



Prevalence and Ecology of Zoonotic Methicillin Resistant *S. aureus* and its Relation to Biofilm Formation

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Abstract | The high zoonotic importance of *Staphylococcus aureus* and the increasing rates of isolation of methicillin resistant traits from both clinical and subclinical cases pose a major threat to human health and animal industry. The aim of this study was to monitor methicillin-resistant isolates of *S. aureus* recovered from milk, human and environmental samples in a small holder dairy unit, detection of their antibiotic resistance and their relation to biofilm formation. Seventy-five milk samples besides 150 different environmental samples including (bulk milk tank swabs, water trough swabs, feeding manager swabs, milk machine swabs, and bedding) and 50 nasal and attendants' hand swabs from animal attendants were collected using stratified random sampling technique. Samples were aseptically cultured for isolation of *S. aureus* that was confirmed using molecular assays. Antibiotic sensitivity pattern and biofilm formation using disc diffusion and Congo red method, respectively were detected. Resistant isolates were screened for *Mec A* and *Ica A* genes. The highest isolation percentage (64.0%) was obtained from manager followed by milk machine swabs (60.0%). All strains showed complete resistance to ceftiofur and ampicillin (100.0% each) and varying degrees of resistance to other used antibiotics. *Mec A* gene was detected in 5 out of 6 examined isolates meanwhile *Ica A* gene was detected in all the tested isolates. It can be concluded that the environment considered the link between animal and human infections through poor standards of hygiene and a possible cross relation between antibiotic resistance particularly to methicillin and biofilm formation was also observed.

Keywords | Antibiotic Resistance, Biofilm, Cow's Environment, MRSA, *S. aureus*

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INTRODUCTION

Staphylococcus aureus is an important bacterial pathogen that causes a variety of diseases in animals and humans ranging from only skin infections to life threatening bacteraemia (Incanni et al., 2013). Milk and its derivatives consider a potential source of infection to human society due to the ability of milk to act as a vehicle to such vicious disease agent (Iqbal et al., 2016).

Over the last decade's methicillin resistant *S. aureus* (MRSA) isolated from both humans and animals have

been reported worldwide (Naimi et al., 2001; Voss et al., 2005). Misuse of antibiotics in the treatment of staphylococcal infections had led to one important complication; the emergence and maintenance of antibiotic resistant traits among pathogenic *S. aureus* strains (Stastkova et al., 2009). Noteworthy that multidrug resistance (MDR) among *S. aureus* strains has become very common which poses a high risk to both human and veterinary medicine (Khan et al., 2007).

Farm animals infected or carrying MRSA strains can easily be involved in the spread of the pathogen not only to the

farm personnel but also to raw food materials intended for further processing (Stastkova et al., 2009). Therefore detection of the resistance pattern is a supportive tool to antibiotic treatment guidelines (Beema and Atindra, 2011).

S. aureus can survive on inanimate objects for prolonged time therefore the environment plays uncontroversial role in the spreading MRSA between animal and human populations (Davis et al., 2012). Milk and dairy products contaminated with antibiotic resistant bacteria including MRSA present a major threat to public health as a result of continuous circulation of resistant pathogens in the environment (Gwida and El-Gohary, 2015).

Biofilm is one feature of some bacteria that protects the microorganism from host defenses and impedes delivery of antibiotics (Gurjala et al., 2011). Virtually it was found that multiple antibiotic resistances increased in MRSA having the ability to form a biofilm causing failure of treatment, chronic and recurrent infections (Diamond-Hernández et al., 2010; Pozzi et al., 2012; Neopane et al., 2018).

The objectives of this study was to monitor the prevalence of methicillin-resistant strains of *S. aureus* recovered from animal, human and environmental samples in a small dairy unit, and to assess the relation between biofilm formation and their resistance pattern to different antibiotics in such strains.

MATERIALS AND METHODS

STUDY AREA AND DESIGN

This study was carried out in a private dairy farm located in Beni-Suef district (coordinates: 29E04'N31E05'E), Egypt during the period from June 2017 to April 2018. The farm consisted of 135 Friesian cows of different production stages. Cows were housed in a partially sheltered yards in 9 groups ($n=15$) according to their production, with earthy floor, each group was provided with a common water trough and a common feeding trough. Cows were milked twice/ day in abreast parlor prepared with 12 milking units. Eventually milk from cows was collected in a bulk tank to be ready for transportation. The hygienic status that prevailed in the farm based on observation and a questionnaire was poor. Seventy-five milk samples besides 150 different environmental samples including (bulk milk tank swabs, water trough swabs, feeding manager swabs, milk machine swabs, and bedding) and 50 nasal and attendants' hand swabs from animal attendants were collected using stratified random sampling technique. Samples were aseptically cultured for isolation of *S. aureus* and the identified isolates were tested for antibiotic sensitivity against seven different antibiotics (ampicillin, amoxicillin clavulanic acid, cefoxitin, cefepime, oxytetracycline, enrofloxacin and kanamycin) using disc diffusion method (CLSI, 2017). Resistant bacteria were tested for

biofilm formation using Cogo Red Agar method then they were screened for MRSA resistance and biofilm formation genes using molecular techniques.

SAMPLE COLLECTION

A total number of 75 milk samples, (approximately 10 mL) were collected from different animals that did not show any signs of clinical mastitis. Sampling collection were done randomly twice/ month throughout the study period. All milk samples were gathered according to National Mastitis Council (1990) and they were kept in ice box to be sent with minimal delay to the laboratory of Hygiene, Zoonoses and epidemiology in the Faculty of Veterinary Medicine, Beni-Suef University. One hundred and fifty environmental samples including bulk milk tank swabs, water trough swabs, feeding manager swabs, milk machine swabs, and bedding. Beside, fifty human samples including attendants' hand swabs ($n= 25$) and nasal swabs ($n= 25$) were collected from the dairy farm workers and attendants. All attendants were men; their ages were ranged from 20–40-year-old, and apparently healthy.

ISOLATION AND IDENTIFICATION OF *S. AUREUS*

One loopful from each milk sample was inoculated on Baird Parker agar plates (BRA, Oxoid, Basingstoke, UK) supplemented with egg yolk tellurite and incubated at 37° C for 24–48 hrs. Typical black colonies with opaque halo were collected and streaked on tryptic soya agar plates incubated at 37° C for 24 hrs, the purified colonies were preserved on tryptic soya agar slopes at 4° C for further identification.

Whereas All the swab samples were pre-enriched on tryptic soy broth (Oxoid, Basingstoke, UK) at 37° C for 18–24 hrs then a loopful from each tube showing turbidity was cultivated on the surface of Baird Parker agar plates (Oxoid, Basingstoke, UK) and incubated at 37° C for 24–48hrs. The purified isolates were identified on the basis of Gram staining, oxidase, catalase and coagulase tests (Biolife, Milan, Italy) according to Quinn et al. (2002). Further identification of the isolates was applied by amplification of *S. aureus* specific *Nuc* gene.

PHENOTYPIC SCREENING OF BIOFILM FORMATION

Biofilm formation was determined qualitatively using Congo red agar assay (CRA) (Osman et al., 2015), depending on the characteristic morphology of *S. aureus* biofilm formation on CRA. Colonies were cultured on CRA plates consisting of; 37 g/L of Brain Heart Infusion agar (BHI, Merck), 0.8 g/L of Congo red dye (Merck, Darmstadt, Germany) and 50 g/L of sucrose (Merck). Isolates were inoculated on CRA plates and incubated aerobically at 37° C for 24 hrs and then plates were kept at room temperature for 48 hrs.

Table 1: Sequences of target genes, amplicon sizes and cycling conditions specific for *S. aureus*

Target gene	Primer sequences	Amplified segment (bp)	Primary denaturation	Amplification (35 cycles)			Final extension	References
				Secondary denaturation	Annealing	Extension		
Nuc	ATATGTATGG- CAATCGTTTCAAT	395	94°C 5 min.	94°C 30 sec.	55°C 45 sec.	72°C 45 sec.	72°C 10 min.	Gao et al., 2011
	GTAAATG- CACTTGCTTCAG- GAC							
Mec A	GTA GAA ATG ACT GAA CGT CCG ATA A	310	94°C 5 min.	94°C 30 sec.	50°C 30 sec.	72°C 30 sec.	72°C 7 min.	McClure et al., 2006
	CCA ATT CCA CAT TGT TTC GGT CTA A							
Ica A	CCT AAC TAA CGA AAG GTA G	1315	94°C 5 min.	94°C 30 sec.	49°C 45 sec.	72°C 1.2 min.	72°C 12 min.	Ciftci et al., 2009

The results of biofilm formation were interpreted according to colonial morphology using a four-color reference scales varying from red to black. Black colonies were considered to be biofilm-producers, while almost-black colonies were considered weak biofilm producers. Red and purple colonies were considered non-biofilm producers (Lira et al., 2016).

ANTIBIOTIC SUSCEPTIBILITY TESTING

The antimicrobial sensitivity test was done by disk diffusion method according to CLSI (2017) using seven antibiotics (ampicillin 10 µg, amoxicillin clavulanic acid 25 µg, cefoxitin 30 µg, cefepime 25 µg, oxytetracycline 30 µg, enrofloxacin 10 µg and kanamycin 3 µg) (Oxoid, Basingstoke, UK).

MOLECULAR ANALYSIS OF MRSA AND BIOFILM GENES

Six resistant strains of *S. aureus* to cefoxitin were randomly selected and submitted for detection of *Mec A* gene that is implicated in methicillin resistance and *Ica A* genes required for synthesis of PIA for biofilm formation (Boye et al., 2007) by molecular analysis, which was applied in biotechnology center in the animal health research institute according to Sambrook and Russel (1989). The primer sequencing and cycling conditions for the target genes were mentioned in Table 1.

RESULTS AND DISCUSSION

Mastitis and in particular subclinical one caused by *S. aureus* is a primary and the most lethal agent affecting our cattle. Increasing concern about the presence of such contagious zoonotic pathogen that cause several pathological conditions to humans and spread between cows during the milking time through contaminated environment and attendants hands (Artursson et al., 2016). Such circumstance

resembles a circle where pathogens, susceptible animals, human, and the environment play a specific role in magnification of the problem.

Table 2: Prevalence of *S. aureus* isolated from different examined samples throughout the study period

Samples/ Swabs	<i>S. aureus</i> prevalence	
	Examined samples (No.)	Positive samples (No.) (%)
Cows:		
Milk sample	75	18 24.0
Humans:		
Nasal swab	25	10 40.0
Attendant's hand	25	7 28.0
Cows' environment:		
Water sample	25	0 0.0
Water trough	25	0 0.0
Feeding manager	25	16 64.0
Bedding	25	0 0.0
Milk tank	25	0 0.0
Milk machine	25	15 60.0
Total	275	66 24.0

S. aureus was isolated from 66 (24.0%) of 275 samples based on cultural and biochemical properties as shown in Table 2, moreover the highest bacterial isolation rate was obtained from feeding manager (environmental sample) followed by swabs from milk machine, nasal swabs, attendants' hand swab (human samples) and milk sample (animal samples) (64.0, 60.0, 40.0, 28.0 and 24.0%, respectively), while no isolation was obtained from bedding, milk tank swabs, water sample or water trough swabs (0.0%). In the

Table 3: *S. aureus* sensitivity isolated from cows, attendants and their environment against seven antibiotics using disc diffusion method *in-vitro*

Antibiotic sensitivity Samples/ Swabs	Exam-ined Samples (No.)	Posi-tive sam-ples (No.)	Antibiotics used																				
			Oxytetracycline (30 µg)		Ampicillin (10 µg)		Enrofloxacin (10 µg)		Cefoxitin (30 µg)		Kanamycin (30 µg)		Cefepime (25 µg)		Amoxicillin clavulonic acid (25 µg)								
			R	I	S	R	I	S	R	I	S	R	I	S	R	I	S	R	I	S			
Milk	75	18	12	4	2	18	-	-	12	2	4	18	-	-	3	-	15	8	-	10	12	-	6
			66.7	22.2	11.1	100.0	0.0	0.0	66.7	11.1	22.2	100.0	0.0	0.0	16.7	0.0	83.3	44.4	0.0	55.5	66.7	0.0	33.3
Nasal swab	25	10	5	1	4	10	-	-	8	2	-	10	-	-	-	3	7	3	2	5	6	3	1
			50.0	10.0	40.0	100.0	0.0	0.0	80.0	20.0	0.0	100.0	0.0	0.0	0.0	30.0	70.0	30.0	20.0	50.0	60.0	30.0	10.0
Hand Swab	25	7	4	1	2	7	-	-	5	-	2	7	-	-	3	-	4	1	2	4	1	-	6
			57.1	14.3	28.6	100.0	0.0	0.0	71.4	0.0	28.6	100.0	0.0	0.0	42.9	0.0	57.1	14.3	28.6	57.1	14.3	0.0	85.7
Milk ma-chine swab	25	15	4	2	9	15	-	-	8	3	4	15	-	-	2	5	8	5	2	8	6	-	9
			26.7	13.3	60.0	100.0	0.0	0.0	53.3	20.0	26.7	100.0	0.0	0.0	13.3	33.3	53.3	33.3	13.3	53.3	40.0	0.0	60.0
Feed man-ager swab	25	16	9	3	3	16	-	-	11	1	4	16	-	-	2	6	7	2	5	9	3	-	13
			56.3	18.8	18.8	100.0	0.0	0.0	68.8	6.25	25.0	100.0	0.0	0.0	12.5	37.5	43.8	12.5	31.3	56.3	18.7	0.0	81.3

*S: Sensitive, I: Intermediate, R: Resistant

Table 4: Prevalence and categorization of biofilm forming isolates of *S. aureus* using CRA obtain from cows, humans and their environment

Biofilm formation Samples / Swabs	Samples		Biofilm Category							
	Examined	Positive	Negative		Weak		Moderate		Strong	
			No.	%	No.	%	No.	%	No.	%
Animal samples Milk	75	18	4	22.2	9	50.0	5	27.8	0	0.0
Human samples (hand & nasal swabs)	50	17	4	23.5	8	47.1	2	11.8	3	17.6
Environmental Samples (milk machine, manager)	50	31	16	51.6	8	25.8	5	16.1	2	6.5
Total	175	66	24	36.4	25	37.9	12	18.2	5	7.6

Table 5: Correlation between antibiotic resistance and biofilm formation in *S. aureus* isolates

Biofilm formation Antibiotic	No. of resistant isolates	Biofilm former		Non-biofilm former	
		No.	%	No.	%
Amoxicillin clavulanic acid	28	10	35.7	18	64.3
Cefepime	19	5	26.3	14	73.7
Kanamycin	10	3	30.0	7	70.0
Cefoxitin	66	42	63.6	24	36.4
Enrofloxacin	44	20	45.5	24	54.5
Ampicillin	66	42	63.6	24	36.4
Oxytetracycline	34	15	44.1	19	55.8

present study frequent distribution of *S. aureus* isolated from different samples revealed that most of the isolates were obtained from the animals' environment in particular milk machine and feeding manager swabs that spot light on the importance of regular cleaning and disinfection inside the farm and this could be attributed to the general standards of hygiene in the farm under study was poor and not sufficient enough to eliminate the risk of infection from the environment that could be the reservoir for both animals and humans in contact. Zakary et al. (2011) concluded that high incidence of *S. aureus* is indicative of poor hygienic measures during production and handling. Attendants' hands are considered as a source of contamination with *S. aureus* in dairy farms (Roberson et al., 1998; Zadoks et al., 2002; Olivindo et al., 2009; Matyi et al., 2013). The results obtained in this study were nearly similar to those reported by Hamid et al. (2017) and Unnerstad et al. (2009).

Concerning the results of antibiotic sensitivity profile (Table 3) of the obtained strains of *S. aureus* were remarkably resistant to two or more of the tested antibiotics (multi-drug resistance) moreover they all were completely resistant to ampicillin and cefoxitin (100.0%), also all samples showed a higher degree of resistance to enrofloxacin mainly the strains recovered from nasal swabs followed by hand swabs, feed manager swabs milk samples and milk machine swab (80.0, 71.4, 68.8, 66.7 and 53.3%, respec-

tively). Variable degrees of resistance were recorded against the other tested antibiotics. On the other strains recovered from milk samples and nasal swabs exhibited high sensitivity mainly to kanamycin (83.3 and 70.0%, respectively) while hand swabs and feed manager swabs exhibited nearly similar sensitivity to cefepime (57.1 and 56.3%, respectively). Antibiotic sensitivity testing had revealed that most of recovered traits of *S. aureus* showed multidrug resistance in particular to cefoxitin and ampicillin (100.0%). The obvious pattern of multidrug resistance of the studied strains of *S. aureus* from animals, humans or environment spot light on a leading problem of possibility cross antibiotic resistance between the three sources although environmental isolates exhibited to some extent lesser degree of resistance compared to animal and human strains and this may be due to the less use of antibiotics to control them as well less use of disinfectants in this farm. Arenas et al. (2017) revealed that the livestock producers could be a source of exposure to multidrug resistant *S. aureus* strains as a result of abuse of antibiotic treatment of animals and unhygienic livestock practices. These results were to some extent in harmony with De Oliveira et al. (2000) and Guerin et al. (2003). On the contrary to the findings in this study Begum et al. (2007) recorded that *S. aureus* was 82.86% and 37.14% resistant to penicillin-G and amoxicillin.

Referring to the prevalence of biofilm forming strains of *S. aureus* using CRA as shown in Table 4 it was found that

out of 66 strain of *S. aureus* 25 (37.9%) were capable of forming weak biofilm whereas 5 (7.6%) and 12 (18.2%) exhibited strong and medium biofilm formation capacity respectively. Meanwhile 24 (36.4%) did not show any biofilm formation. More specifically it was clear that the half of animal samples (milk samples) (50.0%) exhibited weak biofilm capacity, while 47.1% of human samples (nasal and hand swabs) were weak biofilm formers. Concerning the environmental samples (milk machine and feed manager swabs) the majority (51.6%) showed no biofilm formation. The result of biofilm formation pattern of the obtained isolates revealed that 37.9% of all isolates were weak biofilm formers, 18.2% were moderate biofilm forming and 7.6% were strong biofilm formers while 36.4% were non biofilm formers. Similar results were recorded by Eyoh et al. (2014) who identified biofilm production in 35.6% of *S. aureus* isolates. And Chibueze et al. (2017) who reported that 48.2% of the isolates have the potential to form biofilm and 5.4% were strong biofilm producers while 8.9% were moderate producers, 33.9% were weak producers and 51.8% were non biofilm producers. The implications of biofilm forming isolates of *S. aureus* in infection in hospitals environment and hospital personnel act as a steady reservoir that negatively impact the patient health due to biofilm formation inversely influence the antimicrobial therapy (Fatima et al., 2011).

Regarding the relation between antibiotic resistance of the recovered strains and their ability to form biofilm (Table 5) there was an obvious relation between their ability for biofilm formation and antibiotic resistance where 42 (63.6%) out of 66 resistant strain to cefoxitin and ampicillin were able to form biofilm, followed by 20 (45.5%) of resistant strains to enrofloxacin were biofilm formers and 15 (44.1%) of those resistant to oxytetracycline were able to produce biofilm. On the other hand recovered strains that showed the least resistance to kanamycin and cefepime had the least ability to produce biofilm (30.0 and 26.3%, respectively), in other words it was clear that there is a positive relation between antibiotic resistance and the ability for biofilm formation in the same strains of *S. aureus*. Concerning the correlation between biofilm formation and antibiotic sensitivity pattern apposite correlation was noticed between antibiotic resistance and biofilm formation similar results obtained by Eyoh et al. (2014). Also Fitzpatrick et al. (2005) found more MDR strains in biofilm producers than in non-biofilm producers. Biofilm formation in MDR *S. aureus* could be attributed to presence of extracellular polymeric substance that constitute this matrix serving as a diffusional barrier for antibiotics, thus influencing either the rate of transportation of the molecule to the biofilm or the reaction of the antibiotic with the matrix material (Corrigan et al., 2007).

Results of polymerase chain reaction for detection of *Nuc*

gene, MRSA gene (*Mec A*) and biofilm formation gene (*Ica A*) (Figure 1) in the six randomly selected strains (2 environmental, 2 animal and 2 human samples) revealed that *Mec A* was only detected in one environmental sample and all other animal and human samples, while *Ica A* was detected in all of the selected strains. Referring to genotypic detection of MRSA gene (*Mec A*) and biofilm formation gene (*Ica A*) in *S. aureus* strains isolated in this study, there was a correlation between the high prevalence of *Mec* gene, *Ica* gene, and biofilm formation that indicated the important role of biofilm in the pathogenesis of *S. aureus*. In addition, biofilm formation resulted in marked antimicrobial resistance due to the reduction of antimicrobial penetration, slower bacterial metabolic state as well as easier exchange of resistance genes among cells which complicates the therapeutic approaches against *S. aureus* associated infections (Arciola et al., 2001; Yazdani et al., 2006; Cosgrove and Fowler, 2008; Khameneh et al., 2016; Naicker et al. 2016).

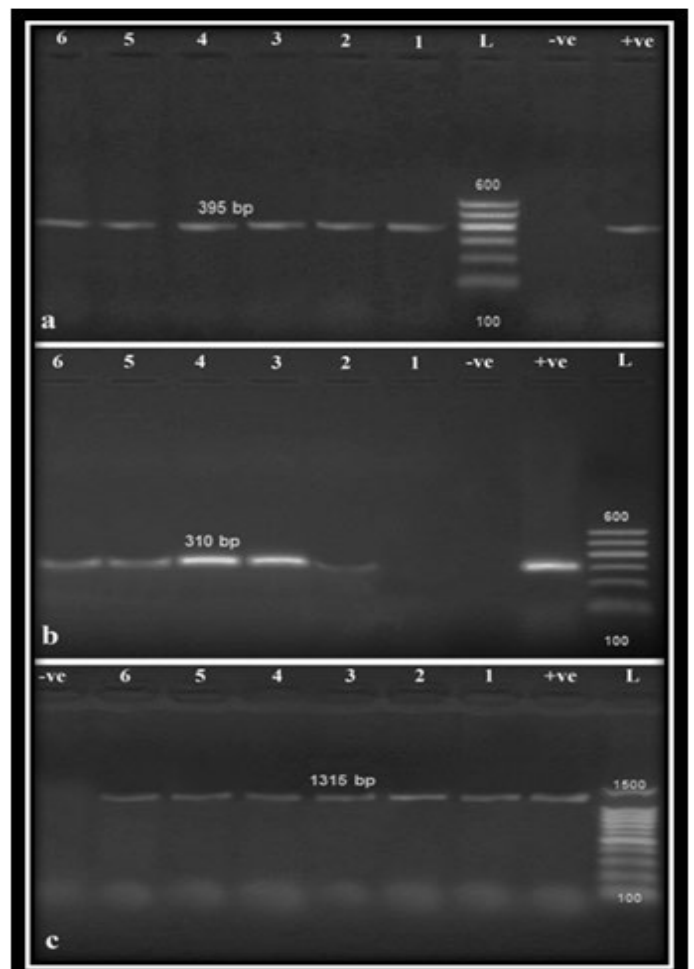


Figure 1: Agarose gel amplification for PCR products specific for *S. aureus* isolates on *Nuc* (a), *Mec A* (b) and *Ica A* (c) genes amplified 395 bp, 310 bp and 1315 bp, respectively. Lane (L): 100 bp Ladder “Marker”, Lanes (1–6): examined samples, Lane Pos: Positive control, Lane Neg: Negative control.

In the light of the study results it can be concluded that the environment considers the link between animal and human infections with MRSA through poor standard of hygiene also animal attendants played a significant role in the circulation of the pathogen in the environment in small dairy units that considered representative to a major sector of dairy industry in Egypt with similar circumstance and facilities. Furthermore, a cross relation between antibiotic resistance, in particular, methicillin and biofilm formation was detected this was reflected by the presence of *Mec* and *Ica A* genes in the same selected isolates. Further studies should be employed to control the dissemination of such MDR, biofilm producers' isolates of *S. aureus*.

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

AUTHORS CONTRIBUTIONS

Authors 1 and 2: shearing the conception and design of the study acquisition of data, analysis and interpretation of data and all the scientific writing, while author 3 drafting the manuscript and grammar revision also writing revision.

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