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Phenotypic and Molecular Characterization of Methicillin-Resistant *Staphylococcus Aureus* Isolated from Bovine Mastitis in Egypt

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ABSTRACT

Bovine mastitis constitutes an economic and serious problem in dairy industry worldwide. This study aimed to determine the phenotypic and molecular characterization of methicillin-resistant *Staphylococcus aureus* isolated from bovine mastitis in Menoufiya governorate, Egypt. A total of 530 mastitic samples (280 clinical and 250 subclinical) were collected and subjected to bacteriological examination. The result revealed that prevalence rate of clinical and subclinical mastitis was 52.83% and 47.16%, while *S. aureus* prevalence rate was 28.57% and 24.4% in clinical and subclinical mastitis respectively. Out of 157 staphylococci isolates on MSA medium, 141(89.9%) were identified as *S. aureus* isolates through biochemical activities and confirmed by amplifying of *nuc* gene at 279 bp. All *S. aureus* isolates confirmed by *nuc* gene (141 isolates) were tested for antibiogram profile against some β-lactams antibiotics (oxacillin, penicillin and amoxicillin/clavulanic acid) that recorded high resistance against these antibiotics. Out of 141 isolates, 128 (90.78%) were confirmed as MRSA strains based on phenotypic resistance to oxacillin and confirmed by molecular detection of the *mec*A gene at 310 bp. In conclusion, these results showed the significance of continuous surveillance of antibiogram pattern of *S. aureus* isolates of mastitis origin to design effective control measures for *S. aureus* mastitis.

Keywords: *Bovine mastitis, mecA, nuc gene, S. aureus, antibiogram*

INTRODUCTION

Staphylococcus aureus is Gram-positive cocci usually found as a commensal of upper respiratory and lower urogenital tract of various species (Johnson, *et al.* 2016 and Vanderhaeghen *et al.* 2010). Mastitis is the most common disease in dairy cattle breeding systems causing severe economic losses include drop in milk production, low milk quality, high culling rate and high treatment costs (He *et al*., 2014; Singh *et al*., 2015). *S. aureus* has the ability to infect various host species, including human and animals. In animals, it causes virtually incurable chronic mastitis (Artursson, *et al.* 2016). Economically, *S. aureus* is the most main udder pathogen that is responsible for 30-40% of all udder infections and 80% of subclinical mastitis resulting in about 35 billion $ losses annually worldwide (Reshi, *et al.* 2015). *S. aureus* has structural or secretory virulence factors that control the *S. aureus* pathogenicity and infection pathway (Quinn, *et al.* 2011). Of these structural virulence factors, protein A, peptidoglycan, capsular polysaccharide and adhesions, while secretory factors include lipase, coagulase, proteases, hyaluronidase and hemolysins (Fagundes & Oliveira, 2004 and Iqbal, *et al.* 2016). Recently, the emerging of many *S. aureus* strains that

showed high resistance to various β-lactam antibiotics including methicillin resistance (Robinson and Enright, 2003). The high resistance to various antimicrobials agents complicated the treatment of infections and led to lose of value of antibiotics. The *mec*A gene that encoded the penicillin-binding protein2a (PBP2a) which responsible for methicillin resistance and β lactams antibiotics in staphylococcus species (Rajabiani, *et al.* 2014). Several molecular techniques as PCR has been applied as a common, rapid, and specific approaches for detection of different DNA genes among many bacterial mastitis (Taponen *et al*., 2009). In recent years, MDR-MRSA strains were predominant and may poses a significant high public health risk as well as the existence of these MDR strains could permitted the transfer resistance genes among different species (Jans, *et al*., 2017). As results of the high prevalence and economic losses of mastitis in dairy cattle in Egypt, this study was planned to determine the phenotypic and molecular characterization of methicillin-resistant *S. aureus* isolated from bovine mastitis in Menoufiya governorate, Egypt.

MATERIALS AND METHODS

Animals and Samples collection:

A total of 530 cows in Menoufiya governorate were examined for mastitis from January to May 2017. Clinical mastitic samples 280 showed abnormities (clots, flakes, blood milk) and 250 subclinical which detected by CMT according to (Quinn, *et al.,* 2011). The milk samples were collected aseptically in a sterile screw-capped bottle from each mammary gland after washing with worm water and soap and cleaning the teats with cotton soaked with 70% ethanol according to (Edmondson and Bramely, 2004) and immediately transfer to the laboratory for bacteriological examination.

Bacteriological isolation and identification:

The samples were firstly processed and then cultured onto mannitol salt agar (Oxoid Ltd.) ® and defibrinated sheep blood agar 7–10% and incubated at 37 C for 48 hours. The suspected colonies of *S. aureus* were tested for biochemical identification through catalase, coagulase, terhalose fermentation test as described by (APHA, 1992), Gram staining, haemolytic activity, DNase agar according to (Murray *et al*., 2003).

Antibiogram profile of S. aureus isolated from bovine mastitis.

The antimicrobial susceptibility test of 141 *S. aureus* isolates was tested in vitro using the disk diffusion method. A suspension of the bacteria was adjusted to a 0.5 McFarland suspension. The following discs were used: Penicillin (P) 100 IU, Oxacillin (OX) 5 µg, Amoxicillin/ clavulanic acid (AMC) 30 µg, (Oxoid)®, followed by incubation at 37 ºC for 24 hours and the results were recording according to the size of inhibition zone together with interpretation of the results according to CLSI (2017).

Molecular characterization of methicillin-resistant S. aureus by PCR.

The genomic DNA was extracted from BHI broth using commertial kits (i-genomic BYF DNA Extraction Mini Kit of iNtRON) ®. The list of primers used to amplify the *nuc*, 16S rRNA and *mec*A genes of *S. aureus* were listed in Table (1). Briefly, the mixture consists of 5 µl of template DNA, 1 µl of each primer, 12.5 µl of 2×EasyTaq ® PCR SuperMix (transbionovo) ® and the final volume was completed to 25ul with distilled water. PCR was performed in Rotor Gene Thermo Cycler. After the run of PCR is completed; about (15 µL) of the PCR products were loaded in agarose gel electrophoresis (1.5%) and visualized under UV light in a gel documentation system.

RESALTS

Prevalence of S. aureus isolated from clinical and subclinical mastitis in Menoufiya governorate.

Five-hundred and thirty cows were examined for mastitis. The clinical examination revealed that 280 (52.83%) cows showed clinical mastitis at which abnormalities in milk were observed (clots, flakes and bloody milk). Another 250 (47.16%) cows were positive with CMT reaction. The results of bacteriological culture revealed that S. aureus prevalence rate was 28.57% and 24.4% in clinical and subclinical mastitis respectively as showed in table (2). Out of staphylococci isolates (157) (141) isolates were biochemically identified as S. aureus through catalase, coagulase, terhalose fermentation test,

hemolytic activity and confirmed by nuc gene amplification at 279bp.

***Antibiogram profile of S. aureus isolates isolated from bovine mastitis**:*

The results in table (3) showed that *S. aureus* isolates exhibit high resistance against Oxacillin, Penicillin, and Amoxicillin/ clavulanic acid with percentage of 88.65%,96.45, and 90.78% respectively. Out of 141 isolates 126 (89.36%) isolates were identified as MRSA strains based on the phenotypical resistance of oxacillin on disk diffusion and molecular detection of *mec*A gene at 310 bp.

Molecular characterization of S. aureus isolated from bovine mastitis.

Phenotypically identified *S. aureus* isolates were examined by 16S universal primer for detection and confirmation of *staphylococcus* species at 756 bp. As well as *S. aureus* isolates were confirmed by amplifying *nuc* gene at 279 bp as illustrated in fig (1, and 2).

Table (1): primer and PCR conditions for 16SrRNA, *mec*A, and *nuc* genes of *S.aureus*.

Gene	Length of amplified product	Primer sequence (5'-3')	Reference
16S rRNA	756 bp	AAC TCT GTT ATT AGG GAA GAA CA CCA CCT TCC TCC GGT TTG TCA CC	Zhang, *et al.*, 2005
***mec*A**	310 bp	GTA GAA ATG ACT GAA CGT CCG ATA A CCA ATT CCA CAT TGT TTC GGT CTA A	Zhang, *et al.*, 2005
Nuc	279 bp	GCG ATT GAT GGT GAT ACG GTT AGC CAA GCC TTG ACG AAC TAA AGC	Shortle, 1983

Initial denaturation step at 95°C for 2 min followed by 30 cycles at 94°C for 30 s, 55°C for 30 s and 72°C for 60 s and a final extension step at 72°C for 7 min.

Table (2): Prevalence of *S. aureus* isolated from clinical and subclinical mastitis in Menoufiya governorate.

Samples	Number of samples		Results of Bacteriological examination of *staphylococci*		*S. aureus*	
	No	%	No	%	No	%
Clinical	280	52.83	100	35.71	80	28.57
Subclinical	250	47.16	57	22.8	61	24.4
	530		157		141	

Table (3): Antibiogram profile of *S. aureus* isolates isolated from bovine mastitis:

Antimicrobials	Resistance		Intermediate		Susceptible	
	No	%	No	%	No	%
Oxacillin (OX)	126	88.65	2	2.13	13	9.22
Penicillin (P)	136	96.45	2	1.42	3	2.13
Amoxicillin/ clavulonic acid (AMC)	128	90.78	5	3.55	8	5.67

*the percentage was estimated according to the total number of isolates as S. aureus (141)

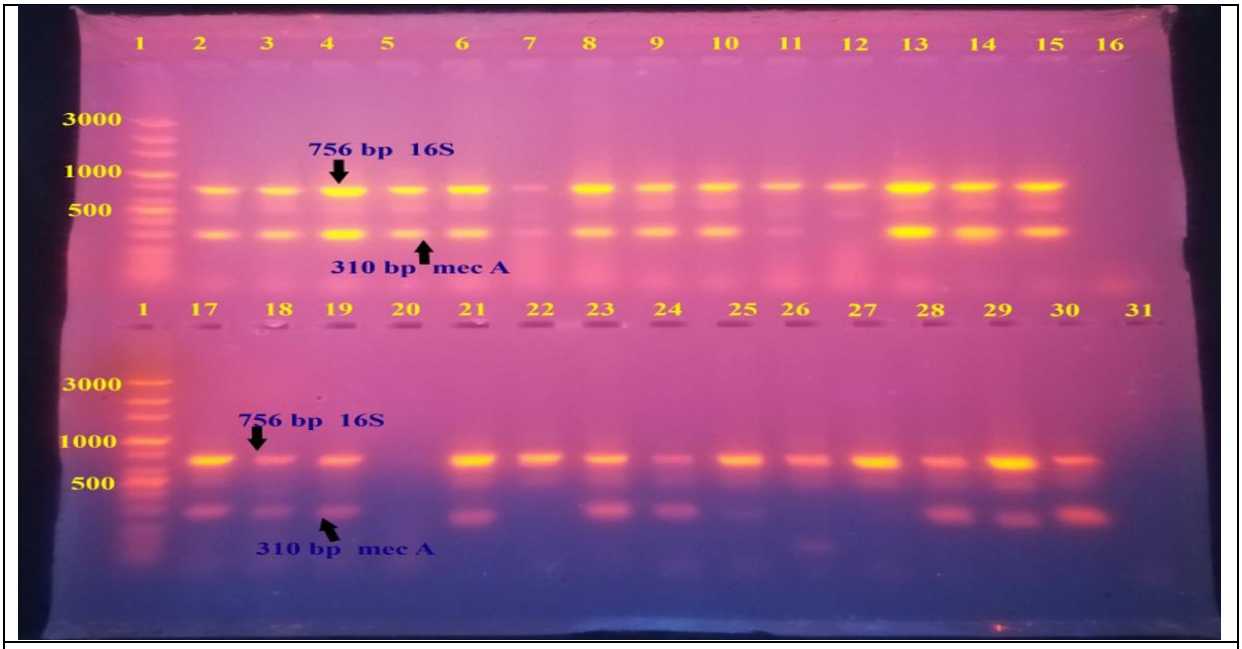

Fig (1): Agrose gel electrophoresis (1.5%) of multiplex PCR for *mec*A gene at 310 bp and 16 S gene at 756 bp. (1) ladder 100bp, (15) positive control and 16: negative control. Positive samples for *mec*A gene (2- 11,13, 14, 17-19, 21, 23, 24, 28-30, while 12,20,22, 25-27, and 31 were negative for *mec*A gene). Meanwhile, positive samples for the 16 S rRNA gene from 1-14, 17-19, and 21-30), while 20 and 31 were neagtive.

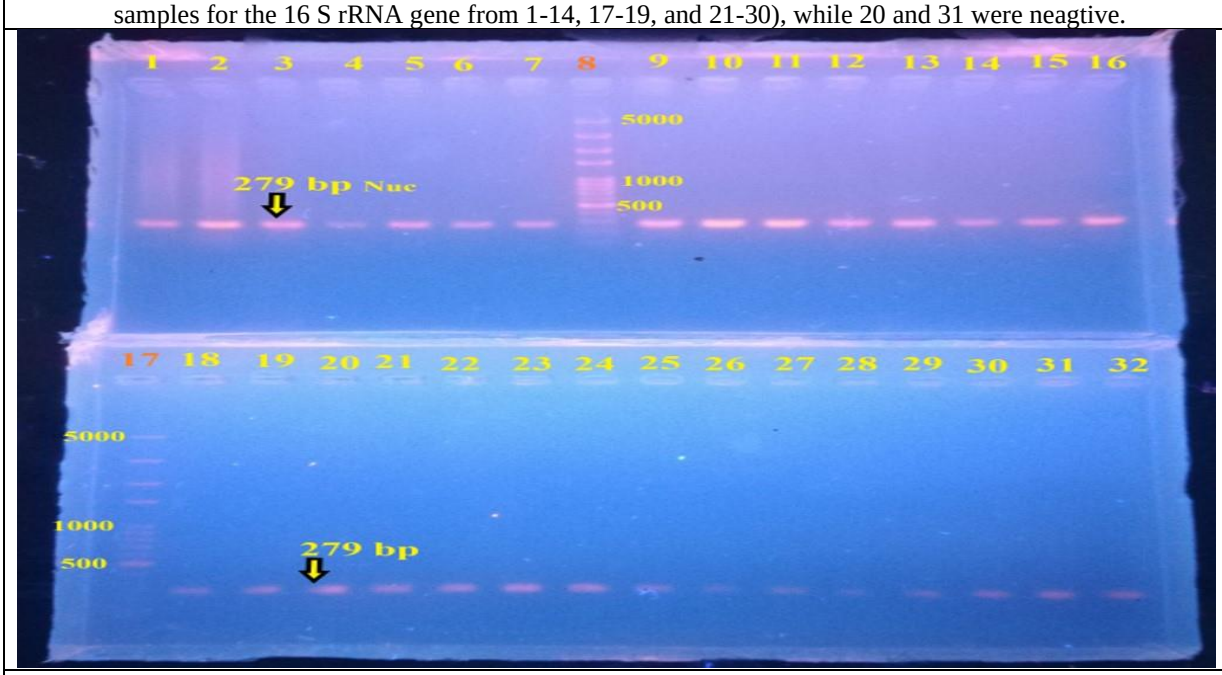

Fig (2): Agrose gel electropherosis (1.5%) of PCR product of the *nuc* gene at 279 bp: 8 and 17 are the ladder 100bp, all the samples are positive for the *nuc* gene.

DISCUSSION

Bovine mastitis is defined as inflammatory infection of the udder tissues caused by bacteria that invade the teat canal. Mastitis is a worldwide dairy problem in cattle herds as it has negative impact on animal milk productivity (Sharma *et al*., 2012). *S. aureus* has the ability to produce a variety of animals and human infections. According to the study of (Taverna *et al*., 2007) about 50% of intramammary infections are cause by *S. aureus*. In the current study, the prevalence rate of clinical and subclinical mastitis among 530 examined cows in Menoufiya governorate was 52.83% and 47.16% respectively. These results are higher than (Mousa *et al*., 2015) reported 20.5% and 32% in clinical and subclinical mastitis respectively in the same governorate. In addition, (Delelesse 2010) recorded lower prevalence rate (10.3%) in Ethiopia from clinical mastitis in cattle. While other study achieved by Karimuribo *et al*., (2008)

revealed higher prevalence rate in subclinical mastitis 75.9%. Furthermore, (Seegers *et al*., 2003) demonstrated that subclinical mastitis is the most common mastitis form in dairy cattle. As well as it represented a source for intramammary infection (Oliver *et al*., 2004) that usually terminated by severe or incurable clinical mastitis form (Reksen *et al*., 2006). In our study *S. aureus* was prevalent in clinical and subclinical mastitis with 28.57%, 24.4%. These was similar contact with (Mousa *et al*., 2015) who isolated *S. aureus* from clinical and subclinical mastitis cases with 29.11% and 21.51%. on the other side, Abdel-Tawab *et al*., 2016; El-Faramawy *et al*., 2019) revealed that *S. aureus* prevalence rate was 45.6% and 46.5% from clinical mastitis cases. The variation in prevalence rates between our study and other researches may attributed to variation in sample size, animal breed, environmental and mange mental conditions.

Regarding to the identification of *S. aureus* isolates through biochemical tests, all the tested isolates were tested by coagulase, catalase and sugar fermentation tests. This was previously applied and described by (Abdeen *et al*., 2015; Elmaghraby *et al*., 2018) who tested all isolates of *S. aureus* by coagulase, catalase and fermentation activities.

The results of the antibiogram pattern of *S. aureus* isolates indicated high resistance against Oxacillin, Penicillin, and Amoxicillin/ clavulanic acid with percentage of 88.65%,96.45, and 90.78% respectively as well as 126 (89.36%) isolates were identified as MRSA strains based on the phenotypical resistance of oxacillin and molecular detection of *mec*A gene. The resistance pattern of *S. aureus* from mastitis origin to most of β lactams antibiotics was previously described in a recent study in Egypt by (El-Faramawy *et al*., 2019) who recorded that 46 isolates of *S. aureus* exhibited higher resistance to Penicillin (95.65%), and Oxacillin (67.39%) and 31 (67.39%) were defined as MRSA strains from 46 *S. aureus* isolates and showed higher resistance to various antibiotics. In addition, resistance to amoxicillin antibiotic was reported by (Umar *et al*., 2013). The existence of MRSA strains from bovine source has been emerged as a serious problem in mastitis treatment and food poisoning outbreaks (Holmes and Zadoks, 2011; Abdeen *et al*., 2021).

The *S. aureus* isolates were identified by 16S universal primer and *nuc* gene for staphylococcus species at 756 bp and 279 bp respectively. Several modern molecular approaches as using of universal primers 16S rRNA has been already applied as powerful method for identification of many bacterial species (Greisen *et al*., 1994). PCR technique is the most sensitive and specific method for detection of the *mec*A gene among the MRSA strains (Okorie-Kanu, *et al*., 2020). Also molecular detection of the *mec*A gene was successfully amplified at 310 bp. Previous report supported the importance of *nuc* gene as gold standard for *S. aureus* identification (David *et al*., 2010). Regarding to the detection of *mec*A gene in most *S. aureus* isolates in our study which was demonstrated in clinical mastitis isolates by (Abdeen *et al*., 2015) who amplified *mec*A gene in all tested *S. aureus* isolates. While lower prevalence of *mec*A gene 16% was recorded in China (Qu *et al*., 2019).

CONCLUSION

S. aureus is one of the most frequent bacterial cause of clinical and subclinical intramammary infection. The results concluded that high resistance to β- lactams antibiotics that represented an obstacle in the treatment of mastitis in dairy cattle particular the MRSA strains. Moreover, the PCR is an efficient tool for detection of the *mec*A gene among the MRSA strains as well as detection of the *nuc* gene for specific identification of *S. aureus* strains. Further studies are needed for continues monitoring of *S. aureus* among the dairy herds to construct ideal preventive measures to control or minimize incidence of mastitis.

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