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Original Research

Genetic diversity of multidrug resistant *Staphylococcus aureus* isolated from clinical and non clinical samples in Egypt

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Abstract: In recent years, the increasing incidence of diseases caused by *Staphylococcus aureus* (*S. aureus*) has been noted in the university hospitals of El-Sharkia and Assuit governorates - Egypt. Therefore, we studied the genetic relatedness of multidrug resistant *S. aureus* isolates from different sources in the above mentioned governorates. One hundred and fifty six *S. aureus* isolates were divided into 5 different groups, 1 non clinical isolates from different food products and 4 different clinical isolates of human and animal sources in the 2 different governorates. Epidemiological characteristics of 156 *S. aureus* isolates were determined by phenotypic methods including quantitative antibiogram typing and biofilm production. Genetic typing of 35 multidrug resistant (MDR) isolates (7 from each group) based on 16S rRNA gene sequence, virulence and antimicrobial resistance gene profiles was done. The genetic relatedness of the highest virulent strain from each group was detected based on different single locus sequence typing and multi-locus sequence typing (MLST). *S. aureus* strains isolated from different sources and geographical areas showed high diversity. The genetic typing revealed different sequence types and different sequences of *coa* and *spa* genes. *S. aureus* isolates were found highly diverse in Egypt.

Key words: *S. aureus*, genetic typing, Egypt, MDR, MLST, single locus sequence typing.

Introduction

Over the past decades, *S. aureus* has emerged as a leading cause of infections for human and animals in both the community and health care settings (1). Previous studies documented high prevalence of *S. aureus* isolated from food products including raw retail meat (2), mastitic cows in Assuit (3) and El-Sharkia governorates (4), also among clinical isolates in Assuit (5) and EL-Sharkia university hospital -Egypt (6). Colonized healthcare workers (HCWs) are capable of developing clinical *S. aureus* infections, transmitting *S. aureus* to patients and introducing *S. aureus* into their families (7).

Antibiotic resistance among *S. aureus* strains is a common phenomenon due to their ability to acquire antibiotic resistance genes. Methicillin-resistance has emerged due to the acquisition of *mec*A gene. Methicillin-resistant *S. aureus* (MRSA) dissemination represents a global problem in both hospitals and communities (8). Recently, infections caused by vancomycin resistant *S. aureus* (VRSA) were reported. Vancomycin resistance is due to the acquisition of *van*A and *van*B genes which result in blocking of the transglycosylation and transpeptidation reactions. Treatment of such strains become more complicated (9). Erythromycin resistance are widely disseminated among many species of bacteria. In *S. aureus*, erythromycin resistance is usually due either to ribosomal modification by 23S rRNA methylases mediated primarily by ermA, ermB, or ermC or to active efflux of the antimicrobial agent by an ATP-dependent pump mediated by msrA (10). The success of *S. aureus* as a pathogen is influenced by the extraordinary ability to express a large repertoire of virulence genes such as *coa* (coagulase gene), *spa (S. aureus* protein A gene), *ica* (intercellular adhesion protein A gene), *tst* (gene encode toxic shock syndrome toxin), *etb* (gene encode exfoliative toxin B) and *sea-see* (staphylococcal enterotoxin genes A-E) which cause harmful toxic effects to the host (11). Accordingly, there is considerable epidemiological interest in the tracking of *S. aureus* strains to gain a clear picture of the dissemination of such strains in the population and the dynamics of clonal spread.

Molecular typing can facilitate the identification of the sources and spread of infections and thus helps to control infections and outbreaks (12). Numerous typing techniques are available to differentiate *S. aureus*. The most reliable typing method is multilocus sequence typing (MLST) (13). This method is based on the sequence analysis of internal fragments of seven housekeeping genes. MLST groups strains into different sequence types (STs) and BURST (Based Upon Related Sequence Types) analysis is then used to group them into clonal complexes (CCs). The level of discrimination provided by MLST is sufficient to provide a relatively detailed picture of the global dissemination of the organism (14). Single locus sequence typing is used to compare sequence variation of a single target gene such as protein A (*spa*) and coagulase (*coa*) genes in MRSA strains. The technique is simple, rapid and highly reproducible (15). The purpose of our work was to study the genetic relatedness of *S. aureus* strains isolated from different sources in Egypt.

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Materials and Methods

Methodology

Specimens collection

A total of 469 different samples were collected from different food products (100 samples), milk from mastitic cows (169 samples) and different human subjects (200 samples) in 2 different geographic areas (El-Sharkia and Assuit governorates-Egypt). They were transported in an ice box and microbiological examination was performed within 2-6 hours.

Phenotypic characterization of staphylococcal isolates

Primary isolation of staphylococcal isolates was carried out into mannitol salt agar (Oxoid, UK). Identification of the isolates was based on standard bacteriological methods including cultural characteristics, Gram's staining and biochemical tests such as O/F (oxidative/fermentative), catalase and tube coagulase tests (16). Furthermore, *S. aureus* isolates were confirmed using the API 20 S identification kit (BioMerieux, Marcy l'Etoile, France). All isolates were stored in 30% glycerol-nutrient broth at –80°C until required. Isolates were classified into 5 different groups according to the type of samples, geographical areas and hosts [non clinical food isolates (group A) and 4 clinical isolates groups: Assuit animal isolates (group B), El-Sharkia animal isolates (group C), Assuit human isolates (group D) and El-Sharkia human isolates (group E)].

Antimicrobial susceptibility testing and quantitative antibiogram typing

The in-vitro activities of various antimicrobials including oxacillin (OX; 1µg), vancomycin (VA; 30 mcg), ceftriaxone (CRO; 30 mcg), sulfamethoxazole/trimethoprim (SXT; 1.25/23.75 mcg), gentamicin (CN; 10 mcg), erythromycin (E; 15 mcg), clindamycin (DA; 2 mcg), ciprofloxacin (CIP; 5 mcg), were determined by Kirby-Bauer disk diffusion method (17). Multidrug resistant strains (MDR which defined as the strain that showed resistance to more than 2 antimicrobials from different classes) were determined and the multiple antimicrobial resistance (MAR) index values for each isolate and each antimicrobial were calculated (18,19). Quantitative antibiogram typing depending on zone diameter (20) and biofilm formation using Congo red agar (CRA) method (21) were used to determine the phenotypic relatedness between isolates within each group. For antibiogram data (diameters of inhibition zones), the Euclidean distance was chosen as a similarity coefficient and the dendogram was constructed. The greater the distance between two organisms, the smaller is the resemblance between them. To define a cutoff distance below which discrepancies are due to casual variability, the antibiograms of all isolates within each group were determined twice on different days and the Euclidean distance between the first and the second determinations were determined and the cutoff point was set as the Euclidean distance of more than 95% of the isolates (20).

Genetic typing

DNA extraction

Seven phenotypic related MDR isolates from each group (35 isolates) were introduced for further genotyping. Total bacterial genomic DNA was extracted using genomic-tip 100/G columns (Qiagen, Germany) and concentration was measured at wave length A260 using the Nanodrop ND-1000 spectrophotometer (NanoDrop Technologies). DNA of *S. aureus* ATCC25923 was used as a positive control for 16S rRNA, *coa* and *spa* genes. Other *S. aureus* strains that were previously tested and were positive for the presence of *mec*A, *van*A, *van*B, *erm*C, *sea*, *seb*, *sec*, *sed*, *see*, *tst*, *etb* and *ica*A genes were used as positive control for these genes. The negative control was DNA of *E-coli* ATCC25922. The negative and positive controls DNA were commercially purchased from the national laboratory for veterinary quality control on poultry production (NLQP), Egypt.

Characterization of S. aureus

The genetic identification of the 35 isolates as *S. aureus* was confirmed by PCR amplification of the *coa* gene and 16S rRNA gene sequence from the purified genomic DNA using the universal primers (22,23). The online software Clustal Omega (EMBL-EBI, Hinxton, UK) was used to perform the multiple sequence alignment and 16S rRNA phylogenetic tree was constructed (24).

PCR amplification of virulence and antimicrobial resistance genes

Multiplex PCRs was used in typing of *S. aureus* based on the occurrence of enterotoxin genes (*sea-see*), *etb* and *tst genes* (25). Meanwhile, s*pa* and *ica*A were amplified by uniplex PCR (26,27). Concerning the antimicrobial resistance genes, *mec*A, *van*A, *van*B and *erm*C were amplified as previously described (28,29,30). PCR amplification was carried out on a PTC-100 TM programmable thermal cycler (Peltier-Effect cycling, MJ, RESEARCH, INC, UK) in a total reaction volume of 25 µl consisting of 12.5 µl of DreamTaq TM Green Master Mix (2X) (Fermentas, USA), 0.1 µl of 100 pmol of each primer (Sigma, USA), 2 µl of the DNA template and water nuclease-free up to 25 µl.

Single locus sequence typing and multilocus sequence typing (MLST)

Genetic diversity of the highest virulent strain (strain that harbor the largest number of virulence genes) from each group was determined using single locus sequence typing based on sequence variations of *spa* and *coa* genes and MLST. A comparative analysis of *spa* and *coa* genes sequences and phylogenetic comparisons of the aligned sequences were performed using the CLUSTAL W multiple sequence alignment program, version 1.83 of MegAlign module of Lasergene DNAStar software Pairwise (31). The MLST genes sequences were compared with the sequences at the MLST website (http://www.mlst.net/) to assign a sequence type (ST) (32). All PCR products were sequenced in Elim Biopharmaceuticals lab. United States.

Results

Phenotypic characterization of staphylococcal isolates

Out of 469 different samples, 156 isolates were confirmed as *S. aureus*; group A (23 isolates), group B (48

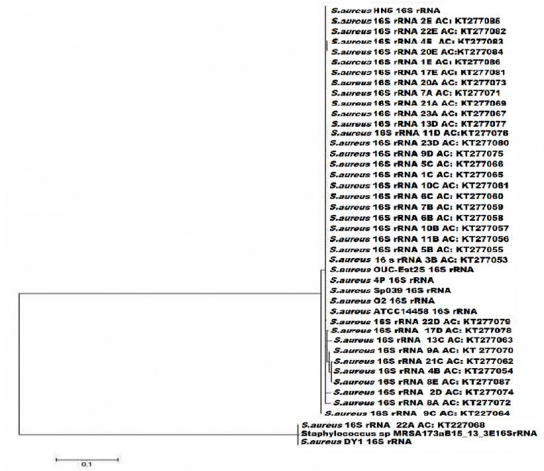

Figure 1. Phylogenetic tree of 35 MDR *S. aureus* isolates based on 16S rRNA gene sequences.

●The genotyping based on 16S rRNA gene sequence of 35 MDR strain showed little discrimination.

●Abbreviations: isolates with code A refers to non clinical isolates, code B refers to animal isolates from Assuit governorate, code C refers to animal isolates in El- Sharkia governorate, code D refers to human isolates in Assuit governorate, code E refers to human isolates in El- Sharkia governorate, while other codes refer to standard 16SrRNA gene possessed by gene bank, AC: Accession numbers of sequenced genes.

isolates), group C (25 isolates), group D (21 isolates) and group E (39 isolates) on the basis of standard bacteriological methods and API 20 S. identification kit. The phylogenetic tree for 35 MDR strains based on the sequence of 16S rRNA gene (accession number KT277053: KT277087), was constructed as shown in Figure 1.

The phenotypic detection of biofilm formation using Congo red agar (CRA) revealed that 26% of group A, 52.3% of group B, 26% of group C, 28.2% of group D and 16% of group E isolates were found positive for biofilm production. Group E showed the lowest number of samples (4 out of 25 isolates), while the highest number of isolates positive for biofilm production was detected among group B (11 out of 21 isolates).

Antimicrobial susceptibility testing

Antibiogram analysis showed that *S. aureus* isolates of each group showed different antimicrobial susceptibility patterns as shown in Figure 2. The highest resistances were detected to gentimycin, ceftriaxone, erythromycin and sulfamethoxazole/ trimethoprim among the human isolates from Assuit university hospital (group D). Meanwhile, Assuit animal isolates (group B) recorded the highest resistance to ciprofloxacin and oxacillin when compared with other groups. Concerning the resistance to clindamycin and vancomycin, the human isolates from El- Sharkia university hospital (group E) and non clinical isolates (group A) recorded the highest percentage of resistance respectively. The dendrogram based on quantitative antibiogram typing and the cutoff distance classified the groups A, B, C, D and E into 5, 4, 7, 10, 6 clusters respectively.

Genetic typing

Thirty five MDR isolates were typed according to MAR indices, virulence and antimicrobial resistance gene profiles as shown in Figure 3. Concerning the occurrence of virulence genes, *coa* was detected in all isolates while none of the isolates harboured *sea, seb and sed*. The prevalence of *ica*A, *spa, etb, tst, sec, see* among isolates of different groups was shown in Table 1.

It was noted that the highest prevalence of toxigenic *S. aureus* isolates (strain that harbour at least one

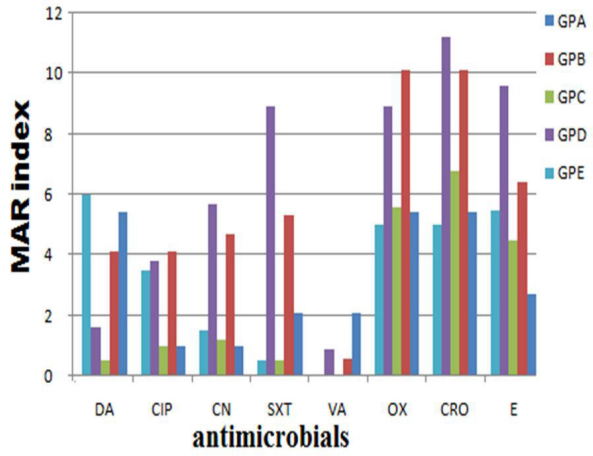

Figure 2. Multiple antimicrobial resistances (MAR) indices of the isolates belonged to different groups to each antimicrobial.

●Vancomycin was the most effective antimicrobials for all groups except non clinical isolates; ciprofloxacin and gentimicin were more effective.

●Antibiotic abbreviations: CRO, ceftriaxone; E, erythromycin; OX, oxacillin; SXT, sulfamethoxazole/ trimethoprim; CN, gentimicin; CIP, ciprofloxacin; DA, clindamycin, ;VA vancomycin.

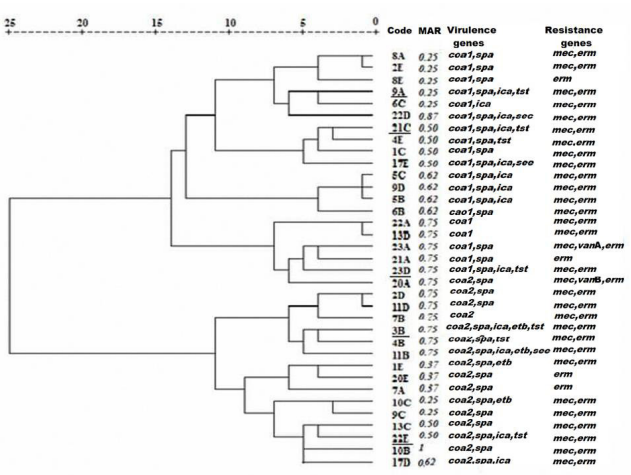

Figure 3. Phylogenetic tree of 35 MDR *S. aureus* isolates based on virulence and antibiotic resistance gene profiles and MAR indices.

●The occurrence of virulence and antibiotic resistance genes and MAR indices proved to be useful tools for rapid and inexpensive discriminatory typing of *S. aureus* isolates**.**

●*coa*1: coagulase (570bp), *coa*2: coagulase(630bp), *spa*: *S. aureus* protein A, *ica*A: intercellular adhesion protein A gene, *tst*: toxic shock syndrome toxin, *etb*: exofolative toxin B, *sec*: *S. aureus* enterotoxin C, *see*: *S. aureus* enterotoxin E, *mec*A: methicillin resistance gene A, *van*A: vancomycin resistance gene A, *van*B: vancomycin resistance gene B, *erm*C: erythromycin resistance methylase C gene.

●Under line codes mean the highest virulent strains that were selected for further genotyping based on multilocus sequencing and single locus sequencing.

Table 1. The prevalence of virulence and antimicrobial resistance genes among different groups.

Virulence & resistance genes	Prevalence among the following groups				
	Non clinical food isolates (7)	Assuit animal isolates (7)	El-Sharkia animal isolates(7)	Assuit human isolates(7)	El-Sharkia human isolates (7)
*ica*A	14.2%	42.8%	42.8%	57.1%	28.5%
spa	85.7%	85.7%	85.7%	85.7%	100%
coa	100%	100%	100%	100%	100%
etb	0.00%	28.5%	14.2%	0.00%	14,2%
tst	14.2%	28.5%	14.2%	14.2%	28.5%
sea	0.00%	0.00%	0.00%	0.00%	0.00%
seb	0.00%	0.00%	0.00%	0.00%	0.00%
sec	0.00%	0.00%	0.00%	14.2%	0.00%
sed	0.00%	0.00%	0.00%	0.00%	0.00%
see	0.00%	14.2%	0.00%	0.00%	14.2%
*mec*A	42.8%	100%	100%	100%	100%
*van*A	14.2%	0.00%	0.00%	0.00%	0.00%
*van*B	14.2%	0.00%	0.00%	0.00%	0.00%
*erm*C	100%	100%	100%	100%	100%

Table 2. Genotypic characterization of the highest virulent strain in each group.

Highest virulent strain belonged to	Genetic characterization						
	Virulence genes	resistance genes	Sequence type	*coa* sequence*	*spa* sequence*	*ica*A sequence*	*tst* sequence*
Group A	*ica*A, *spa*, *coa*, *tst*	*mec*A, *erm*C,	689	**KT248379**	**KT274024**	**KT248383**	KT248387
Group B	*ica*A, *spa*, *coa*, *tst*, *etb*	*mec*A, *erm*C	113	KT211673	KT274025	**KT248384**	KT248388
Group C	*ica*A, *spa*, *coa*, *tst*	*mec*A, *erm*C	80	**KT248380**	**KT274026**	**KT248385**	KT248389
Group D	*ica*A, *spa*, *coa*, *tst*	*mec*A, *erm*C	22	**KT248381**	KT274027	**KT248386**	KT248390
Group E	*ica*A, *spa*, *coa*, *tst*	*mec*A, *erm*C	239	KT248382	**KT274028**	**KT277089**	KT248391

* The accession number of the sequenced genes while underline and bold accession number mean novel sequence (new and first recorded mutation).

toxic or virulence gene) was found in group E (57.1%). Moreover, the highest virulent strain from each group shared in the occurrence of virulence genes such as *coa*, *spa*, *ica*A, *tst* and antimicrobial resistance genes *mec*A, *erm*C.

Concerning MLST and single locus sequence typing, different allelic types and different sequences were revealed. The sequence types and the occurrence of virulence genes were recorded in Table 2. The sequences of 7 housekeeping genes were recorded in gene bank with these accession number KT211639: KT211672.

According to the sequence of virulence genes among the highest virulent strains (accession number KT248379: KT248391 and KT274024: KT274028), the sequence of *coa* and *spa* genes showed high discriminatory power as shown in Figures (4 & 5).

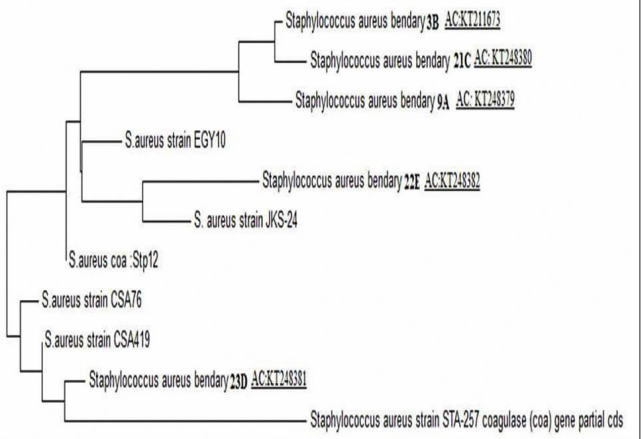

Figure 4. Phylogenetic tree of the selected highest virulent isolates from each group based on *coa* gene sequences.

●Coagulase gene of the highest virulent isolates from each group showed different sequences, *coa* of group A showed new recorded mutations, 10 silent mutations and 5 mis-sense mutations outside the active site. No new mutation was recorded for *coa* of group B and E. Moreover, *coa* of Group C showed new mutations, 4 silent and 3 mis-sense mutations outside the active site. Mean while, group D showed new recorded one mis-sense mutation outside the active site.

●Abbreviation: isolates with code A refers to non clinical isolates, code B refers to animal isolates from Assuit governorate, code C refers to animal isolates in El- Sharkia governorate, code D refers to human isolates in Assuit governorate, code E refers to human isolates in El- Sharkia governorate, while other codes refer to standard *coa* gene possessed by gene bank, AC: Accession numbers of sequenced genes.

Discussion

S. aureus has been recognized as a major pathogen in human and animal infections. Their infections are particularly problematic because of the the wide spread of different *S. aureus* genetic lineages carrying various virulence and antimicrobial resistance genes. Different *S. aureus* genetic clones has been emerged recently in Egypt along different geographical areas (33). In this study we analysed the diversity of 156 *S. aureus* isolates from different sources and areas in Egypt by pheno-genotypic methods.

There was a noticed variation in antimicrobial susceptibility patterns between the different groups. This variation in antimicrobials resistance may be related to the type of antimicrobial agents prescribed for treating

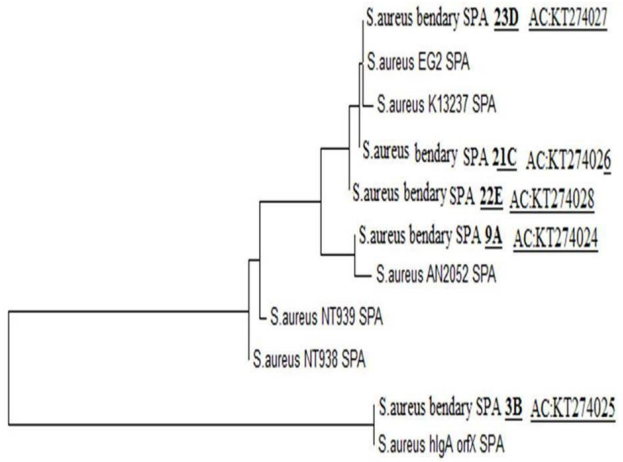

Figure 5. Phylogenetic tree of the selected highest virulent isolates from each group based on *spa* gene sequence.

●Sequences of *spa* gene belonged to group A, C and E showed new recorded one silent mutation meanwhile, spa of group B and D showed no mutations.

●Abbreviation: isolates with code A refers to non clinical isolates, code B refers to animal isolates from Assuit governorate, code C refers to animal isolates in El- Sharkia governorate, code D refers to human isolates in Assuit governorate, code E refers to human isolates in El- Sharkia governorate, while other codes refer to standard *spa* gene possessed by gene bank, AC: Accession numbers of sequenced genes.

various diseases in different geographical areas.

According to the antibiogram results of *S. aureus*, vancomycin showed a maximum activity except for non clinical isolates. Ciprofloxacin and gentimycin were the most effective antimicrobials in contrary to a previous study which showed high level of resistance to gentamicin in South Africa (34). The antimicrobial susceptibility pattern of non clinical isolates was a rather unexpected as recorded in a previous studies which showed that all *S. aureus* strains were susceptible to vancomycin (35). The presence of high percentage of VRSA (17.3%) among non clinical isolates revealed bad hygienic conditions. One possible explanation for the presence of VRSA strains among non clinical isolates might be low food hygiene that allows transmission of *van*A gene from environmental vancomycin resistant enterococci (VRE) and in some case *Bacillus* sp that harbour *van*A gene. Some reports suggest that VRE had spread to the community and the hospitals were not the only source of VRE (36). Moreover, *van*A gene cluster in a vancomycin-resistant bacilli was reported previously through the acquisition of *van*A gene cluster which shows a high degree of homology with that of enterococci (37). The absence of VRSA among group C and E revealed a limited exposure of *S. aureus* strains to the drug due to its limited prescription.

Concerning virulence and antimicrobial resistance genes, both are necessary for bacteria to survive under adverse conditions. Virulence mechanisms are necessary to overcome host defence systems and the development of antimicrobial resistance is essential to enable pathogenic bacteria to survive during antimicrobial therapies. The relation between them depend on many factors such as the bacterial species involved, virulence and resistance mechanisms, the ecological niche and the host (38). In our study, some strains in group A and B showed reverse relationship between the occurrence of virulence genes and antimicrobial resistance as the strains with the highest number of virulence genes showed the lowest level of antimicrobial resistance. Previous studies reported that the acquisition of antimicrobial resistance in *S. aureus* has been associated with loss of pathogenic fitness and also virulence potential (38).

It was noted that *cao* and *spa* genes sequence of *S. aureus* showed high discriminatory power opposite to other virulence genes such as *tst*. This was recorded in previous studies, *coa* and *spa* can be used for quick, preliminary epidemiologic studies for detecting *S. aureus* strains (39). In Egypt the discriminatory power of *coa* and *spa* genotyping was high and it was more useful for local epidemiologic purposes (40).

Multilocus sequence typing (MLST) is a highly discriminatory widely accepted method of DNA sequence based typing. The technique has been compared with other techniques such as pulsed field gel electrophoresis (PFGE) and *spa* typing. A good degree of concordance between results obtained by MLST, PFGE and *spa* typing has been reported (41). MLST is widely used to determine phylogenetic relationships between closely related species. In our study 3 of 5 isolates were found to be belonging to global sequence types; ST80 (group C), ST22 (group D), ST239 (group E). In Portuguese hospitals, ST239 was among the most prevalent MRSA clones (42). This global ST239 was also common in other countries, such as China and Taiwan (43).

ST22 was previously recorded among the two major emerging clones of community-acquired MRSA in India among the ocular Methicillin-resistant isolates (44). Moreover, ST22 clone has been found in healthy young adults without any risk factors in India (45).

Regarding ST80, it was first reported at a university hospital in Greece (46). In the northern Netherlands, the high occurrence of PVL-positive MRSA infection and colonisation between 1998 and 2005 was mainly attributed to ST80 strain (47).

Two isolates had rare STs; ST689 (group A), ST113 (group B). In 2003, one isolate among 101clinical isolates in the south-eastern part of Norway was recorded as sequence type 113 (48). Meanwhile, in two different studies in India, only one ST689 isolates were detected among different clinical isolates (49,50).

S. aureus infections are particularly problematic in Egypt due to the difficulties in controlling and limiting the sources of infections resulting from the wide distribution of large number of *S. aureus* sub-genotypes carrying various virulence and antimicrobial resistance genes along different geographical areas and different hosts. So, further studies are highly recommended to better understand the genetic relatedness and clonal spread of *S. aureus* isolates in Egypt.

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