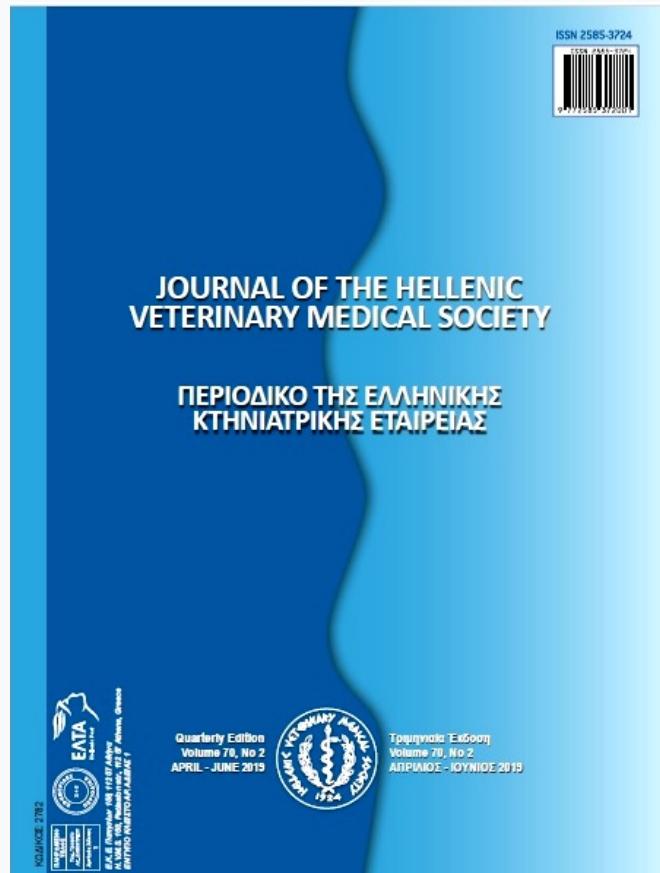


Journal of the Hellenic Veterinary Medical Society

Vol 70, No 2 (2019)



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*A. OREIBY, H. KHALIFA, A. EID, A. AHMED, T.
SHIMAMOTO, T. SHIMAMOTO*

doi: [10.12681/jhvms.20956](https://doi.org/10.12681/jhvms.20956)

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SHIMAMOTO, T. SHIMAMOTO



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To cite this article:

OREIBY A., KHALIFA, H., EID, A., AHMED, A., SHIMAMOTO, T., & SHIMAMOTO, T. (2019). Staphylococcus aureus and bovine mastitis: molecular typing of methicillinresistance and clinical description of infected quarters. *Journal of the Hellenic Veterinary Medical Society*, 70(2), 1511–1516. <https://doi.org/10.12681/jhvms.20956>

***Staphylococcus aureus* and bovine mastitis: molecular typing of methicillin-resistance and clinical description of infected quarters**

A. Oreiby^{a,b}, H. Khalifa^{c,f}, A. Eid^d, A. Ahmed^{a,e}, T. Shimamoto^a, T. Shimamoto^a

^a *Laboratory of Food Microbiology and Hygiene, Graduate School of Biosphere Science, Hiroshima University, Higashi-Hiroshima, Japan*

^b *Animal Medicine Department (Infectious Diseases), Faculty of Veterinary Medicine, Kafrelsheikh University, Kafrel-Elsheikh, Egypt*

^c *Department of Infectious Diseases, Graduate School of Medicine, International University of Health and Welfare, Narita, Japan*

^d *Food Hygiene Department, Animal Health Research Institute, Tanta Branch, Egypt*

^e *Department of Bacteriology, Mycology and Immunology, Faculty of Veterinary Medicine, Kafrelsheikh University, Kafrel-Elsheikh, Egypt*

^f *Pharmacology Department, Faculty of Veterinary Medicine, Kafrelsheikh University, Kafrel-Elsheikh, Egypt*

The first and second authors have an equal contribution to this work.

ABSTRACT. This study targeted *Staphylococcus aureus* (*S. aureus*)-mastitis of bovine as a possible source of live-stock-associated methicillin-resistant *Staphylococcus aureus* (MRSA), to describe clinical signs of mastitis associated with MRSA and methicillin-sensitive *Staphylococcus aureus* (MSSA). The study area was the Gharbia and Kafrelsheikh governorates, in the central and northern regions of the Egyptian Delta. Clinical examination of animals was performed to detect clinical mastitis (CM) and clinically normal cases were tested by the California mastitis test (CMT) to identify subclinical mastitis (SCM). Accordingly, 38 mastitis cases (68 infected quarters) were detected. Milk samples were collected aseptically and were cultured on Baird Parker agar. Fifty nine Gram-positive cocci-shaped isolates were selected and preserved. In addition, 33 *Staphylococcus* spp. isolates originated from bovine mastitis at the same study area were obtained from Animal Health Research Institute (AHRI). The cocci-shaped Gram-positive bacteria and AHRI *Staphylococcus* spp. isolates were used for molecular identification of *S. aureus* and MRSA. Molecular screening had yielded 17 *S. aureus* isolates, from which five isolates (29.41%) were MRSA and 12 isolates (70.59%) were MSSA. The five MRSA isolates were *mecA* positive, but *mecC* negative. Multilocus sequence typing (MLST) of the five MRSA isolates indicated that all were sequence type 1 (ST1). *S. aureus*-associated cases showed different clinical forms of mastitis, including subclinical, acute, chronic, and gangrenous. However, subclinical mastitis was the only detected form associated with MRSA, which may represent a potential hidden risk for humans. Phenotypic antimicrobial-resistance pattern of MRSA isolates showed resistance to all of the tested β -lactam antimicrobials, with marked

Corresponding Author:

Atef Oreiby, Animal Medicine Department (Infectious Diseases),
Faculty of Veterinary Medicine, Kafrelsheikh University,
Kafrel-Elsheikh 33516, Egypt
E-mail address: atef.ibrahim@vet.kfs.edu.eg

*Date of initial submission: 11-07-2018
Date of revised submission: 25-09-2018
Date of acceptance: 10-11-2018*

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 severity of mastitis, it does affect therapeutic success, particularly when β -lactam antimicrobials are used.

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

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 CM or SCM, but the sub-clinical 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 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), health-care-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 and Selim, 2015). Additionally, studies de-

scribing 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 MSSA-associated bovine mastitis.

MATERIAL AND METHODS

Animals, detection of mastitis and sampling

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. Mastitis cases were detected by clinical examination of the animals with special attention to the udder according to Houe et al. (2002), clinically normal cases were tested for SCM by CMT according to Kasikci et al. (2012).

Milk samples were aseptically collected according to Quinn et al. (1994): teats were wiped efficiently with 70% ethyl alcohol, first strip of milk was discarded and a suitable amount of milk (about 5 ml of milk) was collected in a sterile screw-capped Falcon tube. Samples were sent to the laboratory on ice quickly after collection.

Bacteriological examination

Milk 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.

Animal Health Research Institute (AHRI) *Staphylococcus* species Isolates

In addition to the above mentioned putative isolates, another 33 *Staphylococcus* spp. isolates were obtained from AHRI. Such isolates originated from bovine mastitis cases at the same study area, but clinical data of these isolates were not recorded.

Both of the isolated *Staphylococcus* spp. isolates and the obtained AHRI isolates were used in molecular procedures.

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.

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.

Multi-locus sequence typing of MRSA isolates 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/>).

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

Primer	Gene	Sequence (5'-3')	Size (bp)	References
au-F3	<i>Nuc</i>	TCGCTTGCTATGATT GTGG	359	Sasaki et al., 2010
au-nucR		GCCAATGTTCTACCA TAGC		
MecA147-F	<i>MecA</i>	GTG AAG ATA TAC CAA GTG ATT	147	Zhang et al., 2005
MecA147-R		ATG CGC TAT AGA TTG AAA GGA T		
<i>mecA</i>_{LGA251}MultiFP	<i>mecA</i> _{LGA251}	GAAAAAAAGGCTTAGAACGCCTC	138	Stegger et al., 2011
<i>mecA</i>_{LGA251}MultiRP		GAAGATCTTTCCGTTTCAGC		
<i>arcC</i>-Up	<i>Arc</i>	TTGATTCAACCAGCGCGTATTGTC	456	Enright et al., 2000
<i>arcC</i>-Dn		AGGTATCTGCTTCAATCAGCG		
<i>aroE</i>-Up	<i>aroE</i>	ATCGGAAATCCTATTACATTC	456	
<i>aroE</i>-Dn		GGTGTGTATTAATAACGATATC		
<i>glpF</i>-Up	<i>GlP</i>	CTAGGAACGTCAATCTTAATCC	465	
<i>glpF</i>-Dn		TGGTAAAATCGCATGTCCAATT		
<i>gmk</i>-Up	<i>Gmk</i>	ATCGTTTATCGGGACCATC	429	
<i>gmk</i>-Dn		TCATTAACACTAACGTAATCGTA		
<i>pta</i>-Up	<i>Pta</i>	GTTAAAATCGTATTACCTGAAGG	474	
<i>pta</i>-Dn		GACCCTTTGTTGAAAAGCTTAA		
<i>tpi</i>-Up	<i>Tpi</i>	TCGTTCATTCTGAACGTCGTGAA	402	
<i>tpi</i>-Dn		TTTGCACCTCTAACAAATTGTAC		
<i>yqiL</i>-Up	<i>YqiL</i>	CAGCATAACAGGACACCTATTGGC	516	
<i>yqiL</i>-Dn		CGTTGAGGAATCGATACTGGAAC		

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.

RESULTS

Clinical and descriptive aspects of mastitis cases

A total of 38 mastitis cases (68 affected quarters) were detected. Twenty five cases had single diseased quarter, while 13 cases had multiple affected quarters. Different clinical forms of mastitis were noticed as shown in Figure 1.

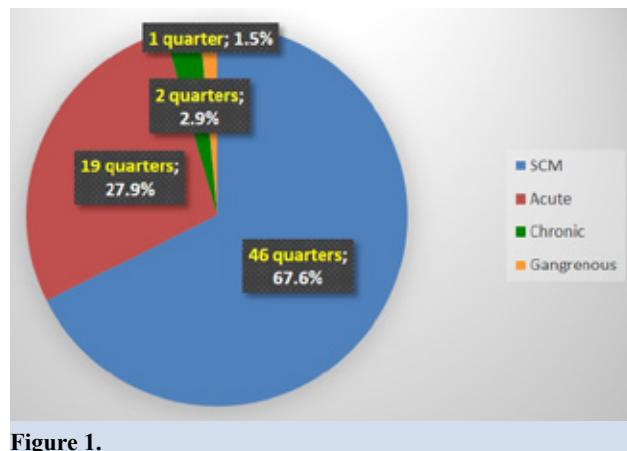


Figure 1.

Subclinical mastitis presented no obvious symptoms except of reduced amount of milk from the affected quarters, the physical characters of milk were normal. On the other hand, acute mastitis showed hotness, pain and enlargement of the affected quarter. Systemic reaction appeared as fever, congested mucous membranes, reduced appetite, increased heart and respiratory rates were recorded in a few cases of acute mastitis. The physical characters of milk were changed; yellowish semi-transparent offensive odor milk were the most common detected changes. Chronic affected quarters were fibrous discharging scanty amount of watery milk, while gangrenous quarter was of blackish discoloration, cold temperature and discharging bloody milk.

Identification of *S. aureus*

Gram-positive cocc-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 coc-

ci-shaped isolates were selected. Molecular screening for *S. aureus* yielded seven isolates from seven individual cases (four subclinical cases, one acute, one chronic and one gangrenous case). In addition, molecular screening of the AHRI isolates resulted in an additional 10 isolates of *S. aureus*. Consequently, 17 *S. aureus* isolates were used in further phenotypic and genotypic 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 2.

Molecular screening/typing of MRSA

Out of the 17 *S. aureus* strains, 12 isolates (70.59%) were MSSA and five (two originated from the clinical cases and three AHRI isolates) strains contained *mecA* (29.41%) and were identified as MRSA ST1. However, *mecC* was absent in all of the *S. aureus* isolates.

Clinical nature of MRSA-associated bovine mastitis

The two cases (AHRI isolates had no clinical data) 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 latter to colonize and infect humans (Juhász-Kaszanyitzky et al., 2007; Alba et al., 2015).

Table 2. 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	<i>mecA</i>
Sa70	AMP, AMC, FOX, CRO	TET, GEN, TEC	<i>mecA</i>
Sa101	AMP, AMC, FOX, CRO, OXA	TET, GEN, TEC	<i>mecA</i>
Sa104	FOX	TEC	
Sa107	AMP	TET, GEN, TEC	
Sa119	AMP	GEN, TEC	
Sa120	AMP	-	
Sa131	AMP, AMC, FOX, CRO, OXA	TET, GEN, TEC	<i>mecA</i>
Sa135	AMP, FOX	TET, TEC	
Sa136	-	TET, TEC	
Sa137	AMP, AMC, FOX, CRO, OXA	GEN, TEC	<i>mecA</i>
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.

The affection of a single quarter, in spite of lack of hygienic milking procedures, in most of the detected mastitis cases indicated the low contagiousness of the involved pathogens. This was confirmed by PCR which showed that most of *Staphylococcus* isolates were not *S. aureus*. In addition, SCM was more prevalent than CM, which agrees with a recent study in Egypt (Elhaig & Selim, 2015), and highlights the importance and widespread nature of SCM.

Staphylococcus aureus was associated with different clinical forms of mastitis. The resulting clinical form of *S. aureus* mastitis is a multifactorial process which is influenced by host's immunity and virulence determinants of *S. aureus* isolates (Haveri et al., 2007). On the other hand, the current study showed that MRSA ST1 was associated only with SCM. This is because 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 LA-MRSA (ST398) in a few CM cases has been reported previously (Vanderhaeghen et al., 2010).

Antimicrobial susceptibility of methicillin resistant and sensitive *Staphylococcus aureus* isolates revealed prominent differences. The MRSA isolates showed resistance to most of the tested antimicrobials

(Table 2). 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, SCM is more prevalent than CM, *S. aureus* associated bovine mastitis was of variable clinical nature (subclinical, acute, chronic and gangrenous), bovine mastitis is a source of LA-MRSA ST1 which is a health risk for humans and source of infection of animals. Additionally, MRSA may be unable to induce severe mastitis, but 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. 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.

CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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