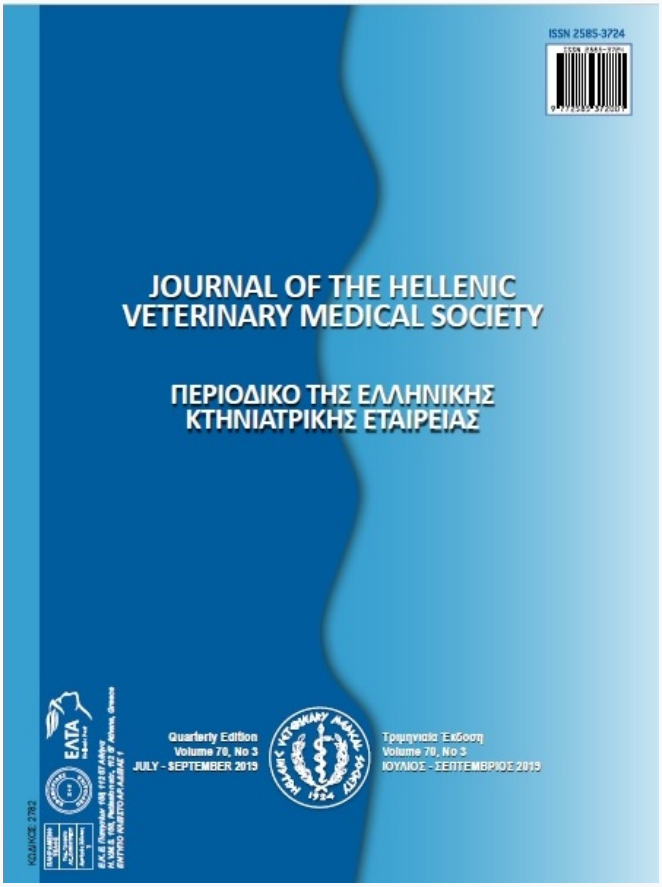
Journal of the Hellenic Veterinary Medical Society

Vol 70, No 3 (2019)



Clinical and molecular characterization of both methicillin-resistant andsensitive staphylococcus aureus mastitis

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doi: 10.12681/jhvms.21805

To cite this article:

OREIB, A., KHALIFA, H., EID, A., AHMED, A., SHIMAMOTO, T., & SHIMAMOTO, T. (2019). Clinical and molecular characterization of both methicillin-resistant andsensitive staphylococcus aureus mastitis. *Journal of the Hellenic Veterinary Medical Society*, *70*(3), 1743–1748. https://doi.org/10.12681/jhvms.21805

J HELLENIC VET MED SOC 2019, 70(3): 1743-1748
ΠΕΚΕ 2019, 70(3): 1743-1748

Research article
Ερευνητικό άρθρο

Clinical and molecular characterization of both methicillin-resistant and-sensitive *staphylococcus aureus* mastitis

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ABSTRACT. This study targeted bovine mastitis as a possible source of livestock-associated methicillin-resistant *Staphylococcus aureus* (MRSA), to identify clinical signs associated with MRSA- and non-MRSA-associated mastitis. Thirty-eight mastitis cases (68 infected quarters) were investigated. Gram-positive cocci-shaped isolates were selected based on Baird Parker agar growth as well as Gram-stained bacterial smears. Molecular screening for *Staphylococcus aureus* (*S. aureus)* yielded 17 isolates, of which five (29.41%) were methicillin resistant. The five isolates were *mecA* positive, but *mecC* negative. Multilocus sequence typing (MLST) indicated that sequence type 1 (ST1) was the identified type of all isolates of MRSA. *S. aureus*-associated cases showed different clinical forms of mastitis, including subclinical, acute, chronic, and gangrenous. Additionally, subclinical mastitis was the only detected condition associated with MRSA, which may represent a potential hidden risk for humans. Phenotypically, isolates of MRSA showed resistance to all of the tested β-lactam antimicrobials, with marked resistance to tetracycline and gentamycin. Based on our knowledge, this is the first report to identify MRSA ST1 in Egypt. Bovine mastitis could be a source for the dissemination of MRSA to humans and other animals. Additionally, while methicillin-resistance may have no effect on the clinical outcome of mastitis, it does affect therapeutic success, particularly when β-lactam antimicrobials are used.

***Keywords*:** *MRSA, Bovine, Mastitis*, *Staphylococcus aureus*

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Date of initial submission:
Date of revised submission:
Date of acceptance:

INTRODUCTION

Staphylococci, particularly *S. aureus*, are common pathogens of mastitis in bovines (Haveri et al., 2007), and the *Staphylococcus spp.* associated mastitis is responsible for considerable economic losses (Lammers et al., 2000). *S. aureus* accounts for 25–30% of all intra-mammary infections (IMI) in cows (Poutrel, 1985). Importantly, *S. aureus* IMI results in a 10–25% milk reduction in infected animals (Anderson, 1983). *S. aureus* can induce either clinical (CM) or subclinical (SCM) mastitis, but the subclinical form is more predominant (Anderson, 1983; Lammers et al., 2000; Akineden et al., 2001). While CM has detectable clinical symptoms, SCM has no detectable symptoms and necessitates screening using the California mastitis test (CMT) (Kasikci et al., 2012). The success in staphylococcal mastitis therapy is dependent on the individual animal, treatment, and pathogen factors (Barkema et al., 2006), and the resistance to antimicrobials is a major factor affecting the cure rates of staphylococcal mastitis (Barkema et al., 2006).

Staphylococci, notably *S. aureus*, have shown resistance to various antimicrobials (Wang et al., 2015), and MRSA strains have gained worldwide attention. MRSA is classified into three categories according to its origin: livestock-associated (LA-MRSA), healthcare-associated (HA-MRSA), and community-associated (CA-MRSA) (Stefani et al., 2012). There is an increasing global interest in LA-MRSA because of its animal and human associated health implications (Graveland et al., 2011). Many sequence types (ST1, ST9, ST97, ST130, ST398, and ST425) of MRSA had been recorded in both cattle and humans (García-Álvarez et al., 2011; Paterson et al., 2012; Spoor et al., 2013; Alba et al., 2015; Cuny et al., 2015). In humans, LA-MRSA strains can colonize tissues, resulting in pneumonia, endocarditis, and other life threatening conditions (Ekkelenkamp et al., 2006; Witte et al., 2007). Molecular epidemiology studies on MRSA in southern Mediterranean countries are limited (Borg et al., 2007). In Egypt, there are very few studies on MRSA strains originating from bovine mastitis (Elhaig &Selim, 2015). Additionally, studies describing the clinical aspects of MRSA- and non-MRSA-associated bovine mastitis are limited. Therefore, this study was intended to screen for MRSA and its sequence types associated with bovine mastitis in Egypt, and to describe the clinical aspects of MRSA- and non-MRSA-associated bovine mastitis.

MATERIALS AND METHODS

Animals and detection of mastitis

Study animals were reared in the Gharbia and Kafrelsheikh governorates, in the central and northern regions of the Egyptian Delta. In these areas, the dairy animals are reared in small groups rather than organized farms. Thirty-eight mastitis cases (37 Friesian-Balady crossbred cows and one Egyptian buffalo) were included in this study. CM cases were detected by clinical examination of the animals with special attention to the udder according to Houe et al. (2002). SCM cases were identified using the CMT (Kasikci et al., 2012).

Sampling

Clinically detected and CMT-positive quarters were aseptically sampled according to Quinn et Al. (1994). Milk samples were sent refrigerated to the laboratory for pathogen isolation.

Bacteriological examination

The samples were centrifuged (1000 *g*/5 min), the supernatant was discarded and sediment was streaked on Baird Parker agar. A 24 h incubation at 37 °C was done according to Silva et al. (2000). Gram-stained Smears of the colonies were examined. The putative *Staphylococcus species* isolates were preserved in glycerol stock at −20 °C until be used in molecular procedures.

In addition to clinical cases isolates, 33 *Staphylococcus species* isolates were obtained from the Animal Health Research Institute (AHRI), Tanta Branch, Egypt. These isolates originated from the study area (Gharbia Governorate) and were isolated from mastitis cases. Clinical data for these isolates was not recorded.

Molecular characterization and typing

For extraction of DNA, Luria-Bertani agar plates were streaked by the isolates and incubated for 24 h at 37 °C. The extraction of DNA was performed by InstaGene matrix (Bio-Rad Laboratories Inc.). The extracted DNA was preserved at −20 °C for use in PCR assays. Primer sequences of *S. aureus*, MRSA and MLST are shown in Table 1.

Table 1. Primers of *S. Aureus*, MRSA and MLST

Primer	Gene	Sequence (5'-3')	Size (bp)	References
au-F3	*Nuc*	TCGCTTGCTATGATT GTGG	359	Sasaki et al., 2010
au-nucR		GCCAATGTTCTACCA TAGC		
***MecA*147-F**	*MecA*	GTG AAG ATA TAC CAA GTG ATT	147	Zhang et al., 2005
***MecA*147-R**		ATG CGC TAT AGA TTG AAA GGA T		
***mecA*$_{LGA251}$MultiFP**	*mecA*$_{LGA251}$	GAAAAAAAGGCTTAGAACGCCTC	138	Stegger et al.,
***mecA*$_{LGA251}$MultiRP**		GAAGATCTTTTCCGTTTTCAGC		2011
***arcC*-Up**	*Arc*	TTGATTCACCAGCGCGTATTGTC	456	Enright et al., 2000
***arcC*-Dn**		AGGTATCTGCTTCAATCAGCG		
***aroE*-Up**	*aroE*	ATCGGAAATCCTATTTCACATTC	456	
***aroE*-Dn**		GGTGTTGTATTAATAACGATATC		
***glpF*-Up**	*GlpF*	CTAGGAACTGCAATCTTAATCC	465	
***glpF*-Dn**		TGGTAAAATCGCATGTCCAATTC		
***gmk*-Up**	*Gmk*	ATCGTTTTATCGGGACCATC	429	
***gmk*-Dn**		TCATTAACTACAACGTAATCGTA		
***pta*-Up**	*Pta*	GTTAAAATCGTATTACCTGAAGG	474	
***pta*-Dn**		GACCCTTTTGTTGAAAAGCTTAA		
***tpi*-Up**	*Tpi*	TCGTTCATTCTGAACGTCGTGAA	402	
***tpi*-Dn**		TTTGCACCTTCTAACAATTGTAC		
***yqiL*-Up**	*YqiL*	CAGCATACAGGACACCTATTGGC	516	
***yqiL*-Dn**		CGTTGAGGAATCGATACTGGAAC		

A PCR assay targeting a 359-bp region of the *S. aureus* thermonuclease (*nuc*) gene was used to detect *S. aureus* as described by Sasaki et al. (2010) with a few modifications. Briefly, a 25-µl reaction was prepared containing 5 µL of DNA, 0.2 mM dNTPs, 1× buffer, 0.5 U of AmpliTaq Gold (Applied Biosystems), and primers (each of 20 pmol). The thermal cycler conditions consisted of 95 °C /10 min, 35 cycles (95 °C/30 s, 56 °C/35 s, and 72 °C/1 min), followed by 72 °C/10 min.

MRSA was identified by PCR targeting 147- and 138-bp regions of *mecA* and *mecC* (*mecA*$_{LGA251}$) as described by Zhang et al. (2005) and Stegger et al. (2012), respectively, with a few modifications. The 25 µL reaction consisted of 5 µL of DNA, 0.2 mM dNTPs, 1× buffer, 0.5 U of AmpliTaq Gold (Applied Biosystems), and primers (20 pmol of each). Mixtures of *mecA* and *mecC* were initially heated at 94 °C for 4 min/15 min, followed by 35/30 cycles of 94 °C/30 s, 52 /59 °C for 30 s/1 min, and 72 °C for 45 s/1 min, respectively. A final extension was conducted at 72 °C/7 min. MLST was performed using seven housekeeping genes according to Enright et al. (2000) and allelic profiles were obtained from MLST web site (http://saureus.beta.mlst.net/).

Antimicrobial susceptibility testing

All *S. aureus* isolates were examined for their susceptibility to ampicillin, tetracycline, ciprofloxacin, gentamicin, sulfamethoxazole and trimethoprim, teicoplanin, ceftriaxone, amoxicillin and clavulanic acid, oxacillin, and cefoxitin. The antibiotic disc diffusion guidelines of The Clinical and Laboratory Standards Institute (2005) were followed up.

RESULTS

Clinical and descriptive aspects of mastitis cases

Most of the studied cases had mastitis in a single quarter (25/38 cases). However, two, three, and four quarters were found affected in four, one, and eight animals, respectively. Of the 68 individual infected quarters, 46 were diagnosed as SCM (67.6%) and 22 as CM (32.4%). Acute mastitis was observed in 19 (27.9%) quarters, with chronic and gangrenous mastitis detected in two (2.9%) and one (1.5%) quarters, respectively.

Identification of *S. aureus*

Gram-positive cocci-shaped bacteria were isolated from 36/38 mastitis cases (94.7%) and 49/68 infected quarters (72.1%). Based on Baird Parker agar growth and Gram-stained smears, 59 gram-positive cocci-shaped isolates were selected. Molecular screening for *S. aureus* yielded seven isolates from seven individual cases. Clinical findings of *S. aureus*- mastitis are shown in Table 2.

Table 2. Clinical findings of *S. Aureus* mastitis

Case number	Isolate name	Type of mastitis	Symptoms
1	Sa28	Chronic	Marked fibrosis of the quarter and yellow watery milk
2	Sa104	Acute	Animal depressed, fluctuation of body temperature between normal and subnormal, marked fibrosis of the quarter and intense bloody milk
3	Sa120	Gangrenous	Coldness and black discoloration of the teat and the quarter base in addition to intense bloody milk
4	Sa119	Subclinical	Normal
5	Sa101	Subclinical	Normal
6	Sa107	Subclinical	Normal
7	Sa69	Subclinical	Normal

Table 3. Phenotypic and genotypic profiles of *S. Aureus* isolates

Isolates	Phenotypic resistance		Genotypic resistance
	β-lactams	**Other antimicrobials**	
Sa28	AMP, FOX	TEC	
Sa69	AMP, AMC, FOX, CRO, OXA	TET, TEC	*mecA*
Sa70	AMP, AMC, FOX, CRO	TET, GEN, TEC	*mecA*
Sa101	AMP, AMC, FOX, CRO, OXA	TET, GEN, TEC	*mecA*
Sa104	FOX	TEC	
Sa107	AMP	TET, GEN,TEC	
Sa119	AMP	GEN, TEC	
Sa120	AMP	-	
Sa131	AMP, AMC, FOX, CRO, OXA	TET, GEN, TEC	*mecA*
Sa135	AMP, FOX	TET, TEC	
Sa136	-	TET, TEC	
Sa137	AMP, AMC, FOX, CRO, OXA	GEN, TEC	*mecA*
Sa140	AMP	-	
Sa144	AMP, AMC, FOX, CRO, OXA	TET, GEN, TEC	
Sa146	AMP, FOX	-	
Sa158	AMP, AMC	-	
Sa164	AMP	-	

TET, tetracycline; CIP, ciprofloxacin; SXT, sulfamethoxazole and trimethoprim; GEN, gentamicin; AMP, ampicillin; AMC, amoxicillin and clavulanic acid; FOX, cefoxitin; TEC, teicoplanin; CRO, ceftriaxone; OXA, oxacillin.

Molecular screening of the AHRI isolates resulted in an additional 10 isolates of *S. aureus*. Consequently, 17 *S. aureus* strains were used in further investigations.

Antimicrobial susceptibility of *S. aureus* isolated strains

A marked resistance of *S. aureus* to teicoplanin and ampicillin was observed. In addition, oxacillin-resistance was evident in five *S. aureus* isolates. *S. aureus* phenotypic and genotypic profiles are listed in Table 3.

Molecular screening/typing of MRSA

Out of the 17 *S. aureus* strains, five (two originated from the clinical cases and three AHRI isolates) strains contained *mecA* (29.41%) and were classified as MRSA ST1. However, *mecC* was absent in all of the *S. aureus* isolates.

Clinical nature of MRSA-associated bovine mastitis

The both cases which were infected by MRSA ST1 had showed SCM, which may indicate that MRSA ST1 may be unable to induce severe mastitis cases.

DISCUSSION

LA-MRSA strains are reported to induce endocarditis, pneumonia, soft tissue and skin conditions in humans (Ekkelenkamp et al., 2006; Witte et al.,

2007). Interestingly, the current study identified a high percentage of MRSA ST1 (29.41%) amongst the *S. aureus* strains isolated from mastitis in bovines. However, larger scale investigations are important to assess LA-MRSA of bovine mastitis origin and its potential risk for humans in Egypt. MRSA ST1 is a wide spread LA-MRSA lineage with a broad host range including humans (Alba et al., 2015). Although it is commonly isolated from pigs, recent studies had recorded MRSA ST1 associated with cattle and dairy farming in some countries such as Italy and Hungary (Juhász-Kaszanyitzky et al., 2007; Alba et al., 2015). Moreover, studies had showed high genetic similarity (90-100%) between human and cattle associated MRSA ST1 and confirmed complete ability of the later to colonize and infect humans (Juhász-Kaszanyitzky et al., 2007; Alba et al., 2015).

Because only a single quarter was infected in most cases, we hypothesize that the involved organisms are not highly contagious, particularly as Egyptian farmers often do not maintain ideal milking hygiene procedures. This was confirmed by the results of the molecular analysis that showed that most of the isolated strains were not *S. aureus*. SCM was also more prevalent than CM, which agrees with previous studies (Elhaig & Selim, 2015), and highlights the importance and widespread nature of SCM.

The clinical outcome of *S. aureus* associated mastitis is a multifactorial process. In addition to host immunity, factors such as exotoxins and other virulence determinants of *S. aureus* can influence the outcome (Haveri et al., 2007). According to the findings of the current study, there is no relationship between MRSA ST1 and the severity of mastitis symptoms. Previous research have shown that most LA-MRSA isolates lack many mastitis-associated virulence factors such as toxic shock syndrome toxin 1, hemolysins, and enterotoxins (Monecke et al., 2007; Walther et al., 2009). Despite this, the involvement of MRSA ST398 in CM cases has been reported previously (Vanderhaeghen et al., 2010). The previous study showed that 2/11 MRSA ST398 isolates were associated with CM; however, most of the isolates were related to SCM.

Antimicrobial susceptibility profiling of the MRSA and non-MRSA isolates revealed prominent differences. The MRSA isolates showed resistance to most of the tested antimicrobials (Table 3). The resistance of MRSA to oxacillin and other β-lactam antimicrobials can be attributed to the existence of *mecA*, which codes for a penicillin binding protein with low affinity for all β-lactams (Hartman & Tomasz, 1984). In conclusion, bovine mastitis is a source of LA-MRSA ST1 which is a health risk for humans and animals. Additionally, while methicillin resistance may not affect the severity of mastitis symptoms, it will affect therapeutic success, particularly when β-lactams are used for treatment.

ACKNOWLEDGMENTS

A. F. O. is supported by a postdoctoral fellowship from the Ministry of Higher Education, Egypt. H. O. K. is supported by a doctoral fellowship from the Ministry of Higher Education, Egypt. A. M. A. is supported by a postdoctoral fellowship (no. PU14012) from the Japan Society for the Promotion of Science. This work was supported in part by a Grant-in-Aid for Scientific Research (no. 25460532) to Tadashi S. from the Ministry of Education, Culture, Sports, Science, and Technology of Japan.

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