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Research Article

Antimicrobial resistance patterns of MDR *Staphylococcus aureus* clinical isolates involved in the lower respiratory tract infections in Egypt

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ABSTRACT

Resistance of *Staphylococcus* (*S.*) *aureus* to the currently used antimicrobials has risen dramatically in the past years creating a medical challenge as therapeutic options became very limited. This study aimed to screen and detect the prevalence of some antimicrobial-resistant genes of *S. aureus* clinical isolates recovered from patients suffering lower respiratory tract infections (LRTI) in Egypt. A total of 231 bacterial isolates were recovered from sputum and bronchoalveolar lavage specimens obtained from patients with LRTI. Thirty-seven isolates (16%) were identified as *S. aureus* where seventeen isolates (46%) showed resistance to ten or more antimicrobials. The antimicrobial susceptibility testing revealed that all the tested isolates were sensitive to vancomycin and linezolid (0%), however, the lowest resistance was observed to doxycycline (3%), and the highest resistance was observed to ciprofloxacin (51%). Sixteen isolates (43%) were found resistant to cefoxitin and harbored the *mec*A gene (100%). However, the *mep*A gene was detected in only 12 isolates (75%). Extended-spectrum β-lactamase (ESBL) including, *ctx-m*, *shv* and *tem* and the *aac(6')-Ib* genes, were detected in 10 (62%) and 8 (50%) isolates, respectively. None of the carbapenem-resistant genes including *kpc*, *imp*, *vim*, *ndm*, and *oxa*, were detected in any isolate. Multiple drug resistance (MDR) is a major health concern limiting the use of common antimicrobials in therapy. Thus, new national guidelines, as well as infection control strategies including antibiotic stewardship, must be implemented in the Egyptian hospitals to limit further spread of antimicrobial resistance.

Keywords: *Lower respiratory tract infections; multiple drug-resistant; Staphylococcus aureus; extended-spectrum β-lactamase; ESBls; MDR*

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1. INTRODUCTION

LRTIs are considered the leading cause of death due to infections and the fifth overall leading cause of death **[1]**. Although *S. aureus* is considered an uncommon cause of community-acquired pneumonia, it is a frequent cause of healthcare encountered pneumonia, accounting for 20-40% of these infections **[2]**. Treatment of *S. aureus* presents a therapeutic challenge as the organism has a remarkable ability to develop resistance. Penicillin was once recommended for treatment of *S. aureus* but resistance rose dramatically and 80% of the hospital-acquired

infections were resistant by 1960 **[3]**. Nowadays almost all communities and hospitals encountered *S. aureus* are resistant to penicillin.

Methicillin-resistant *S. aureus* (MRSA) was first observed in late 1960, this was only less than one year after the introduction of methicillin **[4]**. Resistance to methicillin is mediated by the presence of *the mec*A gene which codes for an altered penicillin-binding protein called PBP2a. This altered protein has a low affinity to β-lactams and it substitutes the normal PBP in the cross-linking of the peptidoglycan chains of the bacterial cell wall **[3]**. The most severe staphylococcal infections are usually caused by MDR organisms which makes the treatment options very limited **[5]**. Generally, bacterial resistance to antimicrobials can be attributed to three main mechanisms: i) alteration of the target site for the antimicrobial; ii) production of antimicrobial inactivating enzymes; iii) decreased uptake (or increased efflux) of the antimicrobial **[6]**. Bacterial resistance to antimicrobials may be chromosomal-mediated, or plasmid-mediated. Plasmid-mediated resistance is more problematic due to the capability of plasmids of horizontal gene transfer **[7]**. This accounts for the rapid spread of antimicrobial-resistant determinants among bacterial species.

Many resistant bacteria can produce enzymes that irreversibly inactivate the antimicrobial agent; such as the aminoglycoside-modifying enzymes and the β-lactamases. One of the most important and widely distributed aminoglycoside-modifying enzymes in staphylococci is the aminoglycoside acetyltransferase; AAC(6')-I **[8]**. Another group of antimicrobial inactivating enzymes of great medical importance is the β-lactamases. More than half of all currently used antimicrobials in therapy belong to the β-lactam group, but their clinical effectiveness is severely limited by the production of these inactivating enzymes **[9]**. Over the years new β-lactamases have emerged and variants of existing enzymes have developed. They are called extended-spectrum β-lactamases (ESBL) due to their enhanced spectrum of activity. They include variants of TEM-1, SHV-1, CTX-M, and others **[10]**. Carbapenems were introduced to overcome the ESBL-producing bacteria, however, shortly afterward bacteria could produce carbapenemases making the therapeutic options very limited **[11]**. Examples of reported carbapenemases include KPC, IMP, NDM, VIM, and variants of OXA-48 **[12]**. Luckily, carbapenemases are only reported in Gram-negative bacteria and none are yet reported in *S. aureus* or any Gram-positive bacteria in literature.

Several experimental data have well documented the role of the multidrug efflux pumps to develop a low level of bacterial resistance to antimicrobials; however, they are a major culprit in the appearance of MDR phenotypes as they can extrude multiple unrelated compounds **[13]**. There are five families of MDR efflux pumps; they differ according to their structure and energy requirements. They are called adenosine-triphosphate (ATP)-binding cassette (ABC) superfamily, the major facilitator superfamily (MFS), the multidrug and toxic compound extrusion (MATE) family, the small multidrug resistance (SMR) family and the resistance-nodulation-cell division (RND) superfamily **[14]**. The first chromosomal encoded multidrug efflux pump described in *S. aureus* was the MepA transporter **[13]**. Nowadays, the treatment of MDR *S. aureus* represents a medical challenge as very narrow therapeutic options are still active.

Therefore, this study aimed to elucidate the antimicrobial-resistant profiles of some *S. aureus* clinical isolates recovered from patients suffering from LRTI in Egypt as well as detection of most important antimicrobial-resistant genes that are

commonly involved in the resistance to the common antimicrobial agents used in the treatment of staphylococcal infections.

2. MATERIALS AND METHODS

2.1. Microorganisms

A total of 231 clinical bacterial isolates were obtained during the period from January 2016 to May 2017 from the microbiology laboratory at Al-Demerdash Hospital, Cairo, Egypt. These isolates were recovered from specimens collected from patients suffering from acute pneumonia. Specimens included sputum collected from either outpatients or patients requiring hospitalization. Only patients who did not receive previous antimicrobial treatment were included in the study. The isolates were identified microscopically by Gram stain and biochemically by catalase and coagulase tests **[15]**. They were further purified by streaking on the surface of fresh mannitol salt agar plates. For short term preservation, the isolates were cultured monthly on fresh nutrient agar slants and kept at 4 °C. For long term preservation, glycerol stocks were prepared and stored at -80 °C. The whole study was approved by the Faculty of Pharmacy, Ain Shams University Research Ethics Committee (ENREC-ASU-Nr. 94) where both informed and written consent were obtained from patients or parents of patients after explaining the study purpose.

2.2. Antimicrobial Susceptibility Test

The antimicrobial susceptibilities were determined by the disk diffusion method on Mueller Hinton agar plates as recommended by the clinical and laboratory standard institute (CLSI) **[16]**. Disks were obtained from Oxoid®, UK and Bioanalyse®, Turkey. The tested antimicrobials were: amikacin (30 µg), gentamicin (10 µg), tobramycin (10 µg), amoxicillin (25 µg), amoxicillin/clavulanic acid (20/10 µg), cefadroxil (30 µg), cefuroxime (30 µg), cefoxitin (30 µg), cefotaxime (30µg), cefepime (30µg), meropenem (10 µg), vancomycin (30 µg), linezolid (30 µg), ciprofloxacin (5 µg), levofloxacin (5 µg), doxycycline (30 µg), tetracycline (30 µg), trimethoprim/sulfamethoxazole SXT (1.25/23.75 µg), azithromycin (15 µg), clarithromycin (15 µg), erythromycin (15 µg) and clindamycin (2 µg). *S. aureus* ATCC 25923 was used for the quality control of this experiment according to CLSI guidelines **[16]**.

2.3. Phenotypic Detection of MRSA Isolates

MRSA isolates harboring the *mec*A-coding gene could be easily detected using cefoxitin (30 µg) disks. Cefoxitin is used as a surrogate for *mec*A-mediated oxacillin or methicillin resistance **[16]**. The test was done according to the CLSI guidelines. Freshly (18 to 24 h incubation period) isolated colonies of the test isolate were suspended in isotonic saline to match the turbidity of 0.5 McFarland standard suspension. Then, the surface of an MHA plate was swabbed in three different directions and along the rim of the plate. A disk containing 30 µg of cefoxitin was placed on the surface of the plate and gently pressed. The plate was incubated at 37 °C for 16 to 18 h. Isolates having inhibition zone (IZ) diameters with cefoxitin ≤ 21 mm are considered *mec*A positive. These isolates were selected for further study.

2.4. Detection of Selected Resistance Genes

Genomic and plasmid DNA was extracted from the MDR isolates using the purchased extraction kits according to the manufacturers' instructions. The genomic DNA was extracted using a Genomic DNA extraction Kit (Thermo Scientific, USA) and the plasmid DNA was extracted using the GeneJet plasmid miniprep kit (Thermo Scientific, USA). The extracted DNA was used as the template in the polymerase chain reaction (PCR) amplification cycles. The PCR

products were detected by agarose gel electrophoresis (AGE) **[17]**. The primers (oligonucleotides) used to amplify the studied resistance genes are listed in **Table 1**.

Table 1 Oligonucleotides used in this study, their target resistance genes, their expected product sizes (bp), their references and the used annealing temperatures (T_a)

Target gene		Primer sequence (5'→3')	Size (bp)	T_a (°C)	Reference
***bla*$_{KPC}$**	P_f	TGTCACTGTATCGCCGTC	1100	50	**[18]**
	P_r	CTCAGTGCTCTACAGAAAACC			
***bla*$_{IMP}$**	P_f	CTACCGCAGCAGAGTCTTTG	587	50	**[19]**
	P_r	AACCAGTTTTGCCTTACCAT			
***bla*$_{VIM}$**	P_f	TCTACATGACCGCGTCTGTC	748	50	**[20]**
	P_r	TGTGCTTTGACAACGTTCGC			
***bla*$_{NDM}$**	P_f	GGTTTGGCGATCTGGTTTTC	621	50	**[21]**
	P_r	CGGAATGGCTCATCACGAT			
***bla*$_{OXA}$**	P_f	GCGTGGTTAAGGATGAACAC	438	50	**[22]**
	P_r	CATCAAGTTCAACCCAACCG			
***bla*$_{CTX-M}$**	P_f	CGCTTTGCGATGTGCAG	550	50	**[23]**
	P_r	ACCGCGATATCGTTGGT			
***bla*$_{SHV}$**	P_f	GGTTATGCGTTATATTCGCC	867	50	**[24]**
	P_r	TTAGCGTTGCCAGTGCTC			
***bla*$_{TEM}$**	P_f	ATGAGTATTCAACATTTCCG	867	50	**[24]**
	P_r	CTGACAGTTACCAATGCTTA			
aac(6')-Ib ***aac(6')-Ib-cr***	P_f	TTGCGATGCTCTATGAGTGG	358	50	**[24]**
	P_r	CGTTTGGATCTTGGTGACCT			
mecA	P_f	AAAATCGATGGTAAAGGTTGGC	533	50	**[25]**
	P_r	AGTTCTGGAGTACCGGATTTGC			
mepA	P_f	ATGTTGCTGCTGCTCTGTTC	718	50	**[26]**
	P_r	TCAACTGTCAAACGATCACG			

Some PCR products were purified using the GeneJET^{TM} purification kit (Fermentas, USA) at Sigma Scientific Services Company, Egypt. Then, they were sent for sequencing at GATC Biotech Company (Germany) through Sigma Scientific Services Company (Egypt) by the use of ABI 3730xl DNA Sequencer. They were sequenced from both the forward and reverse directions, and the obtained ABI sequence files were assembled into the final contigs using Staden Package program version 3 (http://staden.sourceforge.net/).

The resulted contigs were analyzed for the detection of ORFs using FramePlot 4.0 beta (http://nocardia.nih.go.jp/fp4/). The ORFs of the nucleotide and amino acid sequences were analyzed using BLASTn and BLASTp online tools, respectively. The resulted proteins were aligned with other homologous proteins from the GenBank database using Clustal Omega (http://www.ebi.ac.uk/Tools/msa/clustalo/). Finally, some genes were submitted in the GenBank database and their corresponding accession codes were obtained. (NCBI accession code for *mec*A gene: MK341125. NCBI accession code for *mep*A: MK341122).

2.5. Statistical Analysis

Statistical analysis of the data was performed using IBM SPSS Statistics software for Windows v. 20.0 (IBM Corp., USA). Qualitative data were expressed as frequency and percentage. A chi-

square test was used to compare categorical variables. All tests were two-tailed, and *P*-value <0.05 was considered as statistically significant.

3. RESULTS

A total of 231 bacterial isolates were recovered from patients suffering from acute pneumonia. Thirty-seven isolates (16%) were identified as *S. aureus;* their antibiogram analysis is clarified in **Table 2**. From which, 18 isolates (48%) were resistant to ≥3 classes of antimicrobial agents and considered to be MDR **[27]**. Seventeen isolates (94%) of the MDR isolates were resistant to ten or more antimicrobials. Sixteen isolates overall (43%) had IZ diameters with cefoxitin ≤ 21 mm and were considered to be MRSA isolates, these isolates were selected for further study. The antimicrobial susceptibility testing showed that all the tested isolates were sensitive to vancomycin and linezolid (0%). **Fig. 1** shows the approximate percentage of resistance to the different tested antimicrobial agents among the *S. aureus* isolates.

Table 2 Antibiogram analysis of the collected *S. aureus* isolates (n= 37)

	Antimicrobial Agent (conc. 'µg'/disc)	Sensitive		Intermediate		Resistant	
		n°	%	n°	%	n°	%
1	Amoxicillin (25)	20	54	0	0	17	46
2	Coamoxiclav (30)	21	57	0	0	16	43
3	Cefadroxil (30)	20	54	2	5	15	41
4	Cefuroxime (30)	20	54	0	0	17	46
5	Cefoxitin (30)	21	57	0	0	16	43
6	Cefotaxime (30)	20	54	1	3	16	43
7	Cefepime (30)	21	57	0	0	16	43
8	Meropenem (10)	24	65	2	5	11	30
9	Doxycyline (30)	31	84	5	13	1	3
10	Tetracycline (30)	22	59	1	3	14	38
11	Ciprofloxacin (5)	18	49	0	0	19	51
12	Levofloxacin (5)	18	49	1	2	18	49
13	Amikacin (30)	26	70	2	6	9	24
14	Gentamicin (10)	19	51	1	3	17	46
15	Tobramycin (10)	19	51	0	0	18	49
16	Cotrimoxazole (25)	29	78	1	3	7	19
17	Azithromycin (15)	19	51	0	0	18	49
18	Clarithromycin (15)	19	51	1	3	17	46
19	Erythromycin (15)	19	51	0	0	18	49
20	Clindamycin (2)	24	65	0	0	13	35
21	Vancomycin (30)	37	100	0	0	0	0
22	Linezolid (30)	37	100	0	0	0	0

All MRSA isolates (100%) harbored the *mec*A gene responsible for the production of altered PBP2a (NCBI accession code: MK341125). Twelve isolates (75%) harbored the *mep*A multidrug efflux pump gene (NCBI accession code: MK341122). Ten isolates (62%) harbored one or more of the ESBL genes; *ctx-m*, *shv* and/or *tem*. The *aac(6')-Ib* gene was detected in 8 isolates (50%). None of the carbapenem-resistant genes; *kpc*, *imp*, *vim*, *ndm* and/or *oxa*, were detected in any isolate. **Figs. 2** and **3** show the agarose electrophoresis gels of the detected resistance genes. **Table 3** shows the resistance profile of the MRSA isolates along with the detected resistance genes.

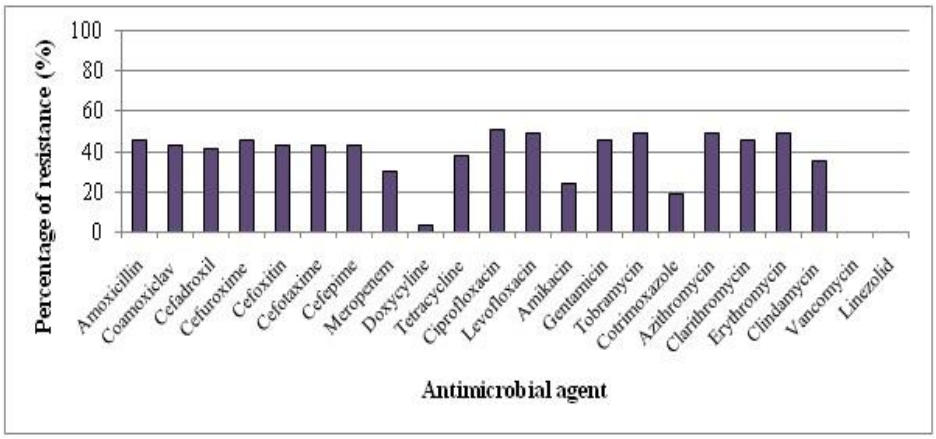

Fig. 1. Approximate percentage of resistance to the different tested antimicrobial agents among the *S. aureus* isolates (n= 37)

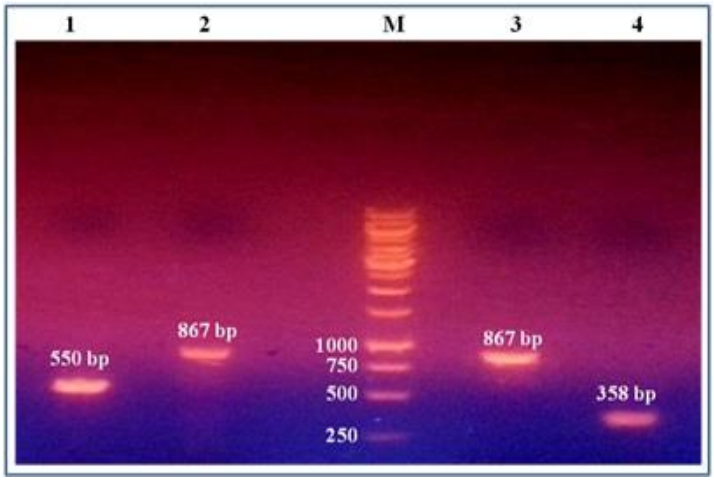

Fig. 2. Agarose gel electrophoresis of the PCR products of the resistance genes isolated from MRSA isolates 43. Lanes: 1, 550 bp PCR product of $bla_{CTX\text{-}M}$; 2, 867 bp PCR product of bla_{SHV}; M, 1 kb DNA ladder (Thermo Scientific, USA); 3, 867 bp PCR product of bla_{TEM}; 4, 358 bp PCR product of *aac(6')-Ib*

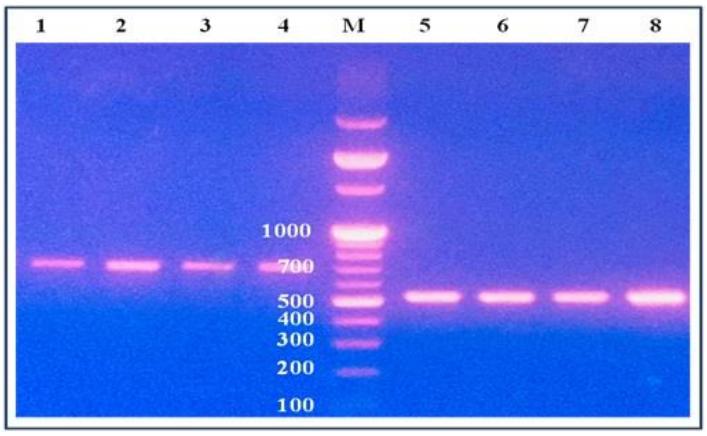

Fig. 3. Agarose gel electrophoresis of PCR products of *mepA* gene (718 bp) and *mecA* gene (533 bp) from different MRSA isolates. Lanes: 1, isolate 26; 2, isolate 43; 3, isolate 130; 4, isolate 141; M, 100bp DNA ladder (Thermo Scientific, USA); 5, isolate 32; 6, isolate 116; 7, isolate135 and 8, isolate 162

Table 3. MRSA isolates resistance profile and the detected resistance genes

Antimicrobial Agent/ Gene	Isolate Code															
	S26	S32	S36	S43	S116	S121	S125	S130	S131	S135	S137	S141	S160	S161	S162	S163
Amoxicillin	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R
Coamoxiclav	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R
Cefadroxil	R	R	R	R	R	R	R	R	R	R	I	R	R	R	R	R
Cefuroxime	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R
Cefotaxime	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R
Cefepime	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R
Meropenem	R	I	R	R	R	R	R	R	R	S	S	I	R	S	R	R
Doxycyline	S	I	S	R	I	S	S	I	S	S	S	I	S	S	S	S
Tetracycline	R	R	S	R	R	S	R	R	R	I	S	R	R	S	S	R
Ciprofloxacin	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R
Levofloxacin	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R
Amikacin	R	R	S	R	R	S	R	R	R	I	S	I	R	S	S	R
Gentamicin	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R
Tobramycin	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R
Cotrimoxazole	S	S	R	S	S	R	S	R	S	S	R	S	S	S	R	S
Azithromycin	R	R	R	S	R	S	R	R	R	R	R	R	R	R	R	R
Clarithromycin	R	R	R	S	R	S	R	R	R	R	R	R	R	R	R	R
Erythromycin	R	R	R	S	R	S	R	R	R	R	R	R	R	R	R	R
Clindamycin	R	R	S	S	R	S	R	R	R	R	R	R	R	R	R	R
Cefoxitin	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R
Vancomycin	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
Linezolid	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
***mec*A**	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
***mep*A**	+	+	-	+	+	-	+	+	+	+	+	+	+	-	-	+
***aac*6'-Ib**	+	+	-	+	-	-	-	+	+	+	-	-	+	+	-	-
ctx-m	+	-	+	+	-	-	-	+	+	-	-	+	+	+	+	+
shv	+	-	-	+	-	-	-	-	-	-	-	-	-	+	+	-
tem	-	-	+	+	-	-	-	-	-	-	-	-	-	-	-	-
kpc	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
imp	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
vim	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
ndm	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
oxa	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

Statistical analysis has shown a statistically significant association between the detection of resistance genes and the phenotypic antimicrobial resistance. Calculation of Pearson Chi-square value showed a significant association between the presence of the multidrug efflux pump; MepA, and resistance to tetracycline and sulfonamides. Also, there was a significant association between the coexistence of *tem* and *aac(6')-Ib* genes and *shv* and *aac(6')-Ib* genes.

4. DISCUSSION

This study aimed to elucidate the antimicrobial-resistant profiles of some *S. aureus* clinical isolates involved in acute pneumonia and to detect the most common antimicrobial-resistant genes. Comparing our results with a previous Egyptian study **[28]**, our findings showed a similar prevalence of *S. aureus* isolated from RTIs. Moreover, a similar, prevalence was also observed with another recent study conducted in Egypt on patients suffering from pneumonia **[29]**. Similarly, none of the isolates were resistant to vancomycin and similar resistance was observed to macrolides. However, we observed a much lower prevalence of MRSA isolates; 43% compared to 81% in the previously mentioned study **[29]**. Much lower prevalence of MRSA isolates was also observed when comparing with a third Egyptian study **[30]**; 43% compared to 77%. While similar resistance was observed with macrolides and ciprofloxacin. On the other hand, our MRSA results and resistance patterns were found similar to another Egyptian study conducted in Upper Egypt **[31]**.

Comparing our results with a systematic review on antimicrobial resistance in African countries **[32]** revealed a similar prevalence of *S. aureus* isolates; 16% in our study compared to 21.5% in the review. However, a much lower prevalence of MRSA isolates was reported in the review; 10.4% compared to 43% in our study. The review reported much higher resistance to amoxicillin, cotrimoxazole, and doxycycline; 78%, 66%, 55% respectively. On the other hand, much lower resistance was reported in the same review to amikacin, cefotaxime, ciprofloxacin, clindamycin, co-amoxiclav, erythromycin, gentamicin, levofloxacin; 3%, 28%, 21%, 11%, 23%, 33%, 18%, 5%, respectively. Similar resistance was reported to cefuroxime and tetracycline. Our study did not detect any isolate resistant to vancomycin as compared to this review which reported 2% vancomycin resistance. The review included more than 144 studies and 149.000 samples from patients all across Africa. Different resistance patterns to the tested antimicrobial agents can be explained by the fact that various patterns of prescribing antimicrobials among the countries will inevitably lead to different resistance profiles.

We compared our results with other developing African countries as we share common practices that lead to the spread of antimicrobial resistance. Resistance in developing countries is attributed to complex factors as the self-prescribing of antimicrobials by the patients, the unnecessary prescribing of antimicrobials by the physicians, the relatively poor quality of the available antimicrobials along with the overall poor hygienic pursuits **[33]**. This leads to comparatively higher levels of resistance compared with the developed countries as well as the extensive spread of MDR isolates making the therapeutic options severely limited. However, antimicrobial resistance is an issue that concerns all countries regardless of their development level; as resistant pathogens easily spread between countries and do not respect border barriers **[32]**.

All of the MRSA isolates were resistant to penicillins and cephalosporins. Besides, all of them (100%) harbored the *mec*A gene responsible for the production of altered PBP2a; this strongly highlights its importance to develop

resistance against methicillin and the other members. The wide prevalence of *mep*A multidrug efflux pump gene (75%) suggests its major role in the development of MDR *S. aureus*. None of the carbapenem-resistant genes; *kpc*, *imp*, *vim*, *ndm* and/or *oxa*, were detected in any isolate and this finding was following other studies that were undertaken worldwide. All amikacin resistant isolates harbored the *aac(6')-Ib* gene which plays a major role in resistance to aminoglycosides. Ten isolates (62%) harbored one or more of the ESBL genes; *ctx-m*, *shv* and/or *tem*.

In conclusion, accurate local periodic reports of the resistance pattern are of great importance to provide the healthcare practitioners with a clear picture and to guide them to more effective antimicrobial prescription patterns. Guided prescription policies must be implemented nationwide to limit the further spread of MDR organisms. Public awareness should also be addressed to limit the high level of antimicrobials misuse and to highlight the importance of hygienic practices.

Declarations

Ethics approval and consent to participate

The whole study was approved by the Faculty of Pharmacy, Ain Shams University Research Ethics Committee (ENREC-ASU-Nr. 94).

Consent to publish

Both informed and written consents were obtained from patients or parents of patients after explaining the study purpose

Availability of data and materials

All data generated or analyzed during this study are included in this published article in the main manuscript.

Competing interests

The authors declare that no competing interests exist

Funding Statement

No funding source was received.

Authors' contributions

The manuscript was drafted and written by SA, KMA and revised by KMA, MAY and NAH. All authors have read and approved the final manuscript.

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