of these infections and the added burden they cause to developing countries in the Eastern Mediterranean and African regions are lacking [5,7].

In this study, *S. aureus* had represented 37.5% of Gram-positive cocci isolates from hospital acquired infections (20.7% were MRSA and 16.8% were MSSA). Matching Egyptian study done by **El Shimy et al.** [7] Variable results were obtained by Chinese study of **Zheng et al.** with 9% for MRSA & 26.8 % for MSSA [16]. Variability in geographic and demographic characters could be the reason for result variations.

Results concerned with age and gender distribution for MRSA and MSSA in our study are matching those obtained by **Sreedharan and Pai**, with common urinary tract infections among females (56%) while wound infections were common among males (44%) [8]. With matched age groups similar to results obtained by **Budzynska et al**. [17]. In recent study; old age, prolonged hospital stay, invasive processing & associated co-morbidities were significant risk factors for MRSA infections in line with recent CDC reports [5].

Methicillin resistant *S. aureus* resistance was more than 40% for ciprofloxacin, erythromycin, clindamycin, tetracycline, doxycycline, nitrofurantoin and sulfa-. Similar results were reported by **Lin et al.** [2] and **Preda et al. [**18]. However, higher rate (67.5%) was reported by **Thapa et al.** [14].

Our results revealed that, 63.2% and 37.5% of *S.aureus* were resistant to erythromycin and clindamycin respectively; with higher resistance in MRSA (84.5%, 45.2%) isolates compared to MSSA isolates (36.8%, 27.9%). Similar results were obtained by **Pardoa et al. [19].** According to macrolide lincosamide streptogramin B (MLSB) phenotyping by D test, 44%, 9.5%,3 1%, 1.2% and 14.3% of MRSA and 25%, 1.5%, 10.3%, 2.9% and 60.3% of MSSA were constitutive cMLSB, inducible iMLSB, MSB, L and S phenotypes respectively with statistically significant difference between MRSA and MSSA regarding different phenotypes. Variable results were noticed in previous studies as **Wang et al.** recorded that 18% and 10.5% of MRSA isolates showed constitutive resistance and inducible resistance phenotype respectively [20]. **Attia et al**. in Egypt reported 18% 10% and 16%c of MRSA isolates were MLSB, iMLSB and MSB respectively [9]. Similarly, **Preda et al.** recorded 9% for inducible clindamycin resistance [18] as well as **Zheng et al.** by 10% [16]. On the other hand, there was no significant association between methicillin resistance in *S. aureus* and neither cMLSB nor iMLSB resistance as reported by **Thapa et al.** [14].

High susceptibility rates among MRSA isolates were noticed in our study to linezolid, streptogramins (100% for each), vancomycin (95.2%) and ceftaroline (90.5%). In agreement with others; **Attia et al.[9], Shariati et al.[21,22]** and **Vecchia et al.** [23]. So, proper antibiotic stewardship program is strongly recommended to keep the benefit of these antibiotics in treatment of MRSA infections with reinforcement of infection control measures to limit the spread of vancomycin non- susceptible isolates within our hospitals and using of more accurate methods in *S.aureus* identification such as MALDI-TOF or PCR as some coagulase-negative *staphylococci* (*CoNS*) can be misinterpreted as *S.aureus* by manual biochemical reactions. Variable vancomycin susceptibility patterns of *S.aureus* were determined in previous Egyptian studies. **Elshimy et al.** [7] had reported VRSA in 4% of MRSA isolates. **Abdel-Maksoud et al.** [24] had reported VISA in 1.2% of hospital acquired MRSA without detection of any VRSA stains. Also, **Soliman et al**. and **Attia et al**. had reported that all MRSA strains were VSSA [3,9].

In this study, beta haemolysis (positive haemolysin) was observed in all MRSA strains. Among them 77 strains (91.7%) carried *hla* gene. Similar *hla* gene prevalence rates were detected [25-27]. On the other hand, lower prevalence rate (37.3%) was detected by **Rasmi et al.** [28].

Regarding screened antibiotic resistance genes, only one of MRSA isolates (1.2%) carried *msrA* gene and nine isolates (10.7%) carried *ermB* gene with remarkable multi drug resistance pattern. However, **Gan et al.** detected *ermB* gene encoding macrolides-lincosamides-streptogramin resistance by 83 % of *S.aureus* isolates [29]. In Egypt, **Attia et al.** could not detect *ermB* at all but could detect *msrA* by 12% [9]. In our study, *Etb* gene was not detected at all. Similar result was reported in previous studies [30,31]. Prospective studies with larger sample size are warranted to support or to verify our findings.

Conclusion

Methicillin and MLS_B resistance among *S. aureus* are concerning. Therefore; great significant efforts should be made for their regular surveillance and accurate detection in hospital settings with strict compliance to infection prevention and control measures to limit their spread. Linezolid, streptogramins, vancomycin and ceftaroline have acceptable susceptibility patterns. Proper antibiotic stewardship program is strongly recommended to keep the benefit of these antibiotics in treatment of MRSA infections. A great diversity in virulence patterns was determined; haemolysin production was detected in almost all MRSA phenotypically and genotypically. While, exfoliative (*etb*) gene was not detected at all. Further analytical studies with larger sample size are recommended to overcome any fundal or numerical limitation

Limitations of the study

We identified *S.aureus* by manual biochemical reactions and assessed vancomycin susceptibility by broth dilution method (home-prepared). Using of more accurate diagnostic methods for proper identification of *S.aureus* and proper differentiation of vancomycin non-susceptible *S.aureus* isolates into VRSA, VISA and hVISA with detection of responsible resistance genes are the subject of further studies by the authors.

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