

Genotypic and Phenotypic Characterization, Antibiotic Resistance and Virulence Patterns of *Staphylococcus aureus* Isolated from Goat Mastitis



Yasser M. Kamel

Infectious Diseases Unit, Department of Animal Health, Division of Animal and Poultry Production, Desert Research Center, Matariya, Cairo, P.O. Box: 11562, Egypt

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Address correspondences to
Rasha M. Elkenany, PhD; Tel: +2
01222203735, E-mail:
ymahmod@kau.edu.sa

ABSTRACT

Objective: To determine the phenotypic and genotypic characterization of the virulence and antimicrobial susceptibility of *S. aureus*.

Design: Descriptive study

Samples: 175 raw goat milk samples

Procedures: A total of 87 *S. aureus* strains was isolated from goat with mastitis in north-west coast of Egypt. PCR technique was used to detect genes coding some virulence and antimicrobial properties while phenotypic antimicrobial susceptibility was determined by the disc diffusion method.

Results: Most *S. aureus* isolates were resistant to amoxicillin (89.7%), penicillin G (88.5%), and ampicillin (86%) while were less resistant to other members of β -lactam group as ceftiofur (17.2%) and ceftiofur (10.3%). The lowest resistant rates were toward fluoroquinolone group members; ciprofloxacin, levofloxacin and ofloxacin in rates 1.2%, 2.3% and 4.5%, respectively. Tetracycline showed the highest level of phenotypic resistance (34.6%) and prevalence of a *tetK* gene (39.1%). The virulence factors, *spa* and *coa* exhibited prevalence rate of 6.9% and 8%, respectively with significant relationship between them.

Conclusion and Clinical relevance: The problem of antimicrobial resistance needs strict measures to limit the indiscriminate use of antibiotics and concerted research efforts to limit the spread of resistant bacteria, which will negatively affect human health and control of the infectious diseases in both humans and animals.

Keywords: *S. aureus*, virulence, antimicrobial resistance, mastitis, goat

1. INTRODUCTION

Staphylococcus aureus (*S. aureus*) is one of the most clinically important opportunistic contagious pathogens of humans and animals. *S. aureus* has particular attention as being one of the most common foodborne pathogen that cause a wide range of clinical infections, including clinical and subclinical mastitis in small ruminants [1]. However, identifying infected goat may be difficult, as unlike cattle, the high number of somatic cells and positive result in the California mastitis test is not certainly dependable indicator of mastitis among small ruminants [2].

It is known that the use of antimicrobial drugs in humans and animals in developing countries is not sufficiently controlled by the competent authorities. As a result, many resistant strains of *S. aureus* can be frequently isolated [3]. Therefore, it is necessary to identify these strains and understand the resistance mechanism in parallel with raising awareness of the importance of this issue in those countries to be wiser in using antibacterial agents to counteract the resistant bacteria in both humans and animals. While the phenotypic and genotypic characteristics of *S. aureus* isolated from bovine milk are well identified, similar data on those isolated from small ruminants are rare [4-6]. Data on *S. aureus* detected in small ruminants related to genetic

characterization, virulence factors and antimicrobial resistance are important to identify different potential mode of transmission, develop and improve management strategies for infected goats' herds, and discover effective therapeutic medications. Consequently, the aim of this study was to identify the virulence characterization and antimicrobial resistance of *S. aureus* isolated from goat's milk at the phenotypic and genotypic levels.

2. MATERIALS AND METHODS

2.1. Sample collection

From small Bedouin goat herds that are living in the north-west coast of Egypt, a total of 175 raw goat milk samples with clinical signs of mastitis; udders inflammation accompanied with or without changing in consistency and/or color of milk were randomly collected. The teats were disinfected with alcohol and the first milk stream was discarded before collecting the milk samples.

2.2. Sample processing and bacterial isolation

The collected raw milk samples were subjected to inoculation onto 5% sheep blood agar plates that were incubated aerobically at 37°C for 24h. Milk samples were considered infected when three or more dissimilar colony types were founded [7].

S. aureus strains were isolated from collected raw milk samples according to the EN ISO 6888-1 standard procedure of the International Organization for Standardization [8] as follows: briefly, milk samples were spread onto Baird-Parker agar plates supplemented with 5% tellurite egg yolk emulsion and incubated aerobically at 37°C for 48 h. The putative colonies were chosen and confirmed by the coagulase test using rabbit plasma and positive colonies were subjected to Gram-staining and biochemical tests (catalase reaction, DNase, beta hemolysis, clumping factor and anaerobic mannitol fermentation). Positive colonies, as a result of these tests, were considered phenotypically as *S. aureus*. All presumptive strains were grown overnight in Luria broth supplemented with glycerol (20% vol/vol), which were then stored at -80°C for further investigations.

2.3. Molecular Identification and characterization of *S. aureus*

Total DNAs from all apparent *S. aureus* isolates were subjected to the conventional boiling method for extraction of total DNA as depicted previously [1] and the extracted DNA was quantified by using Nanodrop spectrophotometer and stored at -20 °C to be used for molecular detection of virulence and antimicrobial resistant genes. All samples DNA and those of *S. aureus* ATCC 29213 (positive control), and *S. epidermidis* ATCC 12228 (negative control) were subjected to PCR procedure to emphasize *S. aureus* isolates via amplifying the *nuc* gene as described previously [10]. Briefly, a total volume of 20µl, containing 2µl of sample DNA, 2µl of 10X PCR buffer, 4µl of dNTPs in concentration 1mM, 1µl of MgCl₂ in concentration 50mM, 1µl of each *nuc* forward and reverse primers in concentration 10 Pmol (primer sequences are shown in table 1), 0.25µl of Taq DNA polymerase in concentration of 5µ/µl and finally 8.75 µl of dH₂O. Amplification was carried out using a thermocycler according to the following PCR steps of an initial denaturation for 5 min at 94°C; followed by 30 cycles of 94°C for 1 min, 50°C for 1 min, and 72°C for 2 min, and a final extension step at 72°C for 10 min. Lastly, ethidium bromide stained PCR products were detected on 2% agarose gel by using electrophoresis. The appearance of the amplified DNA fragments at 270 bp was considered positive result. Molecular grade water and *S. aureus* ATCC29213 were used as negative and positive controls, respectively (Table 1).

2.4. Antimicrobial susceptibility testing

Confirmed *S. aureus* isolates were subjected to Kirby-Bauer disk diffusion method for detection the antibiotic sensitivity [17]. The antibiotics used and their concentration are shown in table 2. Briefly, the sample was inoculated into nutrient broth and incubated for 24 h at 37°C. A loop of 100 ul of growth was streaked onto Mueller-Hinton agar plates. The antibiotic discs were applied onto the dried inoculated agar plates and incubated at 37°C for 24 h. The zones of inhibition were measured, and results were recorded as sensitive (S), intermediate (I) or resistant (R) in accordance with the CLSI guidelines [17].

2.5. Molecular Detection of Virulence, Antimicrobial Resistance Genes

The isolated strains were subjected to multiplex PCR for investigation of virulence and antimicrobial resistance genes according to a previously described method [18]. Briefly, each PCR assay was carried out in a 15 µL comprising 4ul extracted DNA, 0.5 µl of each forward and reverse primer; the details of each primer are summarized in table1, and 3µl of double distilled water. Reactions were proceeded according to the following parameters: initial denaturation at 95 °C for 3 min followed by 36 cycles including denaturation for 30s at 95°C, annealing for 45 s at different temperatures (table 1) according the primer used, extension for 1 min at 72°C and last extension for 5 min at 72°C. The amplified bands were detected on a 2% ethidium bromide stained agarose gel using 1X tris acetate EDTA (TAE) as a buffer. A positive control and negative control were set up with every amplification assay.

2.6. Statistical Analysis

The data were subjected to statistical analysis SPSS (ver. 25), version 25 statistical package. Chi-square test was used to estimate the relationships between the variables, $p < 0.05$ was considered as statistically significant. Evaluation of the strength and direction of the relationship between the antibiotic resistance and virulence genes was detected using two tailed Pearson's correlation test.

3. RESULTS

3.1. Prevalence of *S. aureus* in collected samples

In the current study, a total of 175 collected milk samples from goats were suffering from mastitis, 135 samples were harboring microbial infection. Out of 135 infected milk samples, 87 (64.4%) were confirmed to be *S. aureus* by detection of *nuc* gene PCR methods. Furthermore, four *S. aureus* strains (10.3%, 9/87) were classified as MRSA isolate based on their embracing the *mecA* gene. For determination of association between bacteriological and molecular methods for the detection of *S. aureus*, the results were subjected to the 2-tailed Pearson's correlation test. Results revealed a high significant ($p < 0.001$), positive correlation (69.6%).

3.2. Antimicrobial Resistance Assay

In the present study, all isolated strains of *S. aureus* were tested against various types of antimicrobial agents that are or were using in treatment of *S. aureus* infected cases to determine the phenotypic resistance profile for each isolate, as shown in table 2. **Figure 1.**

Most of *S. aureus* isolates were verified as multi-drug resistant *S. aureus* (MDR-SA), where 72 (82.8%) isolates were resistant to three or more antibiotics, as shown in figure 1. It was found that fluoroquinolone members: ciprofloxacin, levofloxacin, and ofloxacin showed the lowest resistance ratio of 1.2%, 2.3%, and 4.5%, respectively, while amoxycillin, penicillin, and ampicillin displayed the highest resistant in ratios of 89.7%, 88.5%, and 86.2%, respectively. Thirty-five isolates (40.3%) were classified as MRSA by ceftiofur disk diffusion tests. All *S. aureus* isolates were susceptible to linezolid and vancomycin.

Table 1. Sequences of primer sets used to amplify specific virulence and antibiotic resistance genes.

Gene	Primer sequence (5`-3`)	Amplicon size (bp)	Reference
<i>nuc</i>	F-GCGATTGATGGTGATACGGTT R-AGCCAAGCCTTGACGAACTAAAGC	270	11
<i>mecA</i>	F- TCCAGATTACAACCTTCACCAGG R-CCACTTCATATCTTGTAACG	162	12
<i>tetK</i>	F-TCGATAGGAACAGCAGTA R-CAGCAGATCCTACTCCTT	169	13
<i>aac(60)-aph(2`)</i>	F-TAATCCAAGAGCAATAAGGGC R-GCCACACTATCATAACCACTA	227	13
<i>coa</i>	F-CGAGACCAAGATTCAACAAG R-AAAGAAAACCACTCACATCA	730	13
<i>spa</i>	F-CAAGCACCAAAGAGGAA R-CACCAGGTTTAACGACAT	180	14
<i>see</i>	F-TAGATAAAGTTAAAAACAAGC R-TAACTTACCGTGGACCCTTC	170	15
<i>sea</i>	F- CCTTGAAACGTTAAAAACG R-TCTGAACCTTCCATCAAAAAC	127	16

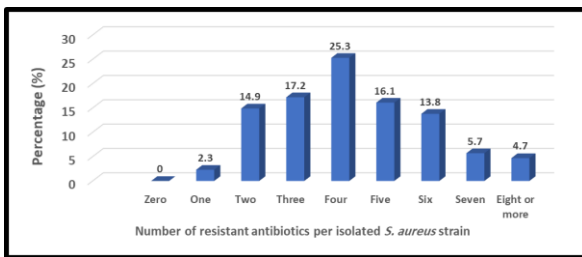


Figure 1. Resistance of *S. aureus* strains to one or more antibiotics.

Table3: Presence of virulence and antimicrobial resistant genes in isolated *S. aureus* strains.

No. of strains containing genes	Antibiotic resistance genes			Virulence related genes			
	<i>aac</i>	<i>tetK</i>	<i>mecA</i>	<i>coa</i>	<i>sea</i>	<i>see</i>	<i>spa</i>
Percent (%)	10.3	39.1	26.4	8	24.1	4.6	6.9

Table 4. Determination of the relationship between the different genetic contents by using Pearson`s correlation and P-values.

	<i>aac</i>	<i>tetk</i>	<i>mecA</i>	<i>coa</i>	<i>sea</i>	<i>see</i>	<i>spa</i>
<i>aac</i>	1	-0.059 (0.243)	0.091 (0.239)	-0.031 (0.601)	-0.087 (0.165)	-0.089 (0.197)	0.045 (0.561)
<i>tetk</i>	-0.059 (0.243)	1	-0.059 (0.243)	-0.071 (0.297)	-0.034 (0.584)	0.025 (0.674)	0.032 (0.654)
<i>mecA</i>	0.091 (0.239)	-0.059 (0.243)	1	0.150* (0.250)	0.015 (0.889)	0.077 (0.335)	0.025 (0.760)
<i>coa</i>	-0.031 (0.601)	-0.071 (0.297)	0.150* (0.250)	1	-0.035 (0.688)	-0.029 (0.713)	0.310* (0.000)
<i>sea</i>	-0.087 (0.165)	-0.034 (0.584)	0.015 (0.889)	-0.035 (0.688)	1	0.320** (0.000)	-0.014 (0.792)
<i>see</i>	-0.089 (0.197)	0.025 (0.674)	0.077 (0.335)	-0.029 (0.713)	0.320* (0.000)	1	-0.088 (0.229)
<i>spa</i>	0.045 (0.561)	0.032 (0.654)	0.025 (0.760)	0.310** (0.000)	-0.014 (0.792)	-0.088 (0.229)	1

*Significant correlation at 0.05
** Significant correlation at 0.01

3.3. Molecular Detection of Virulence, Antimicrobial Resistance Genes

The isolated *S. aureus* strains were examined for a total of nine genes associated with virulence and antimicrobial resistance. Concerning antimicrobial genes, the most established gene was *tetK* (39.1%) referring resistance to tetracycline and followed by the *mecA* gene (26.4%), categorizing these isolates as methicillin resistance *S. aureus* (MRSA). On the other hand, the most prominent genes for virulence was *sea* (24.1%), as shown in table3. Statistical analysis showed up the relationship between the genetic determinants (Table 4).

4. DISCUSSION

S. aureus has been considered an important pathogen that can cause a diverse range of infections particularly zoonotic diseases and the potential transmission of antimicrobial resistant strains especially those expressing a MDR phenotype and possessing many virulence factors including production of enterotoxins (*see* and *sea*) between farm animals and humans through close contact, handling and/or consumption of *S. aureus* contaminated food causing major warnings in both food security and healthcare sectors [19]. Since our isolates were derived from different goat husbandry places, contagious transmission is likely to occur not only within the same herd, but also between different herds, possibly mainly through grazing where they are often grazing in the same areas.

The public health importance of *S. aureus* is evident in outbreaks of poisoning via contaminated dairy products such as those that occurred in Japan and recorded as one of the largest outbreaks of foodborne illness that involved 13,420 infected individuals [20]. Additionally, many cases of dairy-by-products poisoning have also been reported frequently in China [21]. Economically, mastitis often leads to a decrease in milk production and exorbitant treatment costs and may sometimes cause animal executions [20]. The present study identified some virulence characterization and antimicrobial resistance of *S. aureus* isolated from raw goat`s milk that would be helpful to know the antimicrobial resistance and virulence markers which can help in preventing and control the *S. aureus* contamination and protect the end customer. The high significant (p < 0.001) and positive correlation (69.6%) between the conventional microbiological and molecular methods in detection of *S. aureus* indicate the importance of using both methods in parallel for identification and diagnosis of *S. aureus* infection. Although molecular detection methods are less laborious, save time, more sensitive and reliable, it may be cost-effective to start identification using conventional methods and confirm -if it is necessary- by molecular methods. The emerge of one 'superbug' with multiple antimicrobial resistance will increase the challenges facing the food security and healthcare sectors as infections become very difficult-to-treat or even emerging untreatable staphylococcal

infections. For confirmation, the World Health Organization (WHO) has reported that there is a list of infectious diseases that are now difficult to treat, and this is alarming because some antibiotics have become less effective in such a way that this infection is not responding to treatment in some

cases [22]. The unwise use of antimicrobial drugs has a great burden on the health care system where international antibiotic consumption exhibited 65% increase from 2000 to 2015 in developing countries [23].

Table 2. Antimicrobial resistance of isolated *S. aureus* strains.

Antimicrobial group	Antimicrobial	Conc.	No. (%) of isolates with Phenotypic resistance pattern		
			S	I	R
Aminoglycosides	Gentamycin	10 ug	57 (65.5)	(0)	30 (34.5)
	Streptomycin	10 ug	43 (49.4)	16 (18.4)	28 (32.2)
B-lactams	Amoxycillin	25 ug	9 (10.3)	(0)	78 (89.7)
	Ampicillin	10 ug	12 (13.8)	(0)	75 (86.2)
	Cefoxitin	30 ug	52 (59.7)	(0)	35 (40.3)
	Ceftriaxone	30 ug	75 (86.3)	3 (3.4)	9 (10.3)
	Penicillin G	10 IU	10 (11.5)	0 (0)	77 (88.5)
Phenicol	Chloramphenicol	30 ug	70 (80.5)	3 (3.4)	14 (16.1)
Macrolides	Erythromycin	15 ug	68 (78.2)	7 (8)	12 (13.8)
Fluoroquinolones	Ciprofloxacin	5 ug	83 (95.4)	3 (3.4)	1 (1.2)
	Levofloxacin	5 ug	84 (96.6)	1 (1.1)	2 (2.3)
	Ofloxacin	5 ug	75 (86.3)	8 (9.2)	4 (4.5)
Tetracyclines	Tetracycline	30 ug	52 (59.7)	5 (5.7)	30 (34.6)
Sulfonamides and synergistic agents	Cotrimoxazole	25 ug	57 (65.5)	20 (23)	10 (11.5)

Increased recurrent exposure to antimicrobial drugs has created a selective pressure for the emergence of resistant bacteria. This is apparent from our results since most *S. aureus* isolates were resistant to amoxycillin (89.7%), penicillin G (88.5%), and ampicillin (86%) where they are being used injudiciously while were less resistant to other members of β -lactam group as cefoxitin (17.2%) and ceftriaxone (10.3%). The lowest resistant rates were toward the less common antibiotic used in farm animals' treatment as fluoroquinolone group members; ciprofloxacin, levofloxacin and ofloxacin in rates 1.2%, 2.3% and 4.5%, respectively. Our study revealed similar resistance with previous recorded work [24] towards aminoglycosides; gentamycin (34.5%) and streptomycin (32.2%) and tetracycline (34.6%).

Regarding macrolides, lower resistance was reported with erythromycin (13.8%) as compared with the resistance rate (23%) in recent previous study [25]. Detection of livestock associated *S. aureus* (LA-SA) including MRSA and MDR strains among goats have been reported in many international studies [26-28]. In the current study, *mecA* gene had been recognized in 26.4% of the *S. aureus* isolates that were classified as MRSA.

Molecular identification and characterization are essential for the classification and understanding of various microorganisms. As MRSA strains have been isolated from many sources; patients, livestock and wastewater, plenty of studies have been hypothesized to demonstrate and explain genetics of *S. aureus* that includes genes responsible for antibiotic resistance along with virulence genes [29]. The gene *mecA* was the benchmark gene in detection of methicillin resistance, however, many recent studies displayed the failure of *mecA* in detection of MRSA strains [30]. A novel divergent *mecA* homologue methicillin resistance mechanism gene named *mecC* was recently discovered in *S. aureus* [31]. This gene has been recovered

from humans, ruminants and other animals [32], but to the extent of our knowledge" had not recovered yet in Egypt [33]. In the same context, *mecC* was not detected in the tested isolates of our study that phenotypically resist cefoxitin and so, *mecA* gene was considered our target gene for detection of MRSA strains. Moreover, the phenotypic resistance for cefoxitin (40.3%) was significantly higher than those detected depending on genetical basis (26.4%). The high obtained phenotypic MRSA may attribute to one or more genetic mechanisms that were not explored in this study. However, the previous studies established many mechanisms of methicillin resistance such as the penicillin-binding protein 2a (PBP2a) regulated by the *blaZ-blaI-blaR1* and *mecA-mecI-mecR1* systems [34] and also, the expression of the *mecA* gene is highly variable and dependent on different factors. In addition, the *femA* gene encodes proteins affect the expression level of methicillin resistance of *S. aureus* [35]. This may explain the difference in phenotypic and genetic outcomes in our study.

Tetracycline resistance are wide-ranging in bacterial species via conjugation or transposons particularly among those of MDR [36]. Prolonged use paving the way to emergence of tetracycline resistant species which is a serious concern for both human and animal health due to the possibility of transferring the resistant genes to humans from goats and vice versa [37]. In many countries including Egypt, tetracycline is the most used antibiotics for mastitis treatment and routinely added to water as a prophylactic protocol [38], so it is not surprising to get a high level of phenotypic resistance (34.6%) and thus the highest prevalence of a *tetK* gene (39.1%) among all investigated genes. Our results of tetracycline resistance are parallel to some and in contrast with others of previous studies [39-40]. Differences in outcomes can be attributed to differences in resistance gene profiles of isolates where three resistance

mechanisms of tetracycline have been well described: efflux, ribosomal protection, and enzymatic inactivation of tetracycline drugs and there are multiple strain-specific factors in clinical isolates affecting uptake the resistance genes. Moreover, the level of tetracycline-specific pumps expression plays a synchronized role in the overall tetracyclines susceptibility [41]. Based on this result, we can say that we are in critical situation and urgent need to take strict measures to reduce the use of tetracycline.

The prevalence of many virulence-associated genes in *S. aureus* has been investigated worldwide. The current study showed low prevalence but significant relation ($p < 0.05$) between endotoxin genes, *sea* (24.1%) and *see* (4.6%), as shown in table 3. The low frequency of these genes indicates that these isolates are less likely to cause toxin disease [42]. This is consistent with the type of the most samples where were taken from asymptomatic animals. The virulence factors, *spa* and *coa* exhibited prevalence rate of 6.9% and 8%, respectively. These proportions are congruent with previous studies that reported *spa* rate of 9.17% and 9% while was 11% and 5% for *coa* [43-44]. Interestingly, the virulence gene *coa* control the coagulase activity, and the low frequency of this gene indicates that most of the *S. aureus* isolates are coagulase-negative strains (CNS). This result offers an evident that CNS serve as a reservoir of antimicrobial resistance genes that can be exchanged to other bacteria and add more challenges to the control and treatment of mastitis and that could potentially risk on human health [45]. Furthermore, there are co-existence of virulence factors confirmed by the Pearson's correlation that states positive and significant relationships between *coa* and *spa* ($p = 0.000$) as shown in table 4.

Concisely, since our isolates were derived from different goat husbandry places, contagious transmission is likely to occur not only within the same herd, but also between different herds, possibly mainly through grazing where they are often grazing in the same areas. The intricacy of antimicrobial resistance needs synergy of research efforts to determine the interaction between bacterial pathogens in animal-human interfaces, which will help us in controlling the antimicrobial resistance. The findings of our study showed lower rates of the investigated genotypic as compared to the phenotypic characteristics. This can be assigned to that certain phenotypic property needs many genes for its expression. Furthermore, the low frequencies seen for the virulence genes do not certainly mean that the strains have no or limited virulence effect. In this study, *mecC* gene for methicillin resistance couldn't be detected but this may be draw attention for further prevalence studies on large scale and frequent surveillance. Since our isolates were derived from different goat husbandry places, contagious transmission is likely to occur not only within the same herd, but also between different herds, possibly mainly through grazing where they are often grazing in the same areas therefore, The application of strict control systems in animal production systems is increasingly necessary to prevent or reduce the spread of livestock associated *S. aureus* and prevent possible outbreaks as well. The intricacy of

antimicrobial resistance needs synergy of research efforts to determine the interaction between bacterial pathogens in animal-human interfaces, which will help us in controlling the antimicrobial resistance.

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