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Molecular Typing of Methicillin Resistant *Staphylococcus aureus* Colonizing Egyptian Healthcare Workers and Patients

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A B S T R A C T

Methicillin-resistant *Staphylococcus aureus* (MRSA) is a global public health problem. MRSA infection leads to longer hospital stays, increased healthcare cost, and significant morbidity and mortality. We determined the prevalence of MRSA at the Intensive Care Units (ICU) of Minia University Hospital (MUH) and Minia Insurance Hospital (MIH) among Egyptian healthcare workers (HCW) and patients. Also, we examined the MRSA molecular types to determine if the HCW contribute to the spread of MRSA infection. This study included 260 different samples that included 110 nasal swabs from HCW, 60 swabs from endotracheal tube secretion of patients and 90 swabs from the hospitals' environment (roofs, floor, beds, walls, sheets and equipment). The swabs were cultured on mannitol salt agar and MRSA strains were identified by biochemical reactions, antimicrobial susceptibility testing and PCR using specific primers for the *coagulase* and *mecA* gene. MRSA strains were confirmed by PCR through detection of *mecA* gene. Typing of the coagulase-positive strains was performed using RFLP. MRSA colonization was 11.8%, 13.3% and 2.2% among HCW, patients and environmental samples, respectively. Antibiotyping revealed four different antibiotypes. Antibiotype 2 was the most common pattern (8 isolates), 6 of them belonged to the *coA* C2a genotype. Interestingly, RFLP of *coA* gene revealed sharing of strains between HCW and patients. Our results sets a warning that Egyptian HCW could be a possible source or transmission of MRSA infection, which necessitates the application of proper infection control measures.

Introduction

Methicillin-resistant *Staphylococcus aureus* (MRSA) is a global public health problem, which causes significant morbidity and mortality. MRSA has been defined as *Staphylococcus aureus* isolates with oxacillin minimum inhibitory concentration (MIC) of 4 µg/mL or those containing the *mecA* gene. However, the presence of *mecA* gene does not always mean resistance to oxacillin and *mecA*-harboring isolates that are sensitive to oxacillin has been isolated in several parts of the world; reviewed in (Pournaras *et al*., 2015). MRSA infection leads to longer hospital stays and increased

healthcare cost. MRSA is frequently recognized as a hospital-acquired organism. Variants capable of causing infections in the community are known as community-acquired MRSA (CA-MRSA) and represent an emerging and serious public-health issue as well. A study of a large surgical intensive care units' (ICU) cohort in USA over a 15 month period showed that 8% of patients were colonized with MRSA at the time of admission, and that these subjects can serve as a reservoir for the spread of this pathogen (Albrich, 2008). On the other hand, many patients who are not infected on admission may actually become colonized with MRSA. The possibility of MRSA acquisition and carriage by healthcare workers (HCW) is a source of anxiety and concern among HCW and public health authorities. The prevalence of MRSA among HCW was 6.1% and 15.5% in the Middle East and Africa, respectively, reviewed elsewhere. Transmission to patients is then likely to occur during routine patient care. Air-borne transmission appears to be quantitatively related to the number of *Staphylococcus aureus* (*S. aureus*) colonizing the anterior nares. Eradication of MRSA from HCW to limit transmission of MRSA in healthcare settings can be accomplished by topical or systemic antimicrobial agents.

The emergence of antibiotic resistance is considered one of the most important threats to human health in the 21st century. ICU are particularly appropriate for the rapid emergence and spread of MRSA because of the wide variety of pressures; which include frequent use of broad spectrum antibiotics, crowding of patients with high levels of disease acuity in relatively small area. Resistance to most β-lactam antibiotics in Staphylococci is mediated by an altered penicillin-binding protein (PBP), which is encoded by *mecA* genes. There are limited data about the potential transmission of MRSA in the healthcare settings in Egypt. To this end, we examined the patterns of colonization with MRSA among patients and HCW in the ICU, as well as the environment at two Minia Governorate Hospitals and tried to trace the source of infection.

Subjects and Methods

Collection of Samples

This study was carried out at the Microbiology and Immunology Department, Faculty of Medicine, Minia University in the period from April 2014 to November 2014. The study protocol was approved by the IRB and council of the Minia University Faculty of Medicine. A written informed consent was obtained from the study participants. Two-hundred sixty samples were collected from three sources as follows: hospital staff members, patients and environment. Samples were collected form Minia University Hospital (MUH) and Minia Insurance Hospital (MIH). Both hospitals are located in the same city and are ~3 Km apart where samples were collected from various ICU with bed capacities ranging from 8-10 beds with an admission rate of 1000-1500 cases per year. The samples included 110 nasal swabs from HCW (70 from MUH and 40 from MIH), including physicians, nurses, house-keeping staff, and safety workers. All HCW who agreed to participate were enrolled into the study. Also, a total of 90 swabs were collected from the environment of both MUH and MIH (ceilings, floors, beds, walls, sheets and equipment). In addition, 60 swabs were collected from the endotracheal tube secretion from 60 patients (45 from MUH and 15 from MIH) showing signs of pneumonia 48 hours or more following admission. Full history was taken from each HCW and patient, including; age, gender,

and residence. Patients enrolled in our study were those exposed to respiratory tract infections acquired 48-72 h following admission into the hospitals (healthcare-associated infections).

Isolation, Growth and Identification of Bacteria

The swabs were transferred immediately in enrichment broth and were cultured aseptically on nutrient, blood and mannitol salt agar plates, at 37ºC for 24 h. Colonies were confirmed to be *S. aureus* both microscopically and biochemically (catalase, coagulase, β-hemolysis, and sugar fermentation).

Antimicrobial Susceptibility Testing

The following antimicrobial discs were used: Ampicillin (10 µg), Ampicillin/Sulbactam (10 µg), Cephradine (30 µg), Cefoxitin (30 µg), Cefotaxime (30 µg), Cefepime (30 µg), Flucloxacillin (5 µg) and Imipenem (10µg; all from Bioanalyse, Canada). Vancomycin (30 µg) was purchased from Oxoid (England). Methicillin resistance was tested by using the Kirby-Bauer disc diffusion method(9) using oxacillin (1µg) disc on Mueller-Hinton agar (Oxoid, England) with 24 h incubation at 35.8ºC. The results were interpreted according to the guidelines of Clinical Laboratory Standards Institute.

DNA Extraction and PCR amplification

DNA was isolated by using Gene JET Genomic DNA Purification Kit (Thermo Scientific, USA)following the manufacturer's instructions. Primers specific for the *mecA* gene were used to amplify the methicillin resistance gene, which codes for PBP responsible for □-lactam antibiotic resistance. The primer sequences used for the amplification of the*mecA* gene were as follows: F 5'-TAGAAATGACTGAAC GTCCG 3', *mecA*R: 5'-GATGCCATTGTAACTAGCGTT-3'. The thermal cycler was programmed as follows: an initial denaturation step at 95°C for 5 min, followed by 1 min of denaturation at 95°C, 30sec of annealing at 47°C and 30sec of extension at 72°C for 30 cycles and a final extension step at 72°C for 5min. PCR products were resolved on 1% agarose gel with ethidium bromide dye and visualized under a UV trans-illuminator (BiometraGoettingen, Germany).

The presence and molecular type of the coagulase (*coA*) gene was screened in *mecA*-positive strains using specific primers (8)*coA* F:5'-ATAGAGTGCTGGTACAG-3', *co*A R: 5'-GCCATTGTAACTAGCGTT-3'. Molecular typing of the obtained *coagulase* gene products was performed by RFLP after digestion with *Alu*I restriction enzyme (Fast Digest *Alu*I, Thermo Scientific, USA)at the recognition site 5'AGCT , 3'TCGA and cutting occur at : 5'---AG CT---3' and 3'---TC GA---5`. RFLP was done as follows: 1 µL of Fast Digest enzyme, 10 □ Lof PCR products, 2µ□ L of green buffer, and 17 □ L of nuclease-free water were incubated at 37° C for 15 minutes. The resulting RFLP fragments were visualized by electrophoresis on 3% agarose gel using 50 bp ladder (QIAGEN Gelpilot), respectively.

Statistical Analysis

Statistical analyses were performed by the Statistical Package for Social Science (SPSS) version 11.0 (IBM, USA). Chi-square test and student's *t* test were used where appropriate. *p* values of <0.05 were considered significant.

Results and Discussion

Bacterial Isolation and Identification

Out of 260 samples, 50 *S. aureus* isolates (19.2%) were identified, which included 27

isolates (24.5%) from the 110 HCW, 18 isolates (30%) from the 60 patient and 5 isolates (5.6%) from the 90 environmental samples. According to culture, and biochemical characteristics and oxacillin resistance, MRSA was identified in 25 samples (9.6%) out of the 260 samples. These 25 isolates represented a prevalence of 13.6% (15 isolates) among HCW, 13.3% (8 isolates) among patients and 2.2% (two isolates) in the environment, respectively. The mean age of the 110 HCW was 29.2 ± 4.5 years. The frequency of MRSA in HCW regarding gender, job, residence and hospital is shown in Table 1.

Regarding the job, the highest prevalence rates were found among the nursing staff where 29% (20 from 67) were nasal carriers of *S. aureus* and 13 (19.4%) were carrier of MRSA. Also, the number of HCW colonized with *S. aureus* at MUH was 23 (32.9%) while those at MIH were 4 (10%, p=0.007). On the other hand, the mean age of the 60 patients was 61.4 ± 7.4 years with 5 MRSA strains isolated from males (15.2%) and 3 strains isolated from females (11.2%, p>0.05).

Antibiotic Susceptibility Testing and resistance profile of MRSA isolates

All *S. aureus* isolates were subjected to antibiotic sensitivity testing using 10 antimicrobial agents (β-lactams and *Vancomycin*) as described in the Subjects and Methods section. MRSA were detected according to oxacillin resistance with inhibition zone ≤10mm. All of the MRSA isolates were resistant to all the penicillins tested and were all (100%) susceptible to imipenem.Vancomycin resistance rate among the 25 MRSA isolates was 36%. On the other hand, resistance rates to*Cefradine*, *Cefoxitin*, cefepime and *Cefotaxime* were 100, 100, 48%, and 75%, respectively (Table 2). A 100% susceptibility to imipenem and almost half of the isolates were recorded as susceptible to cefepime. As shown in Table 2, MRSA strains showed 4 antibiotypes or resistance profiles (4 different patterns of resistance to β-lactams and Vancomycin). The commonest antibiotype were antibiotype 1 (9 isolates) with resistance to all antibiotics except *Imipenem* and antibiotype 2 that showed resistance to all used antibiotics except *Imipenem*, *Cefepime* and *Vancomycin*.According to this antibiotyping there were shared strains between HCW, patient and environment (Table 2).

Detection of mecA Gene among MRSA isolates

To test the mechanism of resistance of MRSA isolates in our study,*mecA* gene was detected by PCR as described in the Subjects and Methods section. An example of *mec*A PCR amplification among *S. aureus* isolates that were resistant to Methicillin is shown in Figure 1. An amplicon of 154 bp was considered indicative for the presence of *mecA* gene, which is responsible for Methicillin resistance among MRSA isolates. The PCR data indicated that 22 (88%) of MRSA isolates were positive for the *mecA* gene. These included 13 isolates from HCW (86.7%), 7 from patients (87.5%) and all two environment isolates (100%).

PCR Detection of MRSA coagulase (coA) gene

The PCR amplification of the *coA*gene was performed as described in the Subjects and Methods section. An example of *coA* PCR detection among MRSA isolates is shown in Figure 2. The data show that all MRSA isolates (100%) were positive for the *coA* gene. Four different *coA* types were found

based on polymorphisms in the size of the coagulase gene. These were 700±20 bp, 780±20 bp, 980±20 bp, and 500±20 bp. The samples that were MRSA-positive were classified according to the *coagulase* gene product size. It was found that the 780±20 bp type was the most abundant accounting for 86.5%, while the 700±20, 980±20 and 500±20 bp products accounted for the remaining 13.5% (1 isolate or 4.5% of each type).

Typing of coA gene by RFLP and relationship between antibiotypes and coA genotype

The different size *coagulase* gene products were subjected to RFLP analysis by restriction digestion using *Alu*I restriction enzyme as described in Subjects and Methods section. The *co*APCR products, also, yielded different restriction patterns on digestion with the *Alu*I enzyme as shown in Figure 3. The relationship between antibiotype and *co*Agenotyping is shown in Table 3. As shown, a single C1 sample from one HCW showed pattern 1 antibiotype (resistant to all antimicrobial agents used except *Imipenem*). The C2b environmental sample and the C3 sample from patient showed pattern 2 antibiotype (resistant to all antimicrobial agents tested except *Cefipime*, *Imipenem* and *Vancomycin*). The C4 (from environment) showed pattern 3 antibiotype (resistance to all antimicrobial agents used except *Cefipime*, *Cefotaxime*, *Imipenem* and *Vancomycin*).

We found that the remaining 18 samples (from HCW and patient) had the C2a *co*Agenotype and showed 4 different patterns of resistance. These were 6 (33.3%) ofantibiotype1, 6 (33.3%) of antibiotype 2, 2 (11.1%) of antibiotype 3, and 4 (22.2%) of antibiotype4. As mentioned previously, antibiotype 2 was the most common pattern (8 isolates), 6 of them belonged to the common *coA* C2a genotype. The data show that there were shared strains between HCW and patients suggesting that HCW could be the source of infection.

In this study, it was found that 27 (24.5%) out of 110 HCW serving the ICU patients were colonized by *S. aureus* of whom 13 (11.8%) were positive for nasal carriage of MRSA, though they were asymptomatic. MRSA colonization and infection have increased dramatically over the past two decades, evidenced by the increasing number of reported outbreaks in the literature (Sydnor *et al*., 2011). A study in an Indian hospital reported that 1.8% of HCW had colonization with MRSA in the anterior nares(Mathanraj *et al*., 2009).The nasal carriage rate of *S. aureus* found in the present study (24.5%) is similar to that reported in another study, where 34 (28.8%) of the 118 HCW had *S. aureus* and 15 (12.7%) had MRSA, respectively.

A significantly higher colonization with *S. aureus* (p=0.007) was noted at MUH (23 out of 70 HCW; 32.9%) than MIH (4 out of 40 HCW; 10%). This was also true for colonization with MRSA (17.1% vs. 7.5%, respectively), which indicates an increased risk of developing MRSA infections at MUH. The higher frequency of MRSA among HCW and patients at MUH compared to MIH in addition to the presence of the only isolated two MRSA strains from the environment at MUH, all point to the lack of proper infection control measures at MUH and draws the attention towards the strict implementation of appropriate measures at MIH. On the other hand, there was no significant difference in MRSA colonization between males and females. Regarding the job category, we found out that the highest prevalence rate of nasal

carriage of *S. aureus*was among the nursing staff (20 from 67; 29%) and this was also true for MRSA carriage(13 out of 67; 19.4%). These data coincide with another study as the overall rate of MRSA nasal carriage among HCW was 8.9% (10/112)and the frequency was highest (80% or 8/10) among nurse. This may reflect the fact that nurses have a higher level of physical contact with patients compared with other categories of medical staff due to constant exposure to infected patients.

In our study, from 60 patients with nosocomial infection, 18 (30%) had *S. aureus* infection and 8 (13.3%) had MRSA infection. This means that 44% of *S. aureus* infections were caused by MRSA strains. These data are analogous to another study at Minia University that reported a prevalence of *S. aureus* of 29.2% among wound patients, of which 65% were MRSA. The MRSA isolates reported in this study were completely resistant to *Ampicillin* (100%). The present study showed differential resistance rates against *Cephalosporins*; where the isolates were resistant to *Cefradine*, *Cefoxitin*, *Cefotaxime* and cefepimeat rates of 100, 100, 75%, and 48%, respectively (Table 2).On the other hand, 100% of the isolates were susceptible. MRSA is known to be resistant to most beta lactams except next generation molecules.In this regard and similar to our findings, other studies (8,9,15,16), reported high susceptibility to imipenem and partial susceptibility to cephalosporins. Another possible explanation for this susceptibility could be the non-extensive usage of imipenem or cefepime for treatment of MRSA in our hospitals leading to higher sensitivity. In addition, the fact that community-acquired MRSA could be introduced into the hospitals and that they are known to be sensitive to carbapenems, particularly imipenem, requires further investigation and tracing of the isolated strains. Only one study was conducted previously in our region and showed similar susceptibility of MRSA to the used cephalosporins(Ahmed, 2014). In regard to *Cefotaxime*, 75% resistance rate was demonstrated in our study, which is higher than that of another report in which resistance to *Cefotaxime* was 17%. In our study resistance to *Vancomycin* was 36%, which was much higher than a previous study in the same locality among surgical site infected patients, which was 1.5%. This marked rise in the vancomycin resistance could be due to the marked abuse of vancomycin in clinical practice as a drug of choice for MRSA. The isolation of MRSA from ICU in this study; where various risk factors e.g. long hospital stay and the use of broad-spectrum antimicrobials; could help in the emergence of vancomycin resistant MRSA (VRSA).

Methicillin resistance in *Staphylococci* is mediated by the *mecA* gene which encodes for the PBP2A resulting in reduced affinity for the β-lactam antibiotics including the *Penicillinase*-resistant penicillins. In this study, PCR was used to detect *mecA*gene in the 25 MRSA isolates. Twenty two isolates (88%) were positive for *mecA* gene, whereas three isolates (12%) were negative. Of the three isolates, two were resistant to *Oxacillin* at the borderline of the inhibition zone and were thus termed ‘‘moderately resistant *S. aureus* (MODSA). This finding agreed with the published data (Al-Ruaily *et al*., 2011) that showed that out of 15 MRSA isolates, 13 MRSA isolates expressed the *mecA* gene by PCR typing in addition to □-lactamase enzyme production. Regarding the presence of *mecA* gene among the resistant isolates, a report showed that 32 MRSA isolates were initially identified by disk diffusion method, then the PCR confirmed that only 30 isolates (93.8%)

were positive for the presence of *mecA* gene(Araj, 1999). The three isolates that were negative for *mecA* gene could have other resistance mechanism(s) e.g., reduced drug accumulation or altered target site. Another possibility is that we could not detect the *mecA* gene by the used methodology.

Table.1 Frequency of MRSA in HCW regarding gender, residence and job

HCW group (n=110)	**No (%)**	***P* value**
Gender: Male (n=49) Female (n=61)	 6 (12.2%) 9 (14.8%)	0.703
Residence: Rural (n=59) Urban (n=51)	 9 (15.3%) 6 (11.8%)	0.595
Job: Physician (n=30) Nurse (n=67) Worker (n=13)	 2 (6.7%) 13 (19.4%) 0 (0%)	0.094

Table.2 Resistance profile of MRSA isolates. The antimicrobial resistance of the isolates to 10 beta-lactam antibiotics was determined as described in the Subjects and Methods section. Isolates with similar resistance profile were grouped together. R=resistant, S=sensitive.

Antibiotype	Oxacillin	Ampicillin	Flucloxacillin	Ampicillin-sulb	Cefradine	Cefoxitin	Cefipime	Cefotaxime	Imipenem	Vancomycin	**Source of isolates**	**No of isolates N=25 (100%)**
Antibiotype 1	R	R	R	R	R	R	R	R	S	R	Patient and HCW	**9 (36%)**
Antibiotype 2	R	R	R	R	R	R	S	R	S	S	Patients, HCW and Env	**8 (32%)**
Antibiotype3	R	R	R	R	R	R	S	S	S	S	Patients, HCW and Env	**5 (20%)**
Antibiotype4	R	R	R	R	R	R	R	R	S	S	Patient and HCWs	**3 (12%)**

Fig.1 Detection of MRSA mecA gene by PCR among the S. aureus isolates. The gene was amplified as described in the Subjects and Methods section. The lanes shown from left to right are arranged as follows: L: 100 bp ladder; lane 1 negative control, lane 2 positive control, lane 3, 4, 6 and 7 are mecA positive (band at 154 bp) samples while lanes 5, 8, and 9 are negative samples.

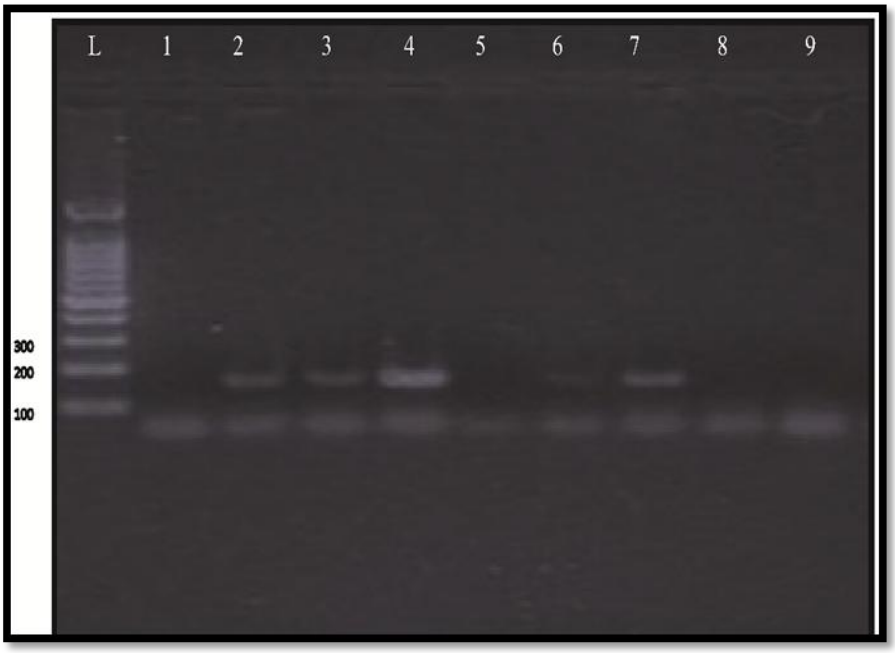

Fig.2 Detection of amplification products of MRSA coA gene by PCR. The PCR amplification of the coA gene was performed as described in the Subjects and Methods section. The lanes (from left to right) after the molecular size marker M: (100 bp ladder) show 4 different PCR product sizes as follows: lane 1: 700±20 bp, lanes 2, 3, 6, 7, 8, 9, and 10: 780±20 bp, lane 4: 980±20 bp, and lane 5: 500±20 bp.

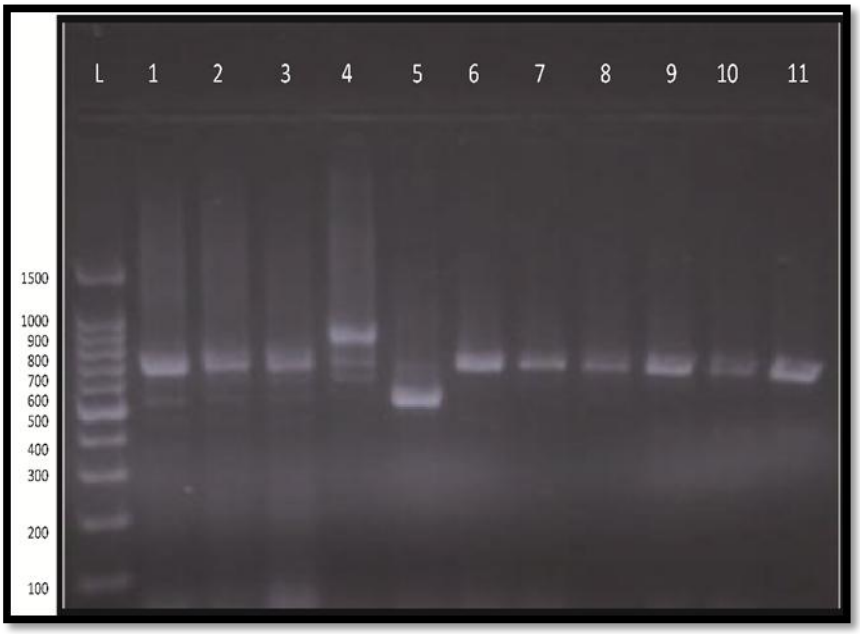

Fig.3 The coA PCR products yielded different restriction patterns on digestion with the enzyme AluI. The different size coagulase gene products were subjected to RFLP analysis by restriction digestion using AluI restriction enzyme as described in Subjects and Methods section. L: 50bp ladder, Lane1 shows coA C1 pattern, Lanes 2, 5, 8, 9, 10, and 11 show C2a pattern, Lane 4, and 7 show C4 pattern, Lane 3 shows C3 pattern while Lane 6 shows C2b pattern.

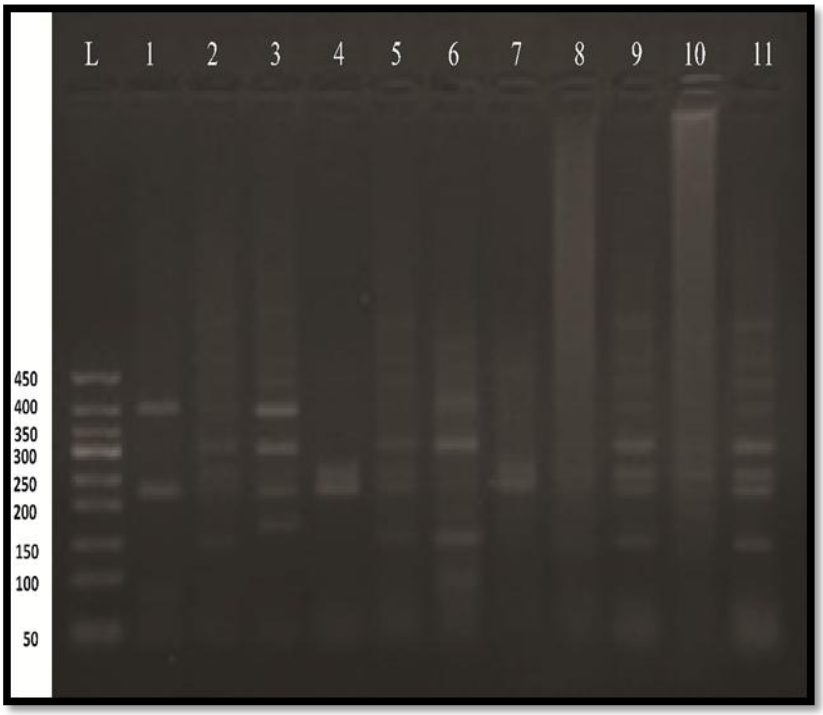

Table.3 Relationship between antibiotype and coA genotype of MRSA isolates

Anti-biotype/ Genotypes	Pattern 1 (n=7)	Pattern 2 (n=8)	Pattern 3 (n=3)	Pattern 4 (n=4)	*p* value
C1	1 (14.3%)	0 (0%)	0 (0%)	0 (0%)	
C2a	6 (33.3%)	6 (33.3%)	2 (11.1%)	4 (22.2%)	
C2b	0 (0%)	1 (12.5%)	0 (0%)	0 (0%)	0.589
C3	0 (0%)	1 (12.5%)	0 (0%)	0 (0%)	
C4	0 (0%)	0 (0%)	1 (33.3%)	0 (0%)	

Five different sized PCR products for the coagulase gene were detected in this study, and RFLP characterization showed that at least five *coA*types of MRSA strains were detected in our study. Of the MRSA-positive samples, we found that the 780±20 bp product was most frequent. This could possibly be an indication of the extent of the spread of this particular MRSA clonal type. Another study in Saudi Arabia found that out of 73 MRSA-positive samples, the 570 bp product was the most frequent. We found that the 18 samples with the C2a *coA* genotype (from HCW and patient) show 4 different patterns of resistance to antibiotics. Among these 18 isolates, it was found that

antibiotype 2 was the most common pattern (8 isolates), 6 of them belong to the common genotype C2a pattern. These findings agreed with that of another report carried on 129 MRSA isolates from 17 different hospitals in various regions of Thailand, in which antimicrobial susceptibility testing with a panel of 10 antimicrobial agents showed 9 different antibiotypes. The antibiotypes 1 and 2 were the most common phenotypes with 44.2% and 35.6% of the isolates, respectively. Coagulase gene typing of MRSA strains showed 4 different genotypes. Coagulase gene PCR-RFLPs exhibited 4 patterns: A, B, C and D, with *Alu*I digested PCR product fragments at 220±20 and 220±20 bp (pattern A); 400±20 and 220±20 bp (pattern B); 420±20 and 220±20 bp (pattern C); and 510±20 and 220±20 bp (pattern D)(Janwithayanuchit *et al*., 2006). The percentages for each pattern were compatible with those from the coagulase gene typing method. The results indicated that antibiotypes 1 and 2 coagulase gene type III and PCR-RFLP pattern C were the epidemic strains, while the rest were sporadic strains. In the current study, there were82% shared strain (C2a) between HCW and patient suggesting a common source of infection.

In conclusion, our results set a warning that HCW could be a possible source for transmission of MRSA infection, which necessitates the application of proper infection control measures.

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Author Disclosure Statement

ALL authors declare that "No competing financial interests exist"

Conflict of Interest: none declared.

Authors contributions

WKMM, NAH, SSH, MAE, and SFA conceived and designed the study. SSH, WKMM, NAH and SFA carried out the experiments and collected the data. WKMM, SSH, MAE, and NAH analyzed and interpreted the data. WKMM, and SFA wrote the manuscript. NAH, and MAE critically revised the manuscript.

List of abbreviations

community-acquired MRSA, CA-MRSA; healthcare workers, HCW; Intensive Care Units, ICU; Methicillin-resistant *Staphylococcus aureus*, MRSA; Minia Insurance Hospital, MIH;Minia University Hospital, MUH; moderately resistant *S. aureus*, MODSA; penicillin-binding protein, PBP; *Staphylococcus aureus*, *S. aureus*

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