Original Research

Characterization of Methicillin Resistant *Staphylococcus aureus* isolated from human and animal samples in Egypt

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Abstract: *Staphylococcus aureus (S. aureus)* has been one of the most problematic pathogens. Methicillin Resistant *S. aureus* (MRSA) has emerged as a major concern for both human and animal. Antibiotic resistance genes dissemination might be possible between human and animal bacteria. The aim of this study is to show phenotypic and genotypic diversity of human and animal MRSA isolates. Antibiogram typing and biofilm production were used as a primary phenotypic typing tool for the characterization of (40) animal and (38) human MRSA isolates. Genetic typing based on sequencing of 16S rRNA gene and virulence gene profiles were done. Antimicrobial resistance profiles of the animal isolates showed little evidence of widespread of resistance, although this was seen in many human isolates. The biofilm production was detected in higher percentage among animal isolates. Based on the genetic typing and multiple antibiotic resistance (MAR) index, the majority of animal isolates clustered into lineages that were not found in human isolates. Animal and human MRSA isolates showed diversity in antibiotic resistance and virulence gene profiles may be due to host adaptation or chances for contamination between the two hosts were not present in our study.

Key words: *S. aureus*, MRSA, genetic typing, 16S rRNA, MAR index.

Introduction

S. aureus is a major nosocomial pathogen that causes a range of diseases, including endocarditis, osteomyelitis, pneumonia, toxic-shock syndrome, food poisoning, carbuncles and boils (1). *S. aureus* can cause a range of infections in livestock and considers the most common etiological agent of contagious bovine mastitis, causing significant losses in the dairy industry (2). The success of *S. aureus* as a pathogen is influenced by both the extraordinary ability to express a large repertoire of virulence factors 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 genesA-E) which cause harmful toxic effects to the host (3). Antibiotic resistance is a common problem among *S. aureus* isolates, multiple resistant isolates can be transmitted easily among patients and visitors making the treatment difficult (4). The pattern of antibiotic susceptibility of methicillin susceptible *S. aureus* (MSSA) and methicillin resistant *S. aureus* (MRSA) isolates differed significantly. The MSSA isolates were susceptible to most of the antibiotics while MRSA isolates were more resistant to many antibiotics (5). People in a healthcare setting are constantly at risk of acquiring, colonizing and spreading MRSA (6). Antimicrobial resistance among bovine *S. aureus* is less common than among human *S. aureus* isolates (7).

Rapid and accurate typing of *S. aureus* is crucial to understand the possibility of transmission of this infectious organism. The traditional phenotypic methods have several drawbacks (8). Virulence genes can be used as an important epidemiological marker for typing of MRSA strains (9). Staphylocoagulase (SC) causes coagulation of plasma and is regarded as a marker for discriminating *S. aureus* from other less pathogenic staphylococci which called coagulase-negative staphylococci (10). Coagulase gene polymorphism analysis may be useful methods for studying clonal relatedness of isolates and for discriminating between MRSA strains (11).

Antibiotic resistance and biofilm-forming capacity contribute to the success of *S. aureus* as a human and animal pathogen (12). All biofilm producing staphylococci were positive for intercellular adhesion protein A locus, which indicates the important role of *ica* genes as virulence markers in staphylococcal infections (13).

previous studies had predicted human-to-bovine transmission by recovering *S. aureus* clones from cattle that are closely related to clones obtained from humans (14). However, other studies suggest that there are differences between strains isolated from bovine and human hosts thereby such transmission is unlikely under natural conditions (15,16,17). The aim of the present study is to investigate phenotypic and genetic diversity of human and animal MRSA isolates depending on antimicrobial resistance profiles, biofilm production, 16S rRNA gene sequencing and occurrence of virulence genes.

Materials and Methods

Sample collection

Three hundred and thirty five different samples were collected from 175 cows suffering from mastitis and 160 patients previously contact mastitic cows from several veterinary hospitals and farms in different areas in

Egypt between February to August 2013. Sample collection followed up the instruction of Centres for Disease Control and Prevention (CDC). All samples from animal origin were milk while those from human subjects were urine (30), pus (75), sputum (46) and CSF (9). 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 onto mannitol salt agar (Oxoid,UK). Single, well-isolated colony from overnight cultures was subcultured onto blood and milk agar for testing the beta haemolysis (18) and pigment production (19). Identification of the isolates was based on standard bacteriological methods including cultural characteristics, Gram's stain and biochemical tests such as O/F (oxidative/fermentative), catalase and tube coagulase tests (20). Furthermore, *S. aureus* isolates were confirmed using the API 20 S. identification kit (BioMerieux, Marcy l'Etoile, France). All isolates were stored with 30% glycerol at –80°C until required.

Antimicrobial susceptibility and resistance determinants

The in-vitro activities of various antimicrobial compounds were tested. Antimicrobial susceptibility profiles of all the confirmed *S. aureus* isolates to a range of antimicrobial agents of different groups using standard antimicrobial disks (Oxoid, UK) 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) and Ciprofloxacin (CIP; 5 mcg) were determined adopting the Kirby-Bauer disk diffusion method (21) on Muller-Hinton agar (Difco, USA) . The inhibition zone diameter around each disk was measured and isolates were categorized as susceptible or resistant based upon interpretative criteria developed by CLSI (Clinical and Laboratory Standards Institute, 2014). According to CLSI criteria, the disk testing is not reliable for evaluating Vancomycin resistance, so minimal inhibitory concentration (MIC) using the broth micro dilution method was performed to determine the susceptibility of *S. aureus* to such glycopeptides antimicrobial agent.

Determination of multiple antibiotic resistance (MAR) index

One of the tools that reveal the spread of resistant bacteria in a given population is multiple antibiotic resistance (MAR) index. The MAR index values for each isolate and each antibiotic were calculated (22,23) using the following formulas:

$$\text{MAR index for isolates} = \frac{\text{Number of antibiotics to which the isolate was resistance}}{\text{Number of antibiotics to which the isolate was exposed}}$$

$$\text{MAR index for antibiotics} = \frac{\text{Number of antibiotic resistance isolates}}{\text{Number of antibiotics x Number of isolates}}$$

Quantitative antibiogram typing

Similarity analysis was performed for antibiogram typing depending on the diameters of inhibition zones. The Euclidean distance was used 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 several isolates were determined twice on different days and similarities between the first and the second determinations were analyzed. The cutoff distance was set up such that >95% of the distances between the first and the second determinations would be smaller than the cutoff. Two isolates were considered to be similar when the Euclidean distance between them was lower than the cutoff value (24).

Phenotypic detection of biofilm formation

Biofilm production of all the *S. aureus* isolates was detected by Congo red agar (CRA) method (25). After incubation, the plates were inspected for the color of the colonies at 24 and 48 hours. A positive result was indicated by black colonies whereas nonproducing isolates developed red colonies. The Congo red dye directly interacts with certain polysaccharides, forming coloured complexes or more likely some metabolic changes of the dye to form a secondary product could play a more important part in the formation of dark colonies.

Genomic DNA extraction

Based on the dendogram, 26 MRSA isolates (3 or 4 isolates from each group) include 12 human and 14 animal strains with the highest MAR index were introduced to further genotyping. Total genomic DNA of the selected isolates were extracted using genomic-tip 100/G columns (Qiagen) and DNA concentration was measured at wave length A260 using the Nanodrop ND-1000 Spectrophotometer (NanoDrop Technologies). Genome of *S. aureus* ATCC25923 was used as positive control for 16S rRNA , *coa* and *spa* genes. Other *S. aureus* genomes that were previously tested and positive for the presence of *mec*A, *sea*, *seb*, *sec*, *sed*, *see*, *tst*, *etb* and *ica*A genes were used as positive control for these genes. Meanwhile, the negative control was genome of *E-coli* ATCC25922. The negative and positive control genomes were commercially provided from national laboratory for veterinary quality control on poultry production (NLQP) in Egypt.

Identification by 16S rRNA gene sequence analysis

PCR gene fragment of 16S rRNA was amplified from the purified genomic DNA using the universal primer (26). Cycling parameters were (i) 94°C for 3 min. (ii) 94°C for 1.5 min. (iii) 55°C for 1 min. (iv) 72°C for 1 min. (v) 36 cycles of steps 2 through 4 inclusive and (vi) 72°C for 10 min. Aliquots of the amplification products were analyzed by agarose gel electrophoresis using 1.0% agarose containing 0.5 μg of ethidium bromide per ml. All PCR products were sequenced in Elim Biopharmaceuticals lab. United States. The results of BLASTn for 16S rRNA DNA sequences were retrieved and aligned with the sequences of bacterial isolates using ClustalW embedded in MEGA 6 (Molecular Evolutionary Genetics Analysis) software (27). The multiple sequence ali-

gnment and 16S rRNA phylogenetic tree was constructed (28). Phylogenetic analysis was conducted based upon 16S rRNA gene data using Maximum Likelihood analyses (ML). Alignment gaps were treated as missing data. ML analysis was conducted using a heuristic search with tree bisection- reconnection (TBR) branch swapping and 100 random addition sequence replicates. Statistical support for the internal branches was estimated by bootstrap analysis based upon 1000 replications.

Detection of Methicillin Resistance

Frozen crude lysates of DNA of the selected 26 *S. aureus* isolates were assayed by PCR. Uniplex PCR protocol was optimized to amplify intrinsic methicillin resistance (*mecA*) gene with the thermocycling conditions set at 94°C for 10 min. followed by 10 cycles of 94°C for 45 s. 55°C for 45 s. and 72°C for 75 s and 25 cycles of 94°C for 45 s. 50°C for 45 s. and 72°C for 75 s. The product was visualized in agarose gels stained with ethidium bromide (29).

PCR amplification of *S. aureus* virulence genes

The virulence genes that carried on chromosomes not on phage (it may acquire or loss in some situation) could be used in typing of *S. aureus* and determine the lineage. *S. aureus coa* , *spa* and *ica*A were amplified by uniplex PCR according to (30,31,32) respectively , *coa* gene was amplified through the following thermal cycle , 35cycles of denaturation at 94°C – 1min. annealing at 60°C – 1min extension at 72°C – 1min. then final extension at 72°C – 5 min. The continuous amplification programme of *spa* gene consisted of 1 cycle at 95°C for 15 min. followed by 40 cycles at 94°C for 20 s. 60°C for 20 s and 72°C for 50 s. The amplification of *ica*A was performed as follows: 92ºC for 5 min of initial denaturation; 30 cycles of 92ºC for 1 min. 49ºC for 1 min and 72ºC for 1 min; and a final extension at 72ºC for 7 min. Detection of *S. aureus* enterotoxin (*sea-see*), *etb* and *tst* genes were performed by multiplex PCR. DNA amplification was carried out with the following thermal cycling profile: an initial denaturation at 94°C for 5 min. was followed by 35 cycles of amplification (denaturation at 94°C for 2 min. annealing at 57°C for 2 min. and extension at 72°C for 1 min.), ending with a final extension at 72°C for 7 min. (33). 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 50 µl consisting of 25 µl of DreamTaq TM Green Master Mix (2X) (Fermentas, USA), 0.2 µl of 100 pmol of each primer (Sigma, USA), 2 µl of the DNA template and water nuclease-free up to 50 µl. Amplicons were loaded onto 1% Agarose Gel containing 1 µg/ml ethidium bromide. The presence and molecular weight of the amplified DNA fragments were confirmed by agarose gel electrophoresis and visualized under UV-light.

Results

Out of 335 clinical samples, 134 *S. aureus* (70 bovine origin and 64 human origin that include 18 sputum, 35 pus, 10 urine and 1 CSF) were recovered. All of the 134 strains grew on mannitol salt agar were gram positive cocci, arranged in grape-like clusters, fermen-
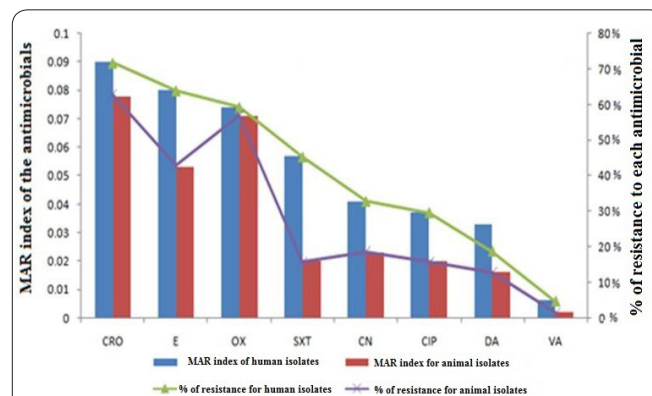
Figure 1. Multiple antimicrobial resistance (MAR) index and percentage of resistance to each antimicrobial. This figure showed high resistance percentage of ceftriaxone against all isolates while vancomycin was considered the most effective drug. Antibiotic abbreviations: CRO, ceftriaxone; E, erythromycin; OX, oxacillin; SXT, trimethoprim sulfamethoxazole; CN, gentamicin; CIP, ciprofloxacin; DA, clindamycin ;VA, vancomycin.

tative , catalase positive, tube coagulase test positive, β-hemolytic and produced the characteristic golden yellow pigments. Further confirmation of the isolates was conducted by API 20 S.

The in-vitro antimicrobial susceptibility profiles of 134 *S. aureus* isolates against antimicrobials of different classes are summarized in Figure 1. Antibiogram analysis showed that *S. aureus* isolates displayed variable antimicrobial susceptibility patterns. Vancomycin showed the maximum overall susceptibility against the isolates. The highest level of resistant isolates was recorded for ceftriaxone (Figure 1). The resistance patterns of *S. aureus* isolates of human and animal origin were found to be highly variable. *S. aureus* strains of human origin were more resistant to most antimicrobial agents as compared to those recovered from animal origin.

From 134 *S. aureus* isolates; 78 (58.2%), 56 (41.8%) MRSA and MSSA respectively were determined by disc diffusion method. Out of 64, 70 human and animal isolates; 38, 40 isolates were MRSA respectively.

MAR index for *S. aureus* isolates of human and animal origin revealed that the level of MDR exhibited by the isolates of human origin is alarming; such that, 62.4% of these isolates were resistant to 3 antimicrobials or more. Conversely, 60% of *S. aureus* isolates of animal origin exhibited non MDR pattern, with only 1 isolate (1.42%) possessed resistance to all antimicrobials. Additionally, the maximum MAR index of *S. aureus* isolate of human origin was 0.87.

According to CLSI, vancomycin resistance was observed in 4.68%, 1.42% of the isolates of human and animal origin respectively with MIC values ranged from 64-256 µg/ml.

S. aureus strains were subjected to quantitative antibiogram cluster analysis using zone diameter to antimicrobials. The cutoff distance of MRSA isolates was 6 thereby the dendrogram based on quantitative antibiogram typing classified the MRSA isolates into 8 clusters from each cluster, the highest MAR index were introduced to further genotyping (Figure 2).

The phenotypic detection of biofilm formation using Congo red agar (CRA) method revealed that 28.3% of *S. aureus* (11.1% human and 17.2% animal isolates) exhibited biofilm formation.
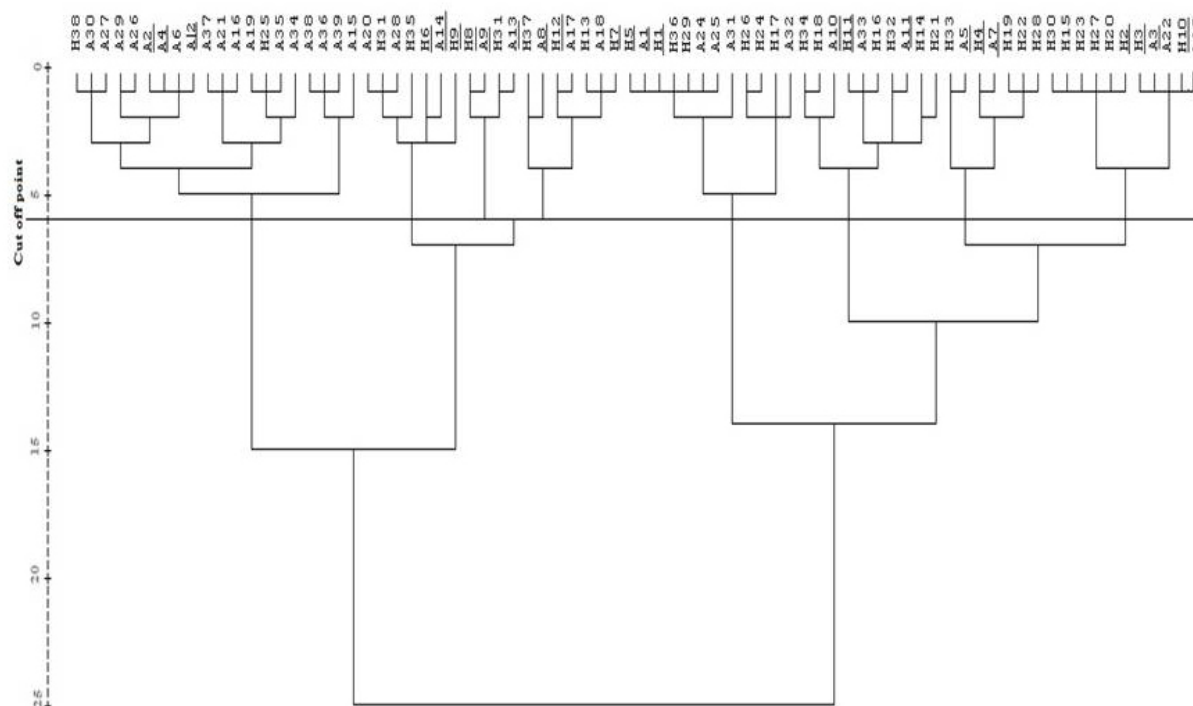
Figure 2. Dendogram deduced from the detailed data of zone diameter of disc diffusion test, depicting clustering and relatedness among 78 MRSA isolates. Degrees of similarity are shown in the scale at top left of the figure and strain numbers are indicated on the right side. Tested strains are indicated by numbers in ascending order for animal isolates from A1 to A40, while human isolates from H1 to H38. Underline codes mean isolates that were selected for genotyping.

Table 1. The percentage of virulence genes among human and animal MRSA isolates.

% of MRSA harbored	Isolates type (NO.)	
	Human isolates (12)	**Animal isolates (14)**
spa	91.6%	85.7%
tst	25%	21.4%
etb	8.3%	21.4%
sea	0.0%	0.0%
seb	0.0%	0.0%
sec	8.3%	0.0%
sed	0.0%	0.0%
see	8.3%	7.1%
ica	50%	42.8%

Genotyping

Results of phenotypic characterization of coagulase production using rabbit plasma were in complete accordance with the PCR findings of these isolates. Amplification of *coa* gene produced 2 different PCR products (570 and 630bp).

On the basis of the amplification of 310 base pair fragments specific for *mec*A gene, all of the selected 26 strains which appeared oxacillin resistant phenotypically by the disk diffusion technique were positive for the presence of *mec*A gene and were therefore confirmed as MRSA strains.

Analysis of MRSA strains for the carriage of *ica*A gene revealed that high percentage of human and animal isolates yielded positive PCR amplification of 1315 base pair product.

All results of phenotypic and genotypic detection of biofilm production were identical except one human and one animal MRSA isolates were phenotypic positive meanwhile genotypic negative.

The percentage of virulence genes among human and animal isolates was recorded in Table 1; *ica*A gene was detected with the highest percentage among all *S. aureus* isolates while *sea*, *seb* and *sed* couldn't be detected.

Genotyping based on phylogenic tree of 16S rRNA sequencing didn't differentiate human and animal MRSA isolates (Figure 3). Another phylogenic tree based on virulence gene profiles and MAR index was constructed (Figure 4). Human MRSA isolates could be discriminated from animal MRSA isolates depending on both phylogenetic trees.

Discussion

Infections due to *S. aureus* are of major importance to veterinary and human medicine. The emergence of MRSA is a global human and animal health problem causing infections in both hospitals and communities. In this study, during our screening for oxacillin resistance marker in clinical *S. aureus* isolates, we observed complete consistency between the phenotypic and genotypic
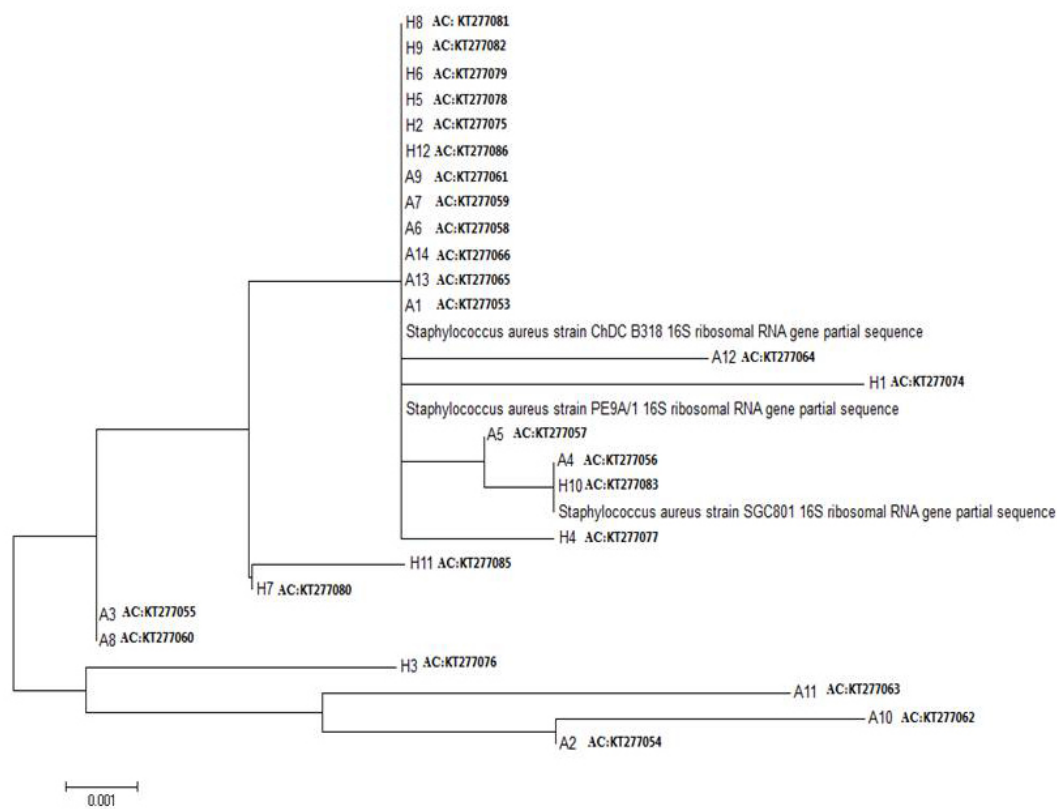
Figure 3. Phylogenetic tree of selected animal and human MRSA isolates depending on 16S rRNA gene sequencing. The genotyping depending on 16S rRNA gene sequencing showed little discrimination. Abbreviations: A. mean animal isolates while H. mean human isolates, other codes refer to standard 16SrRNA gene possessed by gene bank, AC: Accession numbers of sequenced genes.
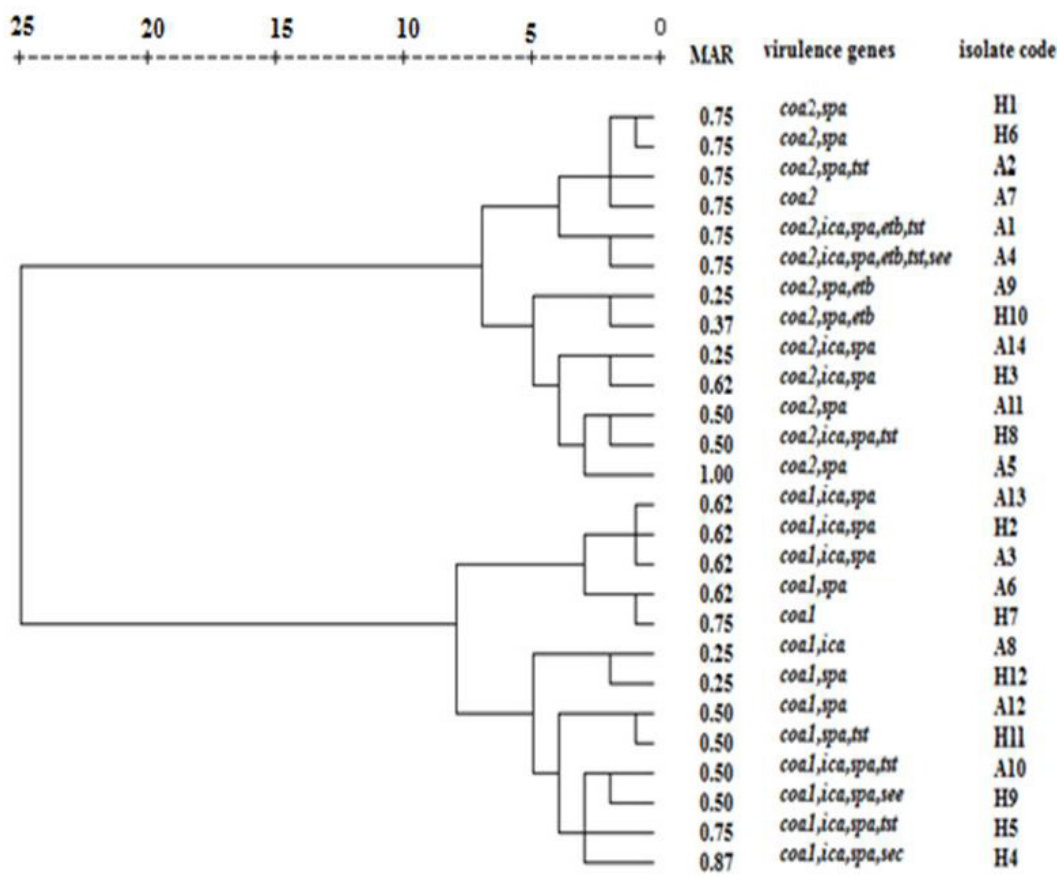
Figure 4. Phylogenetic tree depending on virulence gene profiles and MAR index. The occurrence of virulence genes and MAR index proved to be useful tools for rapid and inexpensive discriminatory typing of human and animal MRSA isolates. Abbreviations: A. mean animal isolates while H. mean human isolates.

(PCR) results as was previously demonstrated (34, 35). It was also noticed that about 59.3% of strains obtained from human infections were MRSA, whereas 57.1% of isolates obtained from bovine mastitis were MRSA.

With regard to the considerable differences between resistance patterns of *S. aureus* isolates of human and animal origin, *S. aureus* strains of human origin were more resistant to most antimicrobial agents as compared to those recovered from animal origin. This was consistent with a previous study conducted in India(36), implying the fact that the indiscriminate use of antibiotics in Egypt has rendered the commonly used antibiotics completely ineffective in the treatment of *S. aureus* infections in human. Increasing levels of methicillin resistance in bovine strains can be explained by the increased use of beta-lactam antibiotics in the treatment of mastitis.

Vancomycin resistance was observed in 4.68% of human isolates with MIC values ranged from 64-256 µg/ml, highlighting a significant public-health hazard regarding the acquisition of vancomycin resistance in human. A similar trend in the level of vancomycin resistant *S. aureus* can be observed in an occasional report in Pakistan (4%) (37), but in contrary to our study, all *S. aureus* strains were sensitive to vancomycin in other studies (38,39). This variation in the drug resistance may be well related to the type of antimicrobial agents prescribed for treating various diseases in different geographical areas.

It is also interesting to note that the MAR indices obtained in this study is a possible indication that a very large proportion of *S. aureus* human isolates have been exposed to several antibiotics. The results showed that majority of human *S. aureus* isolates (62.4%) were MDR with MAR index values greater than 0.2. This suggests that such isolates originated from a high risk source of contamination, where antimicrobial agents are freely available and accessible with high potential for abuse (22,40). Similar MAR index was observed previously in Nigeria (40). Moreover, the high prevalence of MDR among *S. aureus* isolates corroborated an earlier study in India (41).

Moreover, the data presented here showed that there was higher prevalence of virulence genes among the human isolates compared to animal isolates which was detected in a previous study (42). Meanwhile, *ica*A gene showed higher prevalence among animal isolates.

Typing of MRSA is essential to understand epidemiological trends and to initiate infection control strategies. The ability to quickly and reliably differentiate related bacterial isolates is essential for epidemiological surveillance. Modern typing methods based on genotype characterization also attempt to associate bacteria with host-specificity (43,16,44). In our study 16S rRNA sequencing and virulence gene profiles were used as genotyping methods. Some studies showed notable differences in the *S. aureus* clones of human origin and bovine mastitis-associated *S. aureus* clones (36). The present study demonstrated that the strains from human and mastitic cow samples were different, meaning that, the same lineage was not present between humans and animal in the local population. These results might be consistent with the concept of host specialization among *S. aureus* clones or possibility of infection was not pre-

sent for example, the wide application of milk pasteurization in Egypt prevented the exposure of humans to the cow isolates. Other possibility might be the heterogeneous nature of *S. aureus* genome and the ability to acquire and express antibiotic resistance genes as a response to the environment, so according to the types of antibiotics exposure of *S. aureus* in cows, will be the profile of antibiotic resistance genes and the same situation is applied to the human isolates. The wide application of CDC instruction in the large farms and veterinary hospitals from which isolates were isolated could decrease or prevent both human and animal MRSA strain from moving between the two populations. A further study using more MRSA isolates from small farms and using different samples types especially from wounded animal and human in Egypt is highly recommended.

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