

**MOLECULAR CHARACTERIZATION OF KEY VIRULENCE TRAITS
AMONG MULTIDRUG-RESISTANT METHICILLIN-RESISTANT
STAPHYLOCOCCUS AUREUS ISOLATES FROM SOME EGYPTIAN
HOSPITALS**

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ABSTRACT

Methicillin-resistant *Staphylococcus aureus* (MRSA) is an evolving cause of illness and death worldwide. MRSA strains can express a wide range of virulence factors that are implicated in their pathogenicity. The present study aimed to investigate the prevalence of crucial virulence traits encoding genes among MRSA isolates from Egyptian hospitals. A total of 170 *S. aureus* isolates were identified in this study from two Egyptian hospitals. These isolates were recovered from different clinical samples, during the period from September 2017 to December 2018. Of the 170 isolates, 138 (81.2%) were identified as MRSA by conventional microbiological methods and the identification was confirmed by the detection of methicillin resistance encoding gene *mecA*. Antimicrobial susceptibility was determined for MRSA isolates using the Kirby-Bauer disk diffusion method against 16 different antimicrobial agents representing diverse antimicrobial classes. Out of 109/138 (79%) Multidrug-resistant (MDR)-MRSA, fifty MDR-MRSA isolates were selected for further analysis of virulence encoding genes. MRSA isolates were resistant to different classes of antimicrobial agents including β -lactams, aminoglycosides, tetracyclines, macrolides and lincosamides. The antimicrobial resistance patterns among the selected 50 MDR-MRSA isolates revealed that the highest resistance rate was 100% to each of cefoxitin and penicillin, followed by doxycycline (80%), tetracycline (76%), gentamicin (74%), erythromycin (68%), clindamycin (60%) and azithromycin (50%). While the highest susceptibility rate was 88% to linezolid, followed by teicoplanin (66%), and amikacin (60%). Among the selected MDR-MRSA isolates, 52% were strong biofilm producers and 48% were moderate biofilm producers. The 50 MDR-MRSA isolates were screened for the presence of the virulence genes (*icaA*, *icaD*, *cna*, *hla*, *geh*, *tsst-1* and *Luke/D*) that are implicated in their pathogenicity. The highest frequency of virulence genes in the selected MDR-MRSA isolates was 100% to each of *icaD* and *geh*, followed by *hla* (98%), *icaA* (96%), *cna* (92%), *Luke/D* (68%), and *tsst-1* (56%). This study indicates that MRSA infection remains a significant problem in hospitals in Egypt. In addition, this study has verified a high prevalence of virulence factors among MRSA isolates from diverse clinical sources. Therefore, future studies on MRSA should aim to elucidate MRSA epidemiology, study antimicrobial susceptibility profiles, and investigate their virulence factors for effective control measures and better health management.

Key words: MRSA, Virulence Factors, Biofilm, Molecular Characterization.

1. INTRODUCTION

Staphylococcus aureus (*S. aureus*) is the most important human Gram-positive pathogen that can cause both community- and hospital-associated infections (Asghar, 2014). *S. aureus* can cause localized cutaneous infections, pneumonia, endocarditis, food poisoning and bacteremia. In addition, *S. aureus* infections can progress to life-threatening diseases (Goudarzi *et al.*, 2016). Methicillin-resistant *S. aureus* (MRSA) was first identified in 1961, causing life-threatening hospital-acquired infections (Sahebnasagh *et al.*, 2014). The emergence of MRSA has complicated the healthcare management of patients with these infections, increasing the duration of hospital stay and decreasing the efficacy of the available antimicrobials (Li *et al.*, 2019). Methicillin resistance (MR) principally results from the expression of low-affinity penicillin-binding protein PBP2a, encoded by the *mecA* gene which is located on the staphylococcal cassette chromosome *mec* (SCC*mec*) mobile genetic element resulting in resistance to β -lactam antibiotics (Raut *et al.*, 2017).

The success of MRSA as a pathogen is due to its capacity to express a variety of virulence factors that are involved in their pathogenicity. These virulence factors enhance the colonization and invasion leading to severe damage of host cells (Foster *et al.*, 2014). One of the essential virulence traits is the Microbial Surface Components Recognizing Adhesive Matrix Molecules (MSCRAMM) that mediate the initial attachment to host cells and tissues, evasion of immune responses and biofilm formation (Oliveira *et al.*, 2018). Furthermore, MRSA synthesizes a polysaccharide intercellular adhesin (PIA) involved in the biofilm formation that provides protection and resistance to cells within the biofilm (Speziale *et al.*, 2014). The immune evasion is promoted by protein A, an extracellular adherence protein, in addition to cytotoxins such as Panton-Valentine Leukocidins (PVL) and other leukotoxins (Al-Hassnawi, *et al.*, 2013). Other virulence factors include enzymes that destroy tissues and facilitate the spread of the infection such as lipases, hyaluronidases and proteases (Oliveira *et al.*, 2018). In addition, the virulence factors that are related to food poisoning (enterotoxins), toxic shock syndrome (the toxic shock syndrome toxin-1 (*tst-1*), exfoliative toxins A and B and α -toxin (Foster *et al.*, 2014).

Taking into consideration the pathogenic potentials of MRSA and the emergence of antimicrobial resistance, studies should be performed frequently to determine MRSA epidemiology, antimicrobial susceptibility patterns and virulence efficacy. In this respect, this study aimed to the molecular characterization of virulence traits among MDR-MRSA isolates from Egyptian hospitals.

2. MATERIALS AND METHODS

2.1. Isolation and identification of *S. aureus* isolates.

In this study, *S. aureus* isolates were isolated from 492 different clinical samples including 88 blood samples, 76 sputum samples, 43 urine samples, 96 wound swabs, 32 pus of an abscess, 58 eye swabs, 34 intravenous (IV) catheters samples, 53 endotracheal tubes (ETT) samples, and 12 urinary catheters samples. Under medical attention with aseptic precautions, the clinical samples were collected from hospitalized patients at two hospitals in Cairo; Al-Sayed Galal University hospital and Al-Demerdash University hospital during the period from September 2017 to December 2018. These samples were collected from patients admitted to these hospitals by the dedicated members. *S. aureus* isolates were isolated from all clinical samples and were identified according to

Procop *et al.* (2017) using conventional microbiological methods including Gram staining, growth characteristics on nutrient agar, blood agar and mannitol salt agar, and biochemical reactions. The following tests including catalase test, modified oxidase test, bacitracin susceptibility test, coagulase test, deoxyribonuclease (DN-ase) test, novobiocin susceptibility, carbohydrate fermentation, urease production, Voges-Proskauer test and nitrate reduction test were performed.

2.2. Phenotypic detection of MRSA isolates and antimicrobial susceptibilities.

For detection of MRSA, all the isolates were tested for their antimicrobial susceptibility to ceftazidime (30 µg) by disk diffusion method as illustrated in the Clinical and Laboratory Standards Institute guidelines (**CLSI, 2019**). Antimicrobial susceptibility patterns of MRSA isolates were determined using the Kirby-Bauer disk diffusion method (**Bauer *et al.*, 1966**). Sixteen antimicrobial disks (Oxoid, Hampshire, UK), representing different groups of antimicrobial agents, were used including amikacin (AK, 30 µg), azithromycin (AZM, 15µg), ceftazidime (FOX, 30µg), chloramphenicol (C, 30µg), ciprofloxacin (CIP, 5µg), clindamycin (DA, 2µg), doxycycline (DO, 30µg), erythromycin (E, 15µg), gentamicin (CN, 10µg), levofloxacin (LEV, 5µg), linezolid (LZD, 30µg), penicillin (P, 10 units), rifampicin (RD, 5µg), teicoplanin (TEC, 30µg), tetracycline (TE, 30µg) and trimethoprim/sulfamethoxazole (SXT, 1.25/23.75µg). Results were obtained by measuring the inhibition zones (average of 3 readings at 3 different angles) developed around each antimicrobial disk in millimeter (mm), and interpreted as susceptible (S), intermediate (I) or resistant (R) according to CLSI criteria (**CLSI, 2019**). In addition, the isolate was verified MDR when it showed resistance to at least three different antimicrobial classes. Fifty isolates of MDR-MRSA were selected for study.

2.3. Phenotypic detection of biofilm formation ability of MRSA isolates.

Quantitative estimation of biofilm production by MRSA isolates was performed with the micro-plate assay using 96 well polystyrene microtiter plates (Greiner, Germany), according to the method described by **Batistao *et al.* (2016)**. The tested isolates were grown in Tryptic Soy Broth (TSB) at 37°C for 24 hrs. The bacterial culture was then diluted at 1:50 in freshly prepared TSB supplemented with 1% glucose. For negative control, 200 µL TSB without bacteria was used. Aliquots of the prepared bacterial suspension (200 µL) were inoculated into wells of sterile polystyrene microtiter plates and incubated at 37°C for 24 hrs. Next, the bacterial culture was removed, and the wells were washed three times with 250 µL phosphate-buffered saline (PBS) to remove planktonic bacteria and the biofilm was fixed with 200 µL of methanol per well for 15 min. The attached biofilms were stained with 200 µL crystal violet (0.1%) for 10 min, the excess dye was discarded and the wells were washed with distilled water and air dried. Finally, the dye bound to the adherent cells was dissolved in 200 µL of 95% ethanol. The optical density (OD), an index of bacterial biofilm formation, was measured at 570 nm (OD₅₇₀) using an ELISA microtiter plate reader (Bio-Tek Instruments, Highland Park, USA). Each assay was performed in triplicate where the mean OD and standard deviation (SD) for each isolate were calculated. Three standard deviations above the mean OD of the negative control were considered as the cut-off optical density (OD_c) [OD_c = mean OD of negative control + 3 SD of negative control]. The tested isolates were classified into four categories according to their adherence and biofilm formation ability as follows: if $OD \leq OD_c$, the isolate was

classified as non-biofilm producer (-), if $OD_c < OD \leq 2 OD_c$, the isolate was classified as a weak-biofilm producer (+), if $2 OD_c < OD \leq 4 OD_c$, the isolate was classified as a moderate-biofilm producer (++) , and if $4 OD_c < OD$, the isolate was classified as a strong-biofilm producer (+++).

2.4. Molecular identification of the selected MDR-MRSA isolates, and detection of key virulence genes.

2.4.1. DNA extraction and PCR oligonucleotide primers.

Chromosomal DNA was extracted from the selected MDR-MRSA isolates using Gene Jet Genomic DNA Purification Kit (Thermo Scientific, Massachusetts, USA, K0721) according to the manufacturer's instructions. DNA extracts were stored in aliquots of 150 μ L at -20°C . The PCR oligonucleotide primers (**Table 1**) used in this study were the products of Willowfort, Birmingham, UK. The lyophilized primers of the studied genes were reconstituted in nuclease-free water and the concentration of each primer was adjusted to 10 pmole/ μ L.

2.4.2. Molecular identification of MRSA isolates by *mecA*-directed PCR assay.

The DNA extracts of the selected MDR-MRSA isolates were examined for methicillin resistance-encoding *mecA* gene by PCR. The PCR reaction was set up in a total volume of 20 μ L by adding 10 μ L of the Cosmo PCR Red master mix (Willowfort, Birmingham, UK), 1 μ L of each of forward and reverse primer (**Table 1**), 1 μ L of template DNA and the volume was completed to 20 μ L by adding 7 μ L nuclease-free water. The PCR conditions: initial denaturation at 94°C for 5 min, followed by 35 cycles of denaturation at 94°C for 30 seconds, annealing at 49°C for 30 seconds, extension at 72°C for 30 seconds, and final extension at 72°C for 7 minutes (**Maina et al., 2013**).

2.4.3. Molecular detection of target virulence encoding genes.

The selected 50 MDR-MRSA isolates were examined for the presence of some virulence genes. The target virulence genes were intercellular adhesion genes (*icaA* and *icaD*), collagen adhesin-encoding gene (*cna*) gene, α -hemolysin-encoding gene (*hla*) gene, lipase encoding gene (*geh*) gene, toxic shock syndrome toxin-1 (*tsst-1*) gene, and leukotoxins encoding gene (*LukE/D*). The procedures were performed using singleplex PCR assay. The PCR reaction for each gene was set up in a total volume of 20 μ L per reaction by adding 10 μ L of the Cosmo PCR Red master mix (Willowfort, Birmingham, UK), 1 μ L of each of forward and reverse primer, 1 μ L of template DNA and the volume was completed to 20 μ L by addition of 7 μ L nuclease-free water. The PCR oligonucleotide primers and PCR amplicon size of the primers of the tested virulence genes are listed in **Table (1)**, and the PCR cyclic conditions are listed in **Table (2)**.

2.4.4. Detection of amplified PCR products by TBE (Tris-borate-EDTA) agarose gel electrophoresis.

Detection of amplified PCR products by agarose gel electrophoresis was performed according to the procedures described by **Abdollahi et al. (2014)**. PCR products were resolved through TBE agarose gel (0.8%) electrophoresis prepared using molecular biology grade agarose (GIBCO Bethesda Research Lab.; Life Technologies, Grand Island, NY, USA) in $1\times$ TBE buffer (Thermo Scientific, Massachusetts, USA). DNA fragments were electrophoresed (at 100 V and 90 mA for 30 minutes) in the

3. RESULTS

3.1. Identification of MRSA isolates from different clinical specimens.

In this study, a total of 170 isolates of *S. aureus* were isolated from 492 clinical specimens that were collected from the two hospitals of study. The 170 isolates were identified as *S. aureus* by the typical cultural characteristics and biochemical standard methods. Of 170 *S. aureus* isolates, 138 (81.2%) isolates were confirmed phenotypically as MRSA. Out of the 138 MRSA isolates, 109 (79%) were MDR-MRSA. Fifty MDR-MRSA isolates were selected for further investigations as they showed highly multiple resistance profiles. The distribution rate of these 50 MDR-MRSA isolates from different clinical samples was 30% (15/50) from wound swabs, 26% (13/50) from blood, 10% (5/50) from sputum, 8% (4/50) from each of urine and abscess, 6% (3/50) from each of eye swabs and IV catheters, 2% (1/50) from urinary catheters, and 4% (2/50) from endotracheal tubes (**Table 3**). The phenotypically identified 50 MDR-MRSA isolates were further confirmed as MRSA by PCR-based detection of *mecA* gene. The results revealed that all the tested MDR-MRSA isolates harbored *mecA* gene and were confirmed as MRSA (**Figure 3**).

Regarding the geographical source of isolation, the selected MDR-MRSA isolates from both hospitals included in this study were 23/50 (46%) MRSA isolates from Al-Sayed Galal University hospital, and 27/50 (54%) from Al-Demerdash University hospital (**Table 3**). The distribution of the selected MDR-MRSA isolates among clinical samples and units/wards of isolation in hospitals involved in this study is shown in **Table (3)**.

Table (3): The frequency of the selected MDR-MRSA isolates among clinical sources.

clinical source	N. of isolates (%)	Hospitals		Patients' gender		Wards/Units of isolation	
		SUH (%*)	DUH (%*)	Male (%**)	Female (%**)	Type	N. (%**)
Wound swabs	15 (30%)	5 (10%)	10 (20%)	6 (40%)	9 (60%)	Outpatients	9 (60%)
						Surgery	4 (26.7%)
						ICU	2 (13.3%)
Blood	13 (26%)	7 (14%)	6 (12%)	5 (38.5%)	8 (61.5%)	ICU	12 (92.3%)
						Surgery	1 (7.7%)
Sputum	5 (10%)	3 (6%)	2 (4%)	3 (60%)	2 (40%)	ICU	3 (60%)
						Chest	2 (40%)
Urine	4 (8%)	1 (2%)	3 (6%)	3 (75%)	1 (25%)	Outpatients	3 (75%)
						Urology	1 (25%)
Abscess	4 (8%)	2 (4%)	2 (4%)	2 (50%)	2 (50%)	Surgery	4 (100%)
						Ophthalmic	2 (66.7%)
Eye swabs	3 (6%)	2 (4%)	1 (2%)	1 (33.3%)	2 (66.7%)	Outpatients	1 (33.3%)
						ICU	3 (100%)
IV catheters	3 (6%)	2 (4%)	1 (2%)	2 (66.7%)	1 (33.3%)	ICU	3 (100%)
U. catheters	1 (2%)	--	1 (2%)	1 (100%)	--	ICU	1 (100%)
ETT	2 (4%)	1 (2%)	1 (2%)	1 (50%)	1 (50%)	ICU	2 (100%)
Total	50 (100%)	23 (46%)	27 (54%)	24 (48%)	26 (52%)	--	50 (100%)

*Percentage was correlated to the total No. of the selected MDR-MRSA isolates (n = 50).

**Percentage was correlated to the total No. of the corresponding type of clinical source.

SUH: AL-Sayed Galal University Hospital; DUH: Al-Demerdash University Hospital.

3.2. Antimicrobial susceptibility patterns of the selected 50 MDR-MRSA isolates.

In the present study, the antimicrobial susceptibility patterns of the selected MDR-MRSA isolates showed that the highest resistance rate was to each of ceftazidime and penicillin (100%, 50/50), followed by doxycycline (80%, 40/50), tetracycline (76%, 38/50), gentamicin (74%, 37/50), erythromycin (68%, 34/50), clindamycin (60%, 30/50), and azithromycin (56%, 28/50). While the highest susceptibility was to linezolid (88%, 44/50), followed by teicoplanin (66%, 33/50), amikacin (60%, 30/50), and chloramphenicol (56%, 28/50) (**Table 4**).

3.3. The biofilm formation ability of MRSA isolates and the selected 50 MDR-MRSA isolates.

The microplate assay for biofilm formation showed that 126/138 (91.3%) of MRSA isolates were biofilm producers, where 26/126 (20.6%), 56/126 (44.4%) and 44/126 (35%) MRSA isolates were strong, moderate, and weak-biofilm producers, respectively. While 12/138 (8.9%) of MRSA isolates were non-biofilm producers (**Figure 1**). The investigation of biofilm formation ability of the selected MDR-MRSA isolates revealed that 26 (52%) and 24 (48%) of the tested isolates were classified as strong, and moderate biofilm producers, respectively (**Figure 2**).

Table (4): The antimicrobial susceptibility profile of the selected 50 MDR-MRSA isolates.

Antimicrobial agents	Susceptible		Intermediate		Resistant	
	No.	%*	No.	%*	No.	%*
Amikacin	30	60	2	4	18	36
Azithromycin	20	40	2	4	28	56
Chloramphenicol	28	56	--	--	22	44
Ciprofloxacin	22	44	3	6	25	50
Gentamicin	10	20	3	6	37	74
Clindamycin	17	34	3	6	30	60
Doxycycline	6	12	4	8	40	80
Erythromycin	13	26	3	6	34	68
Ceftazidime	--	--	--	--	50	100
Levofloxacin	18	36	10	20	22	44
Linezolid	44	88	--	--	6	12
Penicillin	--	--	--	--	50	100
Rifampicin	21	42	11	22	18	36
Sulfamethoxazole/Trimethoprim	26	52	11	22	13	26
Tetracycline	7	14	5	10	38	76
Teicoplanin	33	66	9	18	8	16

*Percentage was correlated to the total No. of the selected MDR-MRSA isolates (n = 50).

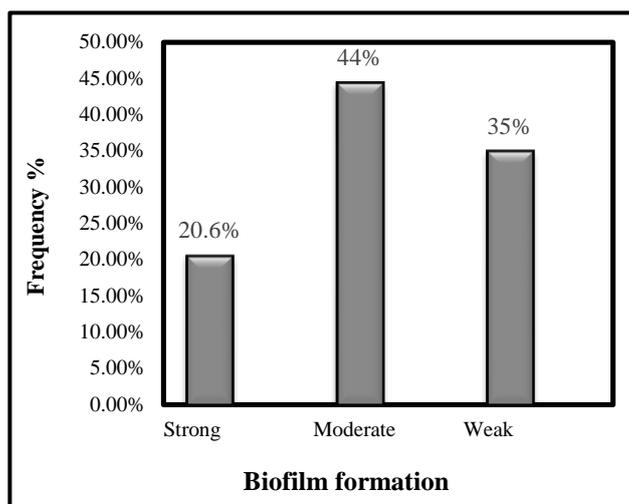


Figure (1): The biofilm formation by the 138 MRSA isolates.

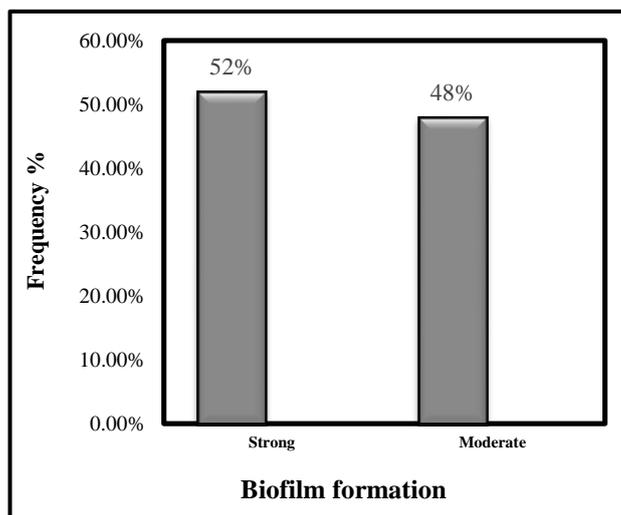


Figure (2): The biofilm formation by the 50 MDR-MRSA.

3.4. Detection of virulence encoding genes in the selected MDR-MRSA isolates.

In the present study, PCR amplification of target MDR-MRSA virulence encoding genes (**Figure 3**) revealed that 50/50 (100%) of tested MRSA isolates harbored three virulence encoding genes, 49/50 (98%) harbored four virulence genes, 48/50 (96%) harbored five virulence encoding genes, 38/50 (76%) harbored six virulence genes, and 20/50 (40%) harbored all tested virulence genes. The highest frequency of virulence genes among the selected 50 MDR-MRSA isolates was 100% to each of *icaD* and *geh*, followed by *hla* (98%), *icaA* (96%), *cna* (92%), *LukE/D* (68%), and *tsst-1* (56%) (**Figure 4**).

It was observed that all virulence genes except *LukE/D* were predominantly present in wound isolates. In wound swabs' isolates, the most predominant virulence genes were each of *icaD*, *hla* and *geh* (15/15, 100%), followed by *icaA* (14/15, 93.3%) and *cna* (13/15, 86.7%), *tsst-1* (9/15, 60%) and *LukE/D* (8/15, 53.3%). Concerning the

tested MRSA from blood, the predominant virulence genes were each of *icaA*, *icaD*, *hla* and *geh* (13/13, 100%), followed by *cna* gene (12/13, 92.3%), *Luke/D* (10/13, 77%) and *tsst-1* (8/13, 61.5%). The frequencies of virulence genes among the selected MDR-MRSA isolates from diverse clinical sources are listed in **Table (5)**.

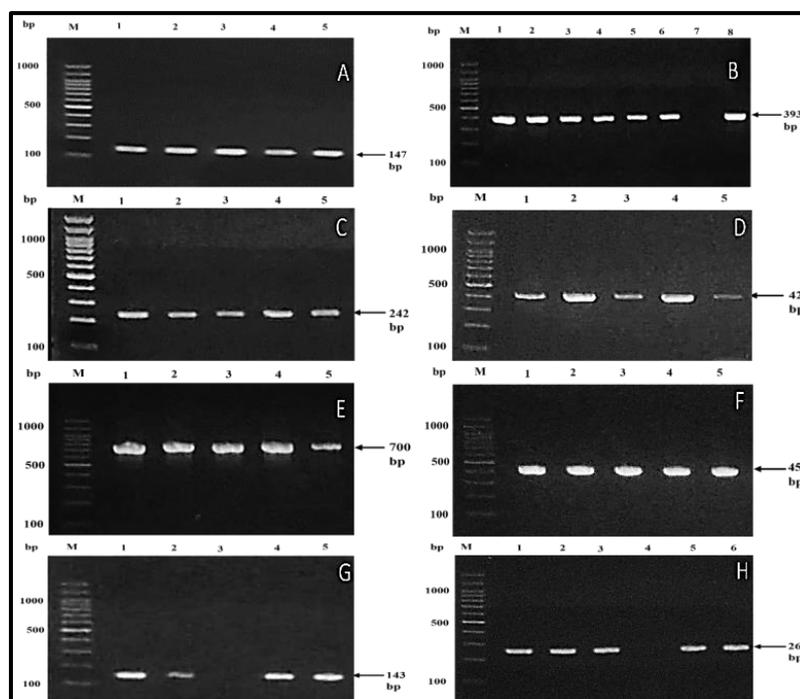


Figure (3): Representative agarose (0.8%) gel electrophoresis of PCR products of amplified *mecA* gene (A), *icaA* gene (B), *icaD* gene (C), *cna* gene (D), *hla* gene (E), *geh* gene (F), *tsst-1* gene (G), *Luke/D* gene (H) from the selected MDR-MRSA isolates, respectively. Lane M; 100-bp ladder size marker, and other lanes in each panel are the gene-directed PCR positive results giving the expected PCR products of 147 bp, 393 bp, 242 bp, 423 bp, 700 bp, 450 bp, 143 bp and 269 bp, respectively.

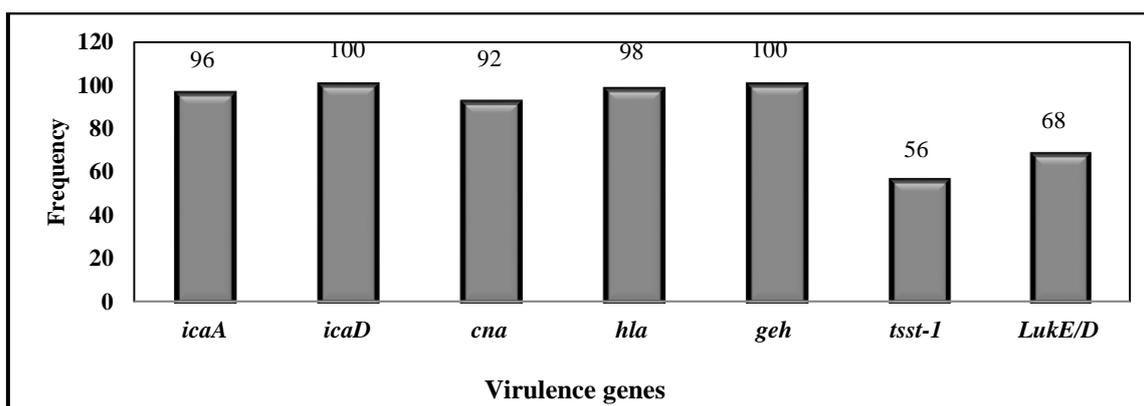


Figure (4): The frequency of virulence genes in the selected 50 MDR-MRSA isolates.

3.5. Virulence genes profiles of the selected MDR-MRSA isolates.

A total of 10 different virulence encoding genes profiles were observed among the selected 50 MDR-MRSA isolates. These patterns were arbitrarily given a number from A – J. The most common virulence genes profile was pattern A (*icaA* + *icaD* + *cna* +

hla + geh + tsst-1 + LukE/D) harbored by 40% (20/50) MDR-MRSA isolates, followed by pattern B (*icaA + icaD + cna + hla + geh + LukE/D*) harbored by 22% (11/50) MDR-MRSA isolates. All virulence genes profiles from A-J are listed **Table (6)**.

A dendrogram that included all virulence patterns was constructed based on the levels of similarity. The ten patterns were categorized into two main clusters and two singletons. Cluster 2 included 4 (40%) patterns with the highest number of isolates (41/50, 82%), and cluster 1 included 4 (40%) patterns that comprised 14% (7/50) isolates. Additionally, two singletons were determined, each included one pattern (10%) with only one isolate (2%) as shown in **Figure (5)**.

Table (5): Frequency of virulence genes among the 50 MDR-MRSA isolates from different clinical sources.

Clinical source	No. of Isolates	<i>icaA</i>	<i>icaD</i>	<i>cna</i>	<i>hla</i>	<i>geh</i>	<i>tsst-1</i>	<i>LukE/D</i>
	N. (%*)	N. (%**)	N. (%**)	N. (%**)	N. (%**)	N. (%**)	N. (%**)	N. (%**)
Wounds	15(30%)	14 (93.3%)	15 (100%)	13 (86.7%)	15 (100%)	15 (100%)	9 (60%)	8 (53.3%)
Blood	13 (26%)	13 (100%)	13 (100%)	12 (92.3%)	13 (100%)	13 (100%)	8 (61.5%)	10 (77%)
Sputum	5 (10%)	4 (80%)	5 (100%)	4 (80%)	4 (80%)	5 (100%)	2 (40%)	4 (80%)
Urine	4 (8%)	4 (100%)	4 (100%)	4 (100%)	4 (100%)	4 (100%)	2 (50%)	3 (75%)
Abscess	4 (8%)	4 (100%)	4 (100%)	4 (100%)	4 (100%)	4 (100%)	1 (25%)	2 (50%)
Eye swabs	3 (6%)	3 (100%)	3 (100%)	3 (100%)	3 (100%)	3 (100%)	2 (66.7%)	3 (100%)
IV catheters	3 (6%)	3 (100%)	3 (100%)	3 (100%)	3 (100%)	3 (100%)	2 (66.7%)	2 (66.7%)
U. catheters	1 (2%)	1 (100%)	1 (100%)	1 (100%)	1 (100%)	1 (100%)	1 (100%)	1 (100%)
ETT	2 (4%)	2 (100%)	2 (100%)	2 (100%)	2 (100%)	2 (100%)	1 (50%)	1 (50%)
Negative (%)*	—	2 (4%)	—	4 (8%)	1 (2%)	—	22 (44%)	16 (32%)

*Percentage was correlated to No. of the MDR-MRSA isolates (n = 50).

**Percentage was correlated to the corresponding total number of isolates from each type of clinical sample.

icaA and *icaD*: intercellular adhesion genes, *cna*: Collagen binding protein, *hla*: Alpha hemolysin, *geh*: Lipase encoding gene, *tsst-1*: Toxic shock syndrome toxin, *Luk*: Leukocidins genes, and U.: Urinary.

Table (6): Distribution of virulence genes profiles among the selected 50 MDR-MRSA isolates from different clinical samples.

Pattern No.	Virulence profile	Total No. of isolates in each pattern (% ^a)	No. of isolates in each clinical source (% ^b)	Clinical source	Hospitals	
					Al-Sayed Galal University Hospital (% ^b)	Al-Demerdash University Hospital (% ^b)
A.	<i>icaA + icaD + cna + hla + geh + tsst-1 + LukE/D</i>	20 (40%)	6 (30%)	Wound	3 (15%)	3 (15%)
			5 (25%)	Blood	2 (10%)	3 (15%)
			1 (5%)	Sputum	1 (5%)	—
			1 (5%)	Urine	1 (5%)	—
			1 (5%)	Abscess	1 (5%)	—
			2 (10%)	Eye swab	1 (5%)	1 (5%)
			2 (10%)	IV catheter	1 (5%)	1 (5%)
			1 (5%)	U. catheter	—	1 (5%)
			1 (5%)	ETT	—	1 (5%)

B.	<i>icaA + icaD + cna + hla + geh + Luke/D</i>	11 (22%)	2 (18.2%)	Wound	1 (9.1%)	1 (9.1%)
			3 (27.3%)	Blood	2 (18.2%)	1 (9.1%)
			2 (18.2%)	Sputum	1 (9.1%)	1 (9.1%)
			2 (18.2%)	Urine	—	2 (18.2%)
			1 (9.1%)	Abscess	—	1 (9.1%)
			1 (9.1%)	Eye swab	1 (9.1%)	—
C.	<i>icaA + icaD + cna + hla + geh</i>	8 (16%)	1 (12.5%)	Wound	—	1 (12.5%)
			3 (37.5%)	Blood	2 (25%)	1 (12.5%)
			2 (25%)	Abscess	1 (12.5%)	1 (12.5%)
			1 (12.5%)	IV catheter	1 (12.5%)	—
			1 (12.5%)	ETT	1 (12.5%)	—
D.	<i>icaA + icaD + cna + hla + geh + tsst-1</i>	5 (10%)	3 (60%)	Wound	1 (20%)	2 (40%)
			1 (20%)	Blood	—	1 (20%)
			1 (20%)	Urine	—	1 (20%)
E.	<i>icaA + icaD + hla + geh + Luke/D</i>	1 (2%)	1 (100%)	Wound	—	1 (100%)
F.	<i>icaA + icaD + hla + geh + tsst-1</i>	1 (2%)	1 (100%)	Wound	—	1 (100%)
G.	<i>icaA + icaD + hla + geh + tsst-1 + Luke/D</i>	1 (2%)	1 (100%)	Blood	1 (100%)	—
H.	<i>icaD + cna + hla + geh + tsst-1 + Luke/D</i>	1 (2%)	1 (100%)	Sputum	1 (100%)	—
I.	<i>icaA + icaD + geh</i>	1 (2%)	1 (100%)	Sputum	—	1 (100%)
J.	<i>icaD + cna + hla + geh</i>	1 (2%)	1 (100%)	Wound	—	1 (100%)

^a Percentage was correlated to the total number of the selected MDR-MRSA isolates (n = 50).

^b Percentage was correlated to total No. of isolates in each virulence pattern.

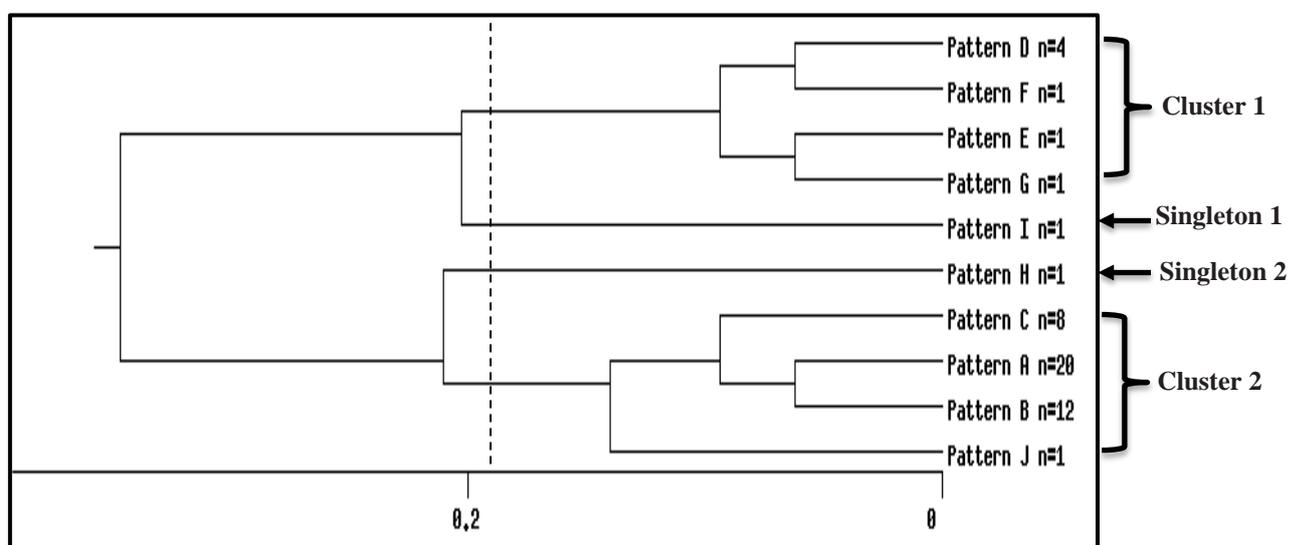


Figure (5): Dendrogram of virulence factors encoding genes patterns of the selected MDR-MRSA isolates. n, number of isolates in each pattern. Cluster analysis was generated with the Dice similarity coefficient and the UPGMA clustering method.

4. DISCUSSION

MRSA has been recognized for its ability to produce a wide range of dangerous infections in humans. This is due to the expression of diverse virulence factors that are involved in the way the disease develops, providing this bacterium the chance to attach to surfaces and/or tissues, evade or invade the immune system (Oliveira *et al.*, 2018). In this respect, this study contributes to the recognition of virulence encoding genes profiles among MDR-MRSA clinical isolates.

In this study, methicillin resistance was identified phenotypically by the cefoxitin disk diffusion method and confirmed by PCR-based amplification of the *mecA* gene; this is consistent with a study carried out by Sahebnaasagh *et al.* (2014). This finding also revealed that the cefoxitin disk was a good predictor of methicillin resistance, as 100% of cefoxitin-resistant isolates harbored *mecA* gene. In this study, considering that detection of the *mecA* gene by PCR is a gold standard method for identifying MRSA isolates, all tested MDR-MRSA isolates harbored *mecA* gene. This finding is consistent with many previous studies which reported that all MRSA isolates harbored *mecA* gene (Merlino *et al.* 2002; Fatholahzadeh *et al.* 2008; Maina *et al.* 2013; Yu *et al.* 2015; Wang *et al.* 2016; Hadyeh *et al.* 2019; Udo *et al.* 2020).

In the current study, 81.2% of *S. aureus* isolates were MRSA. This finding is in accordance with the percentages recorded in many studies which revealed that the percentages of MRSA isolates were 73.3%, 78.2%, and 88%, respectively (Rahimi *et al.* 2009; Sahebnaasagh *et al.* 2014; Hassan *et al.* 2017). While other studies showed higher rates of 89.4% and 93.3%, respectively (Ahmed *et al.* 2011; Goudarzi *et al.* 2016). Regarding the clinical source of the selected MDR-MRSA isolates in this study, their prevalence was significantly different among various clinical specimens. The highest rate of the selected MDR-MRSA isolates was from wound swabs (30%), followed by blood samples (26%), and sputum (10%). This finding agrees with a previous study by Hassan *et al.* (2017) who showed that the highest frequency of MRSA was 32.9% from wounds, 15.9% from burns, and 14.8% from sputum. Also, the results of this study are consistent with the results of many other studies which showed that the highest frequency of MRSA isolates was from wound swabs and blood samples (Fatholahzadeh *et al.* 2008; Goudarzi *et al.* 2016; Gittens-St Hilaire *et al.* 2020).

Regarding antimicrobial susceptibility, in this study, the highest rate of resistance among the 50 MDR-MRSA isolates was to cefoxitin and penicillin 100%, while the highest susceptibility rate was 88% to linezolid, followed by teicoplanin (66%). These findings are consistent with the results revealed by the studies of Fatholahzadeh *et al.* (2008) and Hassan *et al.* (2017).

In this work, the microplate assay for determination of biofilm formation ability of MRSA isolates revealed that 91.3% of MRSA isolates were biofilm producers. Whereas 20.6%, 44.4% and 35% of MRSA isolates were strong, moderate, and weak-biofilm producers, respectively. While 8.7% of MRSA isolates were non-biofilm producers. This finding is relatively consistent with the results of Piechota *et al.* (2018) study which showed that 99.2% of MRSA isolates were biofilm producers, where 39.7%, 47.9%, 11% of MRSA isolates were strong, moderate, and weak biofilm producers, respectively.

In the current study, PCR-based amplification of virulence traits encoding genes, *icaA*, *icaD*, *cna*, *hla*, *geh*, *Luke/D*, and *tsst-1*, revealed that 100% of the selected 50 MDR-MRSA isolates harbored at least three genes, 98% harbored at least four genes,

96% harbored at least five genes, 76% harbored at least six genes and 40% were carriers of all the studied virulence genes. These findings agree with the results of **Wang et al. (2016)** study which reported that all isolates showed carriage of at least four virulence genes and 42.5% of isolates harbored 10 or more virulence genes. In addition, **Yu et al. (2015)** study showed that MRSA isolates carried at least five virulence genes. Our findings are also consistent with the results of **Hassan et al. (2017)** study which reported that 92% of MRSA isolates were carriers of at least three virulence genes.

In the current study, the order of the prevalence of virulence genes among the selected 50 MDR-MRSA isolates from highest to lowest was each of *icaD* and *geh* (100%), followed by *hla* (98%), *icaA* (96%), *cna* (92%), *LukE/D* (68%) and *tsst-1* (56%). These findings are fairly consistent with the results of **Hassan et al. (2017)** study which reported that the prevalence of virulence genes among MRSA isolates was *LukE* (89.7%), *LukD* (87%), *icaD* (81.7%), *geh* (77.3%), *hla* (76.6%), *icaA* (71.5%), *LukS* (60.2%), *LukF* (59%), *cna* (40.9%), and *tsst-1* (4.55%). While the study conducted by **Abdel-Hamed et al. (2016)** showed that the prevalence of virulence genes among MRSA isolates was *lukF* (73.3%), followed by *lukE* (64%), *lukD* (44%) and *lukS* (34.7%).

Biofilm is the most significant factor that contributes to pathogenesis by acting as a barrier to antimicrobial agents and the host immune defense mechanisms which assist persistent bacterial colonization. Several studies have approved that during the late phases of attachment, bacteria attach to each other to form biofilms. This is accomplished through polysaccharide intercellular adhesin (PIA), which is synthesized by the products of *icaABCD* operon (**Moghadam et al., 2014**). In this study, regarding the presence of adhesion-related genes, *icaA* and *icaD* genes, 96% and 100% of MRSA isolates harbored *icaA* and *icaD* genes, respectively. This may indicate that almost all of the tested isolates harbored the *icaADBC* locus. Several studies conducted with MRSA isolated from different clinical sources in different countries showed the concomitant presence of both genes in most of the analyzed MRSA isolates (**Batistao et al. 2016; Wang et al. 2016**). In addition, other studies showed a high prevalence of *icaA*, 94.8% and 96.9%, respectively (**Rodrigues et al. 2013; Yu et al. (2015)**). However, **Hassan et al. (2017)** study revealed lower rates of *icaA* and *icaD* genes of 71.5% and 81.78% among the tested isolates, respectively. In addition, other studies have reported that the prevalence of both genes was 73% and 83%, respectively (**Ghasemian et al. 2015; Machuca et al. 2013**).

The collagen-binding protein encoded by *cna* gene intermediates bacterial adherence to collagen substrates and collagenous tissues, and prevents the classical pathway of complement activation (**Elasri et al., 2002**). The frequency of the *cna* gene in this study was 92%, which is higher than the rates recorded in other studies which reported that the rate of *cna* among MRSA isolates was 32%, 40.89% and 50.6%, respectively (**Yu et al. 2015; Hassan et al. 2017; Yu et al. 2012**)

Hemolysins are pore-forming toxins known to be expressed by most MRSA isolates and have a strong affinity for epithelial cells, macrophages, monocytes, fibroblasts and erythrocytes (**Goudarzi et al., 2016**). In this study, 98% of MRSA isolates were carriers of *hla* gene. The high frequency of *hla* gene agrees with the results of the study conducted by **Udo et al. (2020)** who reported the same result. The results of the present study also agree with the results revealed by **Xie et al. (2016)** and **Wang et**

al. (2016) studies where *hla* gene was detected among 100% of MRSA isolates. The study by **Yu et al.** (2015) recorded a prevalence rate of 95.3% of *hla* gene among MRSA isolates. However, other studies of **El-baz et al.** (2016), **Zarfel et al.** (2013) and **Yu et al.** (2012) reported different frequencies of *hla* gene among tested MRSA isolates of 30.5%, 78% and 80.9%, respectively.

The glycerol ester hydrolase (*geh*) is lipase secreted by MRSA that can catalyze the hydrolysis of the ester bonds between glycerol and fatty acids. This is believed to support the bacteria by contributing to the breakdown of host tissue, liberating nutrients and has been shown to interfere with the host granulocyte function and thus increases bacterial survival against the host defense (**Oliveira et al.**, 2018). The frequency of the *geh* gene in MRSA isolates in this study was 100%, which is higher than that recorded by the study of **Hassan et al.** (2017) and the Malaysian study of **Bitrus et al.** (2016) where 77.24%, and 28.5% of isolates harbored *geh* gene, respectively.

Toxic shock syndrome toxin-1 is a superantigen secreted by some MRSA isolates which encoded by the *tsst-1* gene, a major virulence factor in toxic shock syndrome (**Durand et al.**, 2006). In this study, 56% of MRSA isolates carried *tsst-1* gene. This is consistent with the results of **Khairalla et al.** (2017) and **Goudarzi et al.** (2016) studies which documented 50% and 51.4% of MRSA isolates carried the *tsst-1* gene, respectively. However, **El-baz et al.** (2016) study recorded a higher rate of *tsst-1* gene among MRSA isolates of 64.7%. In contrast, the studies of **Hassan et al.** (2017), **Xie et al.** (2016), **Bitrus et al.** (2016), **Monecke et al.** (2012), **Wang et al.** (2016), **Hadyeh et al.** (2019) and **Al Laham et al.** (2015) revealed lower rates of *tsst-1* among MRSA isolates of 4.55%, 4.8%, 7.1%, 7.48%, 8.8%, 23.2%, and 27.4%, respectively. While the study of **Shukla et al.** (2010) showed that none of MRSA isolates carried *tsst-1* gene.

Leukocidins act by the synergy with two proteins to form pores on cell membranes (**Dinges et al.**, 2000). In the current study, MRSA isolates were inspected for their leukocidins genes (*LukE/D*) with a frequency rate of 68%. This is in accordance with the results of **Abdel-Hamed et al.** (2016) study which showed that the frequency rates of *LukE* and *LukD* were 64% and 44% respectively. However, the study of **Hassan et al.** (2017) revealed higher rates of *LukE* and *LukD* of 89.7% and 87.2%, respectively, and the study of **Udo et al.** (2020) reported 95% and 100% rates of *LukE* and *LukD*, respectively. Also, the studies of **de Almeida et al.** (2013) and **Nelson et al.** (2015) revealed higher distributions of *LukE/D* among MRSA isolates, 82.8% and 95%, respectively. However, the prevalence of *LukE/D* in the study of **Abdallahman and Fakhr**, (2015) was 36.5%.

The genetic analysis of virulence factors combination has brought out ten toxin gene profiles. The most frequent profile associated with MRSA was pattern A (*icaA* + *icaD* + *cna* + *hla* + *geh* + *tsst-1* + *LukE/D*) which was exhibited by 20 isolates (40%). This was followed by pattern B (*icaA* + *icaD* + *cna* + *hla* + *geh* + *LukE/D*) which was exhibited by 11 MRSA isolates (22%). This finding agrees with the results of **Machuca et al.** (2013) study which recorded 10 virulence gene profiles among MRSA isolates. However, another study conducted by **Abdel-Hamed et al.** (2016) showed that 13 different virulence gene profiles were observed among MRSA isolates, while the most common pattern was represented by 16% of isolates. Though, the study by **Hassan et al.** (2017) recorded 49 virulence gene profiles, where the most frequent profile

associated with MRSA was P48 (*LukE* + *LukD* + *LukS* + *Lukf* + *geh* + *cna* + *hla* + *icaA* + *icaD*) carried by 12.5% of MRSA isolates.

In this study, among 10 toxin gene profiles, 6 patterns were exhibited by a single isolate. These findings suggested that MRSA isolates were genetically different. The presence of a single virulence determinant infrequently makes an organism virulent, while the combination of several factors enables bacteria to cause diseases. Analysis of virulence patterns did not allow the clear relation between the distribution of virulence factors and the source of clinical isolates. This finding was supported by the results reported by **El-baz et al. (2016)**.

In this work, the high frequency of virulence factors among isolates from diverse clinical sources indicates that these genes play a determinant role in MRSA virulence and create powerful arguments to re-evaluate the appropriateness of assessing the virulence potential of MRSA in order to control MRSA infections.

5. CONCLUSION

The high spread of MRSA infection is of particular concern, increasing challenge to hospitals, both in the clinical treatment of patients and the prevention of the cross-transmission of these challenging pathogens. Virulence factors are powerful predictors of the pathogenic potential of MRSA infections and these factors have been reported to play a determinant role in MRSA virulence. This study has verified a high prevalence of virulence factors among MRSA isolates recovered from different clinical sources. This current study has revealed no relation between virulence factors and the clinical source of isolation as the virulence factors were detected similarly among MRSA isolates from diverse types of clinical specimens. This high distribution of virulence factors encoding genes among MRSA isolates creates compelling thoughts to reconsider the appropriateness of assessing the virulence potential of MRSA in order to control infections. Regular studies on MRSA should aim to better elucidate MRSA epidemiology, study antimicrobial susceptibility profiles, and to investigate their virulence factors for effective control measures and better health management. Because of the multiple resistance to available antimicrobials and the pathogenic potential of MRSA infections, future researches to find virulence factors inhibitors as a potential alternative therapy for MRSA infections are warranted.

CONFLICT OF INTERESTS

The authors declare no conflict of interest.

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التوصيف الجزيئي لعوامل الضراوة الأساسية في المكورات العنقودية الذهبية المقاومة للميثيسيلين والمتعددة المقاومة للمضادات الميكروبية المعزولة في بعض المستشفيات المصرية

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تعتبر المكورات العنقودية الذهبية المقاومة للميثيسيلين (مرسا) واحدة من أهم مسببات الأمراض والوفيات في جميع أنحاء العالم. هدفت هذه الدراسة إلى دراسة مرسا متعددة المقاومة للمضادات الميكروبية في المستشفيات المصرية وتحديد مدى حساسيتها تجاه المضادات الميكروبية المختلفة، والتوصيف الجزيئي لعوامل الضراوة الأساسية بها بغرض التعرف على مدى انتشار هذه العوامل في عزلات مرسا لتساعد في برامج مكافحة العدوى لهذه البكتيريا الخطيرة.

في هذه الدراسة، تم جمع ١٧٠ عزلة من المكورات العنقودية الذهبية من مصادر إكلينيكية مختلفة من اثنتين من المستشفيات الجامعية، في الفترة من سبتمبر ٢٠١٧ حتى ديسمبر ٢٠١٨، وتم التعرف عليها باستخدام الطرق الميكروبيولوجية القياسية. كما تم التعرف على عزلات مرسا باستخدام طريقة الانتشار من قرص السيفوكستين (٣٠ ميكروجرام). وقد أظهرت النتائج أن عزلات مرسا كانت ١٣٨ (٨١.٢%) عزلة، وتم اختبار حساسيتها للمضادات الميكروبية المختلفة باستخدام طريقة الانتشار من القرص (كيربى باير) باستخدام ١٦ مضادا ميكروبيا مختلفا. هذا وقد بلغت نسبة مرسا متعددة المقاومة لمضادات الميكروبات ٧٩%. تم اختيار خمسين عزلة من المكورات العنقودية الذهبية المقاومة للميثيسيلين ومتعددة المقاومة للمضادات الميكروبية لإجراء الدراسة عليها. وقد تم تأكيد التعرف على عزلات مرسا المختارة باستخدام تفاعل البلمرة المتسلسل (PCR) مستهدفا جين (*mecA*) المسؤول عن المقاومة للميثيسيلين والذي تم اكتشافه في كل العزلات المختبرة.

اعتمادا على نتائج اختبارات الحساسية للمضادات الحيوية فقد أظهرت معدلا مرتفعا للمقاومة للمضادات الميكروبية بين عزلات مرسا المختبرة والتي شملت أغلب المضادات الحيوية المستخدمة في الاختبار مثل البيبتالكتامات، التتراسيكلينات، الامينوجليكوسيدات، والماكروليدات. كما أظهرت النتائج أن عزلات مرسا المختارة كانت مقاومة تماما لكل من البنيسيلين والسيفوكستين (١٠٠%) بينما أظهر اللينزوليد أفضل فاعلية بين المضادات الميكروبية المستخدمة بنسبة ٨٨%، تلاه التيكوبلانين (٦٦%). وبالكشف عن قدرة هذه العزلات لتكوين الأغشية الحيوية (Biofilms) وجد أن كل العزلات المختبرة كانت قادرة على تكوين الأغشية الحيوية، منها ٢٦ (٥٢%) عزلة تم تصنيفها منتجة قوية للأغشية الحيوية و ٢٤ (٤٨%) عزلة تم تصنيفها منتجة معتدلة للأغشية الحيوية.

في الدراسة الحالية، تم الكشف عن وجود بعض من عوامل الضراوة الأساسية لعزلات مرسا المختارة، وقد لوحظ أن ١٠٠% من العزلات كانت تحمل ثلاث جينات من السبعة المختبرة، ٩٨% منها كانت تحمل أربع جينات، ٩٦% منها كانت تحمل خمس جينات، ٧٦% منها كانت تحمل ست جينات، و ٤٠% منها كانت تحمل كل الجينات المختبرة. بالإضافة الى ذلك، قد لوحظ أن الجينات *icaD* و *geh* كانت صاحبة أعلى معدل انتشار بين الجينات المختبرة (١٠٠%)، تلاها *hla* (٩٨%)، *icaA* (٩٦%)، *cna* (٩٦%)، *Luke/D* (٦٨%)، *tsst-1* (٥٦%). كما تم تصنيف العزلات المختبرة بناءا على أنماط جينات عوامل الضراوة المكتشفة إلى عشرة أنماط، كان النمط (أ) هو السائد (*icaA + icaD + cna + hla + geh + tsst-1 + Luke/D*) والذي اشتمل على ٢٠ (٤٠%) من العزلات المختبرة، تلاه النمط (ب) (*icaA + icaD + cna + hla + geh + Luke/D*) والذي تضمن ١١ (٢٢%) من العزلات المختبرة.

تظل عدوى بكتيريا مرسا مشكلة جديرة بالملاحظة والدراسة في المستشفيات المصرية. فقد أظهرت عزلات مرسا احتوائها على عوامل ضراوة بشكل مرتفع ومثير للقلق. فيجب إجراء دراسات بشكل مستمر ومتكرر، حتى في كل بيئة رعاية صحية، لتحديد وبائيات مرسا للتمكن من السيطرة عليها ومنع انتشارها، كما يجب وضع ضوابط وأسس واستراتيجيات للتعامل الصحيح مع المضادات الحيوية، ومنع الاستخدام المفرط والغير منضبط لها لمنع ظهور سلالات بكتيرية مقاومة للمضادات الميكروبية.

الكلمات المفتاحية: المكورات العنقودية الذهبية المقاومة للميثيسيلين، عوامل الضراوة، الغشاء الحيوي، التوصيف الجزيئي.