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Antimicrobial Resistance Genes among *Staphylococcus aureus* Isolated from Bovine Mastitis in Egypt

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Abstract: *Staphylococcus aureus* is usually known as a main infectious pathogen in bovine mastitis. *S. aureus*'s resistance to antimicrobial agents is an increasing global problem; therefore, allergy tests can direct a veterinarian to choose the best suitable antimicrobial agent. This study identified the features of antimicrobial agents and the distribution of antimicrobial genes among *S. aureus* isolated from bovine mastitis in Egypt. Antibiotic susceptibility testing was determined among fifteen *Staphylococcus aureus* isolates collected from cows showing clinical mastitis against 12 chemotherapeutic agents using disc diffusion method. Then distribution of antimicrobial resistance genes of resistant isolates was detected using PCR. Results showed that all *S. aureus* isolates showed complete susceptibility (100%) to: erythromycin, gentamicin, levofloxacin, penicillin-G, piperacillin/ tazobactam, sulfamethoxazole -trimethoprim and tobramycin. However, all the isolates (100%) were resistant to: ceftazidime, oxacillin, streptomycin, tetracycline and vancomycin. PCR detected the high prevalence of *mecA*, *blaz*, *tetk* and *aac (6') aph (2'')* genes and low incidence of *vanA* gene. Conclusion: All *S. aureus* isolates were multidrug resistant; and possess high distribution of antimicrobial resistance genes. Therefore, the susceptibility tests are essential to select the most appropriate antimicrobial agents to controlling antibiotic use in dairy farms and further studies should be investigate to get full reports of antimicrobial resistance genes pattern are essential.

Key words: Antibiotic Resistance · Mastitis · MRSA · PCR · *S. Aureus* · VRSA

INTRODUCTION

Staphylococcus aureus is one of the most common contagious pathogens causing bovine mastitis [1]. It has developed four general resistance mechanisms, including drug trapping, drug target modification, drug enzyme disruption and diaphragm flow pumps, to counteract attack by antimicrobials [2].

Once the mammary gland is successfully infected, *S. aureus* will spread quickly, releasing a variety of toxins and extracellular enzymes and form a biological membrane, which results in increased antibiotic resistance [3, 4].

Biofilm formation is one of the antimicrobial resistance techniques that *S. aureus* uses. This is a significant characteristic of *S. aureus* that defends bacteria from the effects of antimicrobial agents, leading to chronic infection in cows with mastitis [5].

S. aureus resists different types of antimicrobial agents in response to the selective pressure of antimicrobials and treatment options for doctors and veterinarians are limited [6]. Antimicrobial chemotherapy is the primary approach for the treatment of staphylococcal bovine mastitis [7] and susceptibility tests can direct the veterinarian to select the most effective antimicrobial agent [8].

Mastitis caused by emerging methicillin resistant *S. aureus* (MRSA) is increasingly common and it is difficult to treat. The uncontrolled use of antibacterial agents in developing countries could be a reason for the increase of *S. aureus* strains resistant to all types of β-lactam antibiotics that are frequently used for empirical treatment of mastitis [9].

European Food Safety Authority and European Centre for Disease Prevention and Control [10] recommended monitoring food borne pathogens for

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detection of methyl-resistant *S. aureus* (MRSA) and regular monitoring in humans to improve trends in determining the spread or development of these bacteria.

Reports indicate that multiple drug resistance (MDR) *S. aureus* including *S. aureus* methicillin (MRSA) leads to several cases of food poisoning associated with mastitis [11, 12].

More recently, the emergence of strains resistant to methicillin and vancomycin, especially their continued spread between animals and humans, has posed a serious public health threat [13].

In addition to MRSA resistance, it is very important to understand that the pathogenicity of these bacteria depends on a combination of extracellular factors and the ability to form biofilms [14].

Different genetic determinants such as *mecA* and *blaZ* (penicillins), *vanA* (vancomycin), *aacA-aphD* (amino-glycosides), *ermA/B/C* (macrolides), *fusB* (fusidic acid), *tetK/M* (tetracyclines), *rpoB* (rifampicin) and *ileS* (mupirocin) were reasonable for the corresponding antimicrobial resistance mechanisms in *S. aureus* [15].

In addition to producing virulence factors, the *S. aureus* genome demonstrates tremendous flexibility with the acquisition of transportable genetic elements and coding of resistance proteins. One example is the *mecA* gene found in the staphylococcal cassette chromosome mec (SCCmec) [16]. The *mecA* gene encodes an alternative penicillin binding protein, PBP2a [17], making the bacterial strain resistant to methicillin (MRSA) and all other -lactam antibiotics) [16].

Later, over 90% of the *S. aureus* strains were resistant to penicillin. The increase in this resistance led to the discovery of methicillin drugs, a semi synthetic penicillin that is virtually resistant to the genetic differences of β-lactamase enzyme. During development, strict bacteria that cannot be treated with antibiotics continue until the first bacterial strain of Methicillin-resistant *S. aureus* (MRSA) was isolated in 1961. Since then, MRSA become a dangerous endemic organism worldwide and listed on the top of the serious problems with a negative impact on public health. MRSA mediates by the presence of a new penicillin-binding protein (PBP), PBP-2a, which is expressed by an external *mecA* gene [18, 19].

Therefore, the aim of this study was to evaluate the antimicrobial resistance of *S. aureus* isolates of bovine mastitis cases in Egypt and to identify MRSA and VRSA by *mecA* and *vanA* genes respectively, as well as the distribution of *blaz, tetk* and *aac (6') aph (2'')* genes among the isolates.

MATERIALS AND METHODS

Isolation and Identification of *S. aureus*: A total of eighty-five samples of raw milk were collected from cows presenting with clinical mastitis from different farms in and around Giza city during the period from June to August 2019. Isolation of *S. aureus* was carried out according to method described by Singh and Prakash [20].

S. aureus confirmatory biochemical tests were performed using catalase test, coagulase test, acetoin production, DNase test, oxidase and D-mannitol fermentation. Isolates that were positive in all these tests except oxidase test which was negative were classified as *Staphylococcus aureus* [21, 22]. The confirmed *S. aureus* isolates were kept and stored in BHI with 40% [v/v%] glycerol at -80°C.

Antimicrobial Susceptibility Tests: Antibiotic susceptibility testing of all *S. aureus* isolates was determined by modified Kirby-Bauer disc diffusion method on Mueller-Hinton agar (Oxoid) [23]. Concisely, bacterial inoculum (~0.5 McFarland, from each strain) was swabbed on a separate Mueller Hinton agar (Oxoid) to obtain a lawn of confluent growth. The antibacterial discs (Oxoid Limited, UK) used in this study were ceftazidime-fortum (30 µg) CAZ, erythromycin (15µg) E, gentamicin (10 µg) CN, levofloxacin (5 µg) LEV, oxacillin (1 µg) OX, penicillin-G (10 µg) P, piperacillin/ tazobactam (110 µg) TZP, streptomycin (10 µg) S, sulfamethoxazole - trimethoprim (25 µg) SXT, tetracycline (30 µg) TET, tobramycin (Nebcin) (10 µg) TOB and vancomycin (30 µg) VAN were placed on inoculated plate followed by incubation at 37°C for 18 h. Assessment of inhibition around antibiotic discs were interpreted according to breakpoints provided by Clinical and Laboratory Standard Institute [24].

DNA Extraction: It was applied according to QIAamp DNA mini kit instructions (QIAamp DNA Mini Kit, Catalogue no.51304) with modifications from the manufacturer's recommendations. Concisely, 200 µl of the sample was added to 200 µl of lysis buffer, the mixture was incubated at 56°C for 10 min. Then, 200 µl of (96%) ethanol was added to the lysate. Next, the sample was centrifuged following the manufacturer's recommendations. Nucleic acid was eluted with 100 µl of elution buffer supplied with the kit.

PCR Amplification: Resistant *S. aureus* isolates to ceftazidime-fortum, oxacillin, streptomycin, tetracycline and vancomycin were subjected to PCR for detection of

Table 1: Oligonucleotide sequence of the used primers

Genes	Sequences	Amplified products	References
mecA	GTA GAA ATG ACT GAA CGT CCG ATA A CCA ATT CCA CAT TGT TTC GGT CTA A	310 bp	[25]
vanA	CATGACGTATCGGTAAAATC ACCGGGCAGRGTATTGAC	885 bp	[26]
blaZ	ACTTCAACACCTGCTGCTTTC TGACCACTTTTATCAGCAACC	173 bp	[27]
aac(6')aph (2'')	GAAGTACGCAGAAGAGA ACATGGCAAGCTCTAGGA	491 bp	
tetK	GTAGCGACAATAGGTAATAGT GTAGTGACAATAAACCTCCTA	360 bp	

Table 2: Temperature and time conditions of the primers used in PCR amplification.

mecA	94°C	94°C	50°C	72°C		72°C
	5 min.	30 sec.	30 sec.	30 sec.	35	7 min.
vanA	94°C	94°C	50°C	72°C		72°C
	5 min.	30 sec.	40 sec.	45 sec.	35	10 min.
blaZ	94°C	94°C	54°C	72°C		72°C
	5 min.	30 sec.	30 sec	30 sec	35	7 min.
tetK	94°C	94°C	54°C	72°C		72°C
	5 min.	30 sec.	40 sec.	40 sec.	35	10 min.
aac(6')aph (2'')	94°C	94°C	54°C	72°C		72°C
	5 min.	30 sec.	40 sec.	45 sec.	35	10 min.

mecA, vanA, blaz, tetk and aac (6') aph (2'' genes. The following primers were used from Metabion (Germany) as shown in Table (1) and their PCR conditions was presented in Table (2).

Primers were utilized in a 25- µl reaction containing 12.5 µl of Emerald Amp GT PCR Master Mix (2X) (Thermo Scientific), 1 µl of each primer (20 pmol concentration), 4.5 µl of PCR grade water and 6 µl of DNA template.

Negative controls were performed simultaneously with each test reaction by replacing the template DNA with sterilized water in the PCR mixture. Twenty µl of each uniplex PCR product were loaded to the gel. Each amplification product was separated by electrophoresis in a 1.5% agarose gel (Applichem, Germany, GmbH) in TBE buffer according to Sambrook *et al*. [28] with slight modification. The reaction was performed in an applied biosystem 2720 thermal cycler. For gel analysis, 20 µl of the PCR products were loaded in each gel slot.

Ethidium bromide staining (0.5µg/ml) allowed the visualization of DNA fragments with gel documentation system and the data was analyzed through computer software. Bands determination was possible with Gel Pilot 100-bp DNA ladder with size range: 100-600 bp and 100-1000 bp (cat. no. 239035 and cat. no. SM0243 respectively) supplied from QIAGEN, USA.

RESULTS

Isolation and Identification of *S. aureus*: Result analysis revealed that, out of total 85 samples of raw milk isolated from mastitis cows 15 isolates (12.75 %) was *S. aureus* according to morphological, cultural characteristics and biochemical tests.

Antimicrobial Susceptibility Tests: All *S. aureus* isolates showed complete susceptibility (100%) to erythromycin, gentamicin, levofloxacin, penicillin-G, piperacillin/ tazobactam, sulfamethoxazole -trimethoprim and tobramycin. On the other hand, all the isolates (100%) were resistant to ceftazidime- fortum, oxacillin, streptomycin, tetracycline and vancomycin.

Prevalence of Antimicrobial Resistance Genes: PCR detected a high prevalence rate of β-lactam resistance genes among *S. aureus* isolated from mastitic cows. Fourteen *S. aureus* isolates (93.3%) harbored the *mecA* gene and 15 (100%) possessed the *blaZ* gene as shown in (Table 3) and (Figs 1 and 2) respectively. While, only two isolates (13.3%) were positive for *vanA* gene as shown in (Table 3) and (Fig. 3).

Tetracycline and aminoglycoside resistance genes were of high prevalence in *S. aureus*, where *tetK* and *aac (6') aph (2'')* were detected in all isolates (100%) for both genes as shown in Table (3) and Figs (4 and 5) respectively.

Table 3: Prevalence of antimicrobial resistance genes among *S. aureus* isolates from clinical mastitis cows.

Sample	*mecA*	*vanA*	*blaZ*	*tetK*	*aac(6')aph (2")*
1	+	-	+	+	+
2	+	+	+	+	+
3	+	+	+	+	+
4	+	-	+	+	+
5	-	-	+	+	+
6	+	-	+	+	+
7	+	-	+	+	+
8	+	-	+	+	+
9	+	-	+	+	+
10	+	-	+	+	+
11	+	-	+	+	+
12	+	-	+	+	+
13	+	-	+	+	+
14	+	-	+	+	+
15	+	-	+	+	+

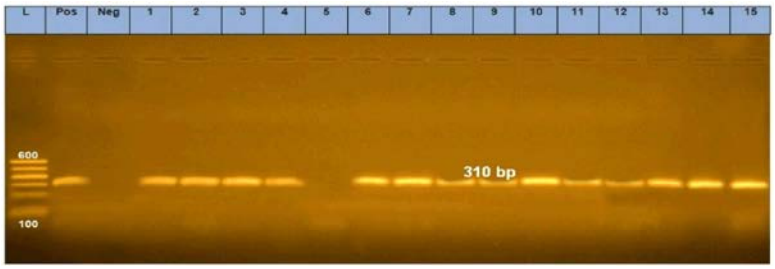

Fig. 1: PCR results for amplification of *mecA* gene at 310 bp among *S. aureus* isolates. Lane L: 100-bp DNA ladder (Gel Pilot 100 bp ladder cat. no. 239035), lane Pos: positive control, lane Neg: negative control and lanes 1-15: positive *S. aureus* isolates for *mecA* gene except isolate 5 was negative for *mecA* gene.

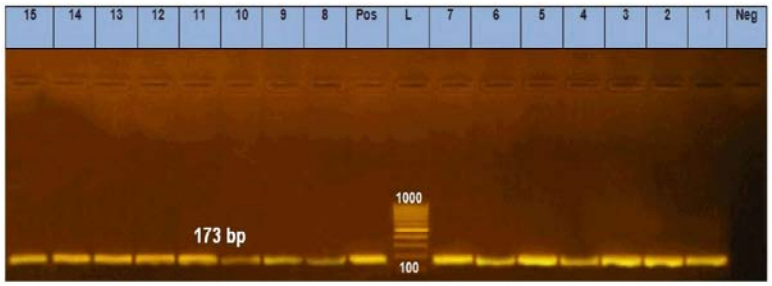

Fig. 2: PCR results for amplification of *blaZ* gene at 173 bp among *S. aureus* isolates. Lane L: 100-bp DNA ladder (Gel Pilot 100 bp ladder cat. no. SM0243), lane Pos: positive control, lane Neg: negative control and lanes 1-15: positive *S. aureus* isolates for *blaZ* gene.

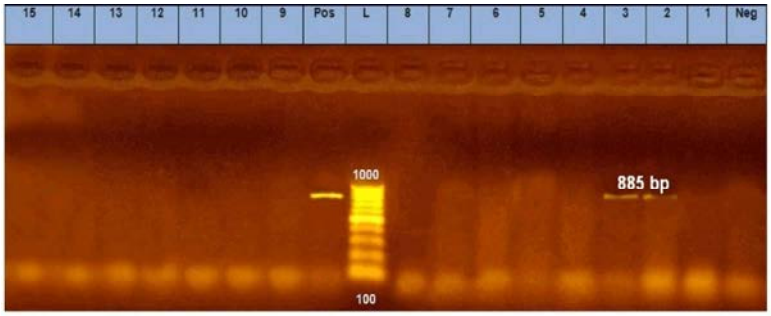

Fig. 3: PCR results for amplification of *vanA* gene at 885 bp among *S. aureus* isolates. Lane L: 100-bp DNA ladder (Gel Pilot 100 bp ladder cat. no. SM0243), lane Pos: positive control, lane Neg: negative control and lanes 1-15: negative *S. aureus* isolates for *vanA* gene except isolates 2 and 3 were positive for *vanA* gene.

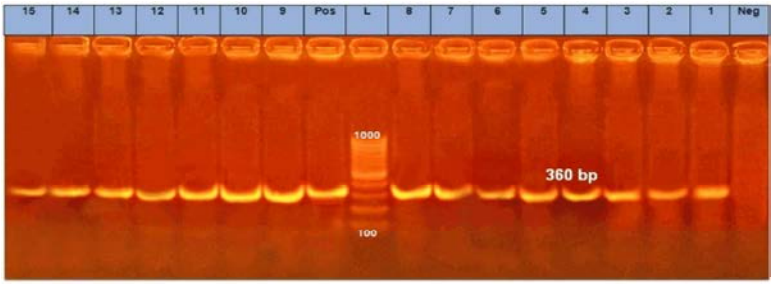

Fig. 4: PCR results for amplification of *tetK* gene at 360 bp among *S. aureus* isolates. Lane L: 100-bp DNA ladder (Gel Pilot 100bp ladder cat. no. SM0243), lane Pos: positive control, lane Neg: negative control and lanes 1-15: positive *S. aureus* isolates for *tetK* gene.

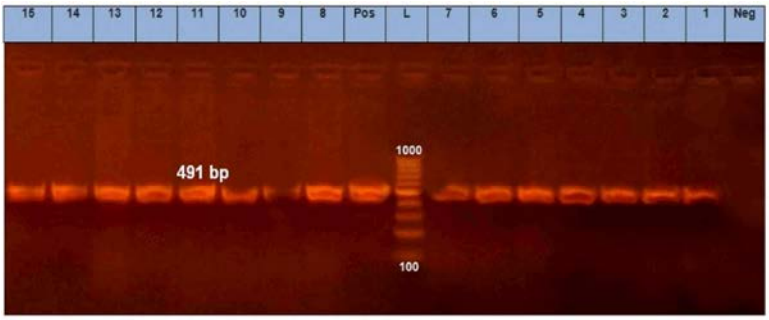

Fig. 5: PCR results for amplification of *aac (6') aph(2")*gene at 491 bp among *S. aureus* isolates. Lane L: 100-bp DNA ladder (Gel Pilot 100 bp ladder cat. no. SM0243), lane Pos: positive control, lane Neg: negative control and lanes 1-15: positive *S. aureus* isolates are for *aac (6') aph(2*")gene.

DISCUSSION

Staphylococcus aureus is often recognized as the main infectious pathogen in bovine mastitis [29]. Left untreated, it poses a serious problem in the dairy herds with significant economic consequences, mainly due to the low quality and quantity of milk production [30].

S. aureus' resistance to antimicrobial agents is a growing global problem. The identification of antimicrobial sensitivity profiles is necessary not only for successful treatment but also for checking the frequency of resistant strains in specific environmental niches [31, 32].

This study examined the antimicrobial susceptibility profiles of twelve antimicrobial drugs against fifteen isolates of *Staphylococcus aureus* isolated from eighty-five clinically mastitis cows in Egypt and identified resistance related genes of these isolates.

All isolates were performed according to Singh and Prakash [20] with slight modification and were confirmed biochemically according to Lancette and Tatini [21] and Garcia [22].

According CLSI [24] all *S. aureus* isolates had full susceptibility (100%) to: erythromycin, gentamicin, levofloxacin, penicillin-G, piperacillin/ tazobactam sulfamethoxazole -trimethoprim and tobramycin.

On the other hand, all the isolates (100%) were resistant to: ceftazidime- fortum, oxacillin, streptomycin, tetracycline and vancomycin.

Several reports indicate that members of the penicillin group are the most resistant antibiotic to S. aureus isolated in milk samples from cows infected with bovine mastitis [33-38].

Our study was fully supported by Costa *et al*. [39] who reported that gentamicin, amoxicillin and norfloxacin were the most effective antimicrobial agents against isolates, while the lowest were penicillins, streptomycin and ampicillin.

The antimicrobial susceptibility profiles of *S. aureus* were determined and high levels of resistance to penicillin followed by erythromycin and tetracycline were recorded by Yang *et al.* [40].

Yang *et al*. [40], determined the antimicrobial sensitivity profiles of *S. aureus* and recorded high levels of penicillin resistance followed by erythromycin and

tetracycline. This data is the same as that in other reports [41, 42]. In particular, more than half of *S. aureus* isolates were penicillin-resistant (84.09%), a characteristic that is similar to other data previously reported in China [43].

The antimicrobial agents, such as cefazolin, ciprofloxacin, enrofloxacin, erythromycin, kanamycin, oxacillin, tetracycline and vancomycin can be used to control *S. aureus* infection [33, 38]. Our study was diverse from these reports, where we found that oxacillin and vancomycin were not effective to control the *S. aureus* infection.

The ability of an organism to resist and grow against two or more antimicrobials is defined as multiple drug resistance. Bezina *et al.* [44] reported that isolates showed an inverse relationship between the nature of MDR and the number of antimicrobials applied.

This observation matches with the finding of Sori *et al.* [45] who demonstrated MDR pattern of 25, 10.45 and 7% for two, three and four types of drugs, respectively. However, it differs from Teshome *et al*. [46] who recorded MDR of 34.8% of the isolates for three and 8.7% for two antimicrobials. Pechere [47] explained the difference that was detected in the MDR pattern due to the chemical composition similar of a group of drugs and the mechanism of action may show cross resistance from bacteria despite the number of drugs involved.

This difference may occur due to the difference in the practice of milking, the purpose of antibiotic use and inappropriate therapeutic treatment by non-professionals [44].

In our study, PCR detected the high prevalence of blaZ, tetK and aac (6') aph (2") genes (100% each) mecA (93%) antibiotic resistance genes among *S. aureus* isolates. While two S. aureus isolates had vanA gene (13%).

MRSA prevalence rate differs between studies worldwide; some Egyptian studies have reported much higher rates of occurrence [48], whereas a low detection rate was seen in most African countries [49].

All the MRSA isolates were multidrug resistant [50]. Combined tetracycline, macrolide and aminoglycoside resistance as well as β-lactams has been reported in MRSA strains isolated from livestock or milk and dairy products [51-53].

This study is consistent with these reports, as all MRSA strains of mastitis milk contain at least three antimicrobial genes, such as β-lactams, tetracycline and aminoglycoside.

Knowledge of distribution of antimicrobial resistance genes among pathogenic and non-pathogenic udder microbes is a key to understand evolution of multi-drug resistant agents in dairy cattle. All *Staphylococcus* isolates carried at least one antimicrobial resistance gene and nearly all isolates (98%) carried the blaZ gene, indicative of penicillin resistance [54].

The bacterial resistance mechanisms are so complex that the presence or absence of a specific resistance gene does not certainly indicate that a specific isolate is resistant or sensitive to the corresponding antimicrobial agent [55]. We matched with this study, where we determined that two isolates possessed *vanA* resistant gene, while all the fifteen isolates were resistant to vancomycin.

CONCLUSIONS

Mastitis is considerable a serious problem in dairy herds with important economic losses. *Staphylococcus aureus* is one of the most common infectious pathogens causing bovine mastitis world wild. Therefore, our study examined the antimicrobial susceptibility profile and prevalence of antimicrobial resistance genes of *S. aureus* isolates of cow's raw milk with clinical mastitis from different farms in Giza city. Our results revealed that antimicrobial agents, such as: erythromycin, gentamicin, levofloxacin, penicillin-G, piperacillin/ tazobactam, sulfamethoxazole -trimethoprim and tobramycin, can be used to control bovine mastitis caused by *S. aureus*. In contrast, there was resistance to: ceftazidime- fortum, oxacillin, streptomycin, tetracycline and vancomycin. These results may assist veterinarians for choosing antibiotic therapy for treating bovine mastitis and confirmed the importance of controlling antibiotic use in dairy farms.We detected high distribution of antimicrobial resistance genes among *S. aureus* isolated from clinically mastitic cows. However, further investigations to get full reports of antimicrobial resistance genes pattern are essential.

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