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Characterization of methicillin susceptible and methicillin-resistant *Staphylococcus aureus* from healthy cattle and buffaloes in a linked community

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ABSTRACT

Objective: To give updated information regarding the occurrence of methicillin-susceptible and methicillin-resistant *S. aureus* isolated from dairy cattle and buffalos in a linked study population.
Design: Descriptive study.
Samples: The study comprised 360 samples (240 of animal origin and 120 from humans). Three different types of samples (including teat swabs, milk and feces, 80 each) were collected from animals in addition (nasal swabs, hand swabs and stool specimens, 40 each) were collected from contact persons.
Procedures: The collected samples were examined by stander techniques.
Results: *S. aureus* was identified in 59.3% (73/123) from the examined farm dairy cattle. MRSA was not determined in any of the examined cows' samples while for buffaloes, it was detected in 63.2% (12/19), 64.7% (11/17) and 40 % (4/10) in milk, teat swabs and fecal samples, respectively. For smallholding cattle and buffaloes, MRSA was detected in the above mentioned samples and in relation to the recovered *S. aureus* isolates (at cefoxitin 4µg/ml) in the following pattern: 22.2% (4/18), 15% (3/20) and 18.8% (3/16) and 20% (3/15), 100% (11/11) and 43.8% (7/16), respectively. However, in contact persons the percentage was 85 (34/40), 82.5 (33/40) and 90 (36/40) from nasal swabs, hand swabs and stool specimens, respectively.
Conclusion and clinical relevance: The results herein confirmed that cows, buffaloes and their contact workers could play a significant role in the transmission of MRSA, whereas the detection of MRSA in the raw milk, teat swabs and feces of cows and buffaloes may create the opportunity for the transmission of such bacteria.

Keywords: *S. aureus*; MRSA; Cattle; Buffalo; linked study

1. INTRODUCTION

Staphylococcus aureus (*S. aureus*) is a ubiquitous zoonotic pathogen with clinical relevance for both humans and animals **[1-2].** The bacterium is a common colonizer and is considered a part of the natural commensal flora of humans and animals and colonizing approximately 30–50 % of human population **[3]**. It can cause either minor infections in humans such as superficial skin and soft tissue infections or life-threatening conditions including necrotizing fasciitis, pneumonia, septicemia, food poisoning, postoperative wound infections, and nosocomial infections **[4]**.

S. aureus has received a special interest in animal since they are considered a major pathogen of mastitis in dairy cattle and buffaloes **[1,5]**.Its ability to cause a multitude of infections is probably due to the expression of various toxins, virulence factors, as well as cell wall adhesion proteins **[2]**. On the other side, its low cure rate and the ability of the organism to persist in the mammary gland in the form of undetected subclinical infections as well as its resistance to antibacterial therapies make it a unique pathogen that necessitate continuous monitoring **[1, 6].**

The resistance of *S. aureus* to antimicrobials remains a global setback and can complicate the treatment of infections especially in both resource-limited and developed countries **[7].**The resistance of the bacterium to the used antibacterial agents is a serious problem and is strongly related to the improper and excessive use of antimicrobials either for the treatment of animal ailments or as growth promoters in food producing animals.

Methicillin -resistant *S. aureus*, which initially emerged as human nosocomial infections, has also spread in dairy animals in different countries **[8]**. It has been reported that the acquisition of the *mec*A gene is the cause of resistance to methicillin. This gene encodes an alternative penicillin-binding protein, called PBP2A that exhibits a low affinity for beta-lactam antibiotics **[9]**. The existence of MRSA strains in the environment could be a possible source of MRSA infection in dairy farms, because they can survive for several months in the surrounding environment **[10].**

The epidemiology of MRSA has changed due to the increasing appearance of livestock-associated MRSA (LA-MRSA), which has been detected in food producing animals and workers who in close contact with MRSA colonized animals **[11]**. In Egypt, there has been limited information regarding the existence of MRSA strains in cows and buffaloes

as well as their contact workers in a linked community. Hence, the present study was set to fill in the gap and provide updated information regarding the occurrence of methicillin-susceptible and methicillin- resistant *S. aureus* isolates in a linked study population including commercial and small holding cattle and buffaloes as well as their contact owners.

2. MATERIAL AND METHODS

2.1. Samples collection and preparation

The study was performed during the period from December 2019 till October 2020 and comprised 360 samples (240 from animal origin and 120 from humans). Samples of animal origin (*n* = 240) were collected from seemingly healthy cattle (*n* = 40) and buffalo cows (*n* = 40) that were raised at Dakahlia and Damietta governorates in different geographic locations. From each animal, three different types of samples (including teat swabs, milk and feces) were collected. The cows were dairy animals and were either reared as smallholders (22 cattle; 17 buffalo) or belonged to commercial farms (18 cattle; 23 buffalo). On the other side, samples of human origin (*n* = 40) included nasal swabs, hand swabs and stool specimens, 40 each were collected from contact keepers. The study was performed in accordance with the ethical committee of Mansoura University and follows the guidelines for the Care and Use of Agricultural Animals in Research and Teaching, 3rd ed. (http://www.fass.org/). The investigated animals were selected based on convenience and a consent to participate in sampling procedure was obtained from the contact owners. For more clarity, the detailed information about the sampling and processing for each type of sample were given below.

2.2. Isolation and Identification of S. aureus

The collected samples were inoculated into tryptone soya broth (TSB) with 70 mg of NaCl /ml and were incubated at 37°C for 24hrs. After incubation, a loopful (i.e. approximately 10 µL) from each of the incubated broth was streaked onto selective media for *S. aureus*, Baird Parker agar base (Oxoid, CM 275) supplemented with 5% egg yolk potassium tellurite and were incubated at 37°C for 24-48hr **[1]**.Colonies with typical growth of staphylococci (i.e. black, shiny, convex colonies) were picked up and streaked on Baired Parker agar for purification and incubated at 37°C for 48 hrs. The cultivated colonies were checked for purity and confirmed as *Staphylococcus* spp. using biochemical tests **(**i.e. coagulase test, catalase test and mannitol fermentation test**)**. The selective purified colonies were finally preserved as glycerol stock at -20°C for further identification.

2.3. Detection of methicillin-resistant S. aureus (MRSA)

The biochemically identified *S. aureus* strains were cultured on mannitol salt agar plates containing cefoxitin at two different concentrations (2 µg and 4 µg/ ml). The growing colonies were tentatively considered as MRSA and were kept in glycerol at -20°Cfor further investigations**[12]**. Strains were classified as MRSA if they grow in parallel at both cefoxitin concentrations.

2.4. Molecular Characterization of MRSA strains

All biochemically suspected MRSA isolates were substantially examined by PCR for *nuc* and the positive samples were further tested for *mec*A gene.

2.4.1. DNA extraction

Bacterial lysates were obtained by the method previously mentioned by **[13]**. Three to five colonies of purified biochemically suspected MRSA strains were picked up and transferred to a sterilized tube containing 100 µl of sterilized distilled water, then heated for 15 minutes at 95°C in heat block. The boiled lysates were then centrifuged at 13000 rpm for 10 minutes. The supernatants were transferred to sterile Eppendorf tubes, kept at -20°C, and used as DNA templates.

2.4.2. Method of DNA amplification

The polymerase chain reaction (PCR) assays were performed in individual reactions using an applied Biosystems 96-well Thermal Cycler to detect (*nuc* and *mec*A). The sets of primer sequences and corresponding amplicon sizes are showed in Table 1. PCR reactions were performed in a total volume of 20 µL consisting of 10 µL of 2X Red master Mix (Applied Biotechnology Co., Egypt), 3 µL DNA templates, and 1 µL of each primer and completed to 20 µl with DNA/RNA free water. PCR reactions and thermal conditions used were performed, according to **Sallam et al [14]**, with an initial denaturation at 94˚C for 2 min, 35 cycles were performed (98˚C for 10 seconds, 58 ˚C for 30 second and 68˚C for 1 min), and final extension at 68˚C for 7 min.An aliquot of each amplified PCR product was electrophoresed in ethidium bromide stained 1.5% agarose gel for 30 minutes at 100 V then visualized and photographed by an ultraviolet transilluminator.

2.5. Antibiotic resistance profiles in MRSA strains

The susceptibility to cefoxitin (30 µg), penicillin G (10 units), clindamycin (2 µg), gentamycin (10 µg), kanamycin (30 µg), sulphamethoxazol (1.25/23.75 µg), ciprofloxacin (5 µg), chloramphenicol (30 µg), vancomycin (30 µg) and fusidic acid (5-10 µg) was determined by the disk diffusion method as described in Clinical and Laboratory Standards Institute guidelines **[15]**.

3. Results and Discussion

In Egypt, there are two different production systems: the first one is an efficient, and specialized commercial dairy farms, while the second is smallholders which keep buffaloes, cows, or both in a small-scale production. Given that milk and dairy products are an essential part of food in Egypt, diseases in dairy animals that are caused by bacterial pathogens

including *S. aureus* can not only affect milk production but also pose a potential health hazard **[1].** In the present study, *S. aureus* was identified in 59.3% (73/123) from the examined farm dairy animal samples; whereas it was recovered from all milk samples (18/18), 50% (9/18) teat swab but not determined in fecal samples of dairy cows. The findings regarding the occurrence of *S. aureus* in cow's milk was higher than those reported by other researchers [5, 16-17] who found the following contamination rate in milk samples (6.39%; 36.3% and 72.8%). In addition, the recovery rate of *S. aureus* in teat swabs was nearly similar to that given by EL-Gohary and others **[16]** who detected *S. aureus* in 159 out of 372 (42.7%) from farm cows teat swabs. While in the present study *S. aureus* did not recover from any of farm cows' fecal samples. In another study, *S. aureus* was detected in one sample out of 40 (2.5%) fecal samples of farm cows **[18]**. On the other side, the overall occurrence of *S. aureus* in the examined farm buffalos' samples was 66.75 % (46/69) and the recovery rate in the examined milk samples, teat swabs and feces were 82.6% (19/23), 73.9% (17/23) and 43.5 % (10/23), respectively. MRSA was identified in the previously examined samples (at 2 µg/ml cefoxitin) with the percentage of 66.7 (12/18) and 11.1% (1/9) in milk and teat swabs, respectively but was not detected in any of the examined fecal samples. In addition, MRSA was not determined in any of the examined cows' samples when using cefoxitin at a concentration 4µg/ml but for buffaloes samples, MRSA was determined at the concentration of 2µg/ml of cefoxitin in 63.2% (12/19), 64.7% (11/17) and 40% (4/10) in milk samples, teat swabs and feces, respectively; while at the concentration of 4µg/ml it was 10.5% (2/19), 41.2% (7/17) and 30% (3/10). *S. aureus* was detected recently in Egypt in 31 out of 88 (35.2%) mastitic milk samples of farm buffaloes **[17]**. The distribution of MRSA in milk samples of farm cows was in harmony with that previously reported by Huber and others **[19]** who failed to identify MRSA in bulk tank milk and raw milk cheese but reported MRSA in 1.4% (2/142) of mastitic cow's milk. In different studies from Egypt, the occurrence of MRSA in milk of farm cows was 41% (111/271) and 37.7% (20/53) **[16, 17]**, respectively. While MRSA was not detected in teat swabs of farm cows using cefoxitin with concentration 4µg as showed in Table 2. Similar result was detected by Lim and their colleagues **[10]** who failed to determine MRSA in teat swabs of farm cows. On the other hand, MRSA was not detected in feces of farm cows using cefoxitin at concentration of 2µg and 4µg (Table 2).Similar result was detected by Lim et al.[18]who failed to detect MRSA in feces of farm cows.

The high detection rate of *S. aureus* from the cow's milk could be attributed to existence of several sources of contamination such as unclean utensils, towels and unclean milkers' hands. It might also be attributed to improper housing, bad handling of animals and bad sanitation [**17]** as well as contaminated surfaces including stainless steel, aluminum and glass which act as reservoir for *S. aureus* in dairy environment. Also *S. aureus* can get access to the milk either by direct excretion from infected udders, or via environmental contamination during the handling and processing of raw milk. Likewise, the relaxation of sphincter muscles of teats in older animals could also enable the bacterium to reach udder.

For smallholder animals, *S. aureus* was identified in 96/117 with the percentage of 82% in the examined cows' samples and the recovery rate was 81.8% (18/22) in milk samples. Low detection rate (41.6% **;** 3.7% **;** 7.6%) was reported in several studies in Egypt **[20, 5, 1]**. Similar pattern of occurrence was given by Kotb and Sayed **[21]** who isolated *S. aureus* with the percentage of (100) 5/5 from milk samples of smallholder mastitic cows and in 14/25 (56%) milk samples of smallholder healthy cows. While, the occurrence of *S. aureus* in fecal samples of smallholder cows was 72.7% (16/22), which is higher than that reported by EL-Gohary et al. **[20]** who reported that the occurrence of *S. aureus* in fecal samples of smallholder cows was 21.3% (16/75).

The occurrence of *S. aureus* in milk samples of smallholder buffaloes (15/17, 88.2%) was higher than the results obtained by other researchers in Egypt either from healthy animals or from those with mastitis. For example, in 2015 El-Ashker et al. **[5]**,and Elhaig and Selim **[22]** detected *S. aureus* with the percentage of 8.3 and 36.3 in smallholder buffaloes with mastitis, respectively. *S. aureus* was also detected in 16/17 (94.1%) fecal samples of smallholder buffaloes which is higher than those reported by EL-Gohary et al.**[20]** who isolated *S. aureus* with the percentage of13.3 (8/60) in fecal samples.

MRSA was detected among the recovered *S. aureus* isolates (using cefoxitin at concentration of 2µg/ml) in 27.8% (5/18), 90% (18/20) and 56.3% (9/16) in smallholders cows' milk, teat swabs and feces, respectively. While, it was 22.2% (4/18), 15% (3/20) and 18.8% (3/16) in the former samples at 4µg/ml cefoxitin. In buffaloes, *S. aureus* was determined in 88.2% (15/17), 64.2% (11/17) and 94.1% (16/17) in the above-mentioned samples, respectively. By using 2µg/ml cefoxitin, MRSA was detected in 20% (3/15), 100% (11/11) and 43.8% (7/16) in milk samples, teat swabs and feces, respectively; while at the concentration of 4µg/ml cefoxitin it was 6.7% (1/15), 9.1% (1/11) and 12.5% (2/16) (Table3). The distribution of MRSA in smallholder cow's milk was in harmony with that previously reported in Egypt by El-Ashker et al.**[1]** who identified MRSA in milk samples with the percentage of (28.6) 12/42. In another study in Egypt, **[21]** reported higher detection rates of MRSA in milk samples (36%) 9/25 from healthy cows and (80%) 4/5 from mastitis cow's milk samples. While the distribution of MRSA in milk samples of smallholder buffaloes was lower than the result obtained in Egypt by Kotb and Sayed **[21]** who identified MRSA with a percentage of 16.6 (5/30) from milk samples which obtained from healthy buffaloes and in 37.5% (3/8) from mastitis buffaloes milk

samples. It is difficult to compare the obtained results with other studies in the context of *S. aureus* and MRSA occurrence in buffalo's teat swabs and feces due to the lack of studies related to these issues. Taken altogether, it has been suggested that the discrepancies of our detection rates of *S. aureus* from other similar studies could be attributed to many factors including the number of collected samples from each category of animal, seasons and localities. In addition, it becomes clear that *S. aureus* can be found in high percentage and this could be attributed to poor hygiene and bad farm management **[23]**. Regarding the high detection rate of MRSA, Algammal et al. mentioned that the uncontrolled use of antibiotics in Egypt to treat mastitis leads to the emergence of MRSA and the widespread of multi drug resistance *S. aureus* in bovine species to different β-lactam compounds **[17]**.

Table 1. Oligonucleotide primers sequences used for amplification of *S. aureus* strains.

Primer	Primer sequence	Amplicon size (bp)	Reference
Nuc	F: 5'-GTGCTGGCATATGTATGGCAATTG-'3 R: 5' -CTGAATCAGCGTTGTCTTCGCTCCAA-'3	660	**Sallam et al. (2015)**
*mec*A	F: 5'-GATTGGGATCATAGCGTCA-'3 R: 5'-CAGTATTTCACCTTGTCCG-'3	1200	

Table 2. Frequency distribution of *S. aureus* and MRSA in farm animals.

Species	Type of samples	No. of samples	*S. aureus*		MRSA Cefoxitin 2 µg		MRSA Cefoxitin 4 µg	
			No.	%	No.	%	No.	%
Cows	**Milk**	18	18	100	12	66.7	-	-
	Teat swabs	18	9	50	1	11.1	-	-
	Feces	18	zero	0	-	-	-	-
	Total	54	27	50	13	48.1	-	-
Buffaloes	**Milk**	23	19	82.6	12	63.2	2	10.5
	Teat swabs	23	17	73.9	11	64.7	7	41.2
	Feces	23	10	43.5	4	40	3	30
	Total	69	46	66.7	27	58.7	12	26
Total		123	73	59.3	40	54.8	12	16.4

Table 3. Frequency distribution of S. *aureus* and MRSA in smallholders' animals.

Species	Type of samples	No. of samples	*S. aureus*		MRSA Cefoxitin 2 µg		MRSA Cefoxitin 4 µg	
			No.	%	No.	%	No.	%
Cows	**Milk**	22	18	81.8	5	27.8	4	22.2
	Teat swabs	22	20	90.9	18	90	3	15
	Feces	22	16	72.7	9	56.3	3	18.8
	Total	66	54	81.8	32	59.3	10	18.5
Buffaloes	**Milk**	17	15	88.2	3	20	1	6.7
	Teat swabs	17	11	64.7	11	100	1	9.1
	Feces	17	16	94.1	7	43.8	2	12.5
	Total	51	42	82.4	21	50	4	9.5
Total cows and buffaloes		117	96	82	55.2	64.6	14	14.9

Regarding the occurrence of *S. aureus* in human samples, our findings demonstrated that 103/120 (85.8%) were recovered from the examined human samples; whereas 85% (34/40), 82.5% (33/40) and 90 % (36/40) were determined from nasal, hand swabs and stool specimens, respectively. In several studies in Egypt **[24,22,25,16]** low detection rates were reported (36.7%) 11/30, (40%) 20/50, (53.8%),and (70.4%) 19/27, respectively. However, the occurrence of *S. aureus* in human hand swabs was similar to that reported by Kamal and other researcher **[26]**. For stool specimens, our finding was higher than those reported previously **[27;20].** The authors identified *S. aureus* in 20% (5/25) and 45% (45/100) of the examined stool specimens. For the distribution of MRSA in the examined human samples, it was identified in 35.3% (12/34), 33.3% (11/33) and 61.1% (22/36) at the concentration of 2 µg/ml and in 20.6% (7/34), 21.2% (7/33) and 27.8% (10/36) at concentration 4 µg/ml from nasal, hand swabs and stool specimens, respectively. In Egypt, nearly similar result (31.6%, 6/19) was reported from nasal swabs of dairy farm worker **[16]**. Nevertheless, a higher finding (40%, 16/40) was recorded from meat handlers **[28].** On the contrary, low detection rate (7%, 3/43) was reported in Korea **[10]**. For the

stool specimens, high recovery rate of MRSA was detected from contact workers (44.8%, 26/58)**[29]**. It becomes clear that the occurrence of MRSA in workers and animals represent great public health concern whereas theses resistant bacteria may spread to the environment causing hazards not only on health care or human health but also disseminates to food channel**.**

In the present study 50/360 (13.9%) were phenotypically characterized as MRSA. However, only 28 isolates (56%) harbored *nuc* gene. Out of the molecularly identified *S. aureus* strains (n =28), 19 isolates (67.9%) harbored *mec*A gene and were confirmed as MRSA. In Egypt, a previous study carried out by El-Jakee et al**.[30]** the authors detected *mec*A gene in (57.1%) 4/7 of the examined bovine milk. Kamal et al. couldn't detect *mec*A gene in any of the examined hand swabs from dairy workers **[26]**.While AL-Ashmawy et al. found that (75%) 30/40 of dairy animals' milk samples were expressed *mec*A gene **[31]**. Ismail and others found that all 16 isolates that phenotypically confirmed as MRSA were expressed to *nuc* gene and *mec*A gene**[28]**.

For the antibiotic susceptibility testing, table 5 demonstrated that MRSA isolates (n=19) showed high resistance to penicillin G and cefoxitin (100% each) followed by kanamycin (89.5%), fusidic acid (68.4%) and gentamicin (57.9%).Whereas the tested MRSA strains showed susceptibility to ciprofloxacin (100%) followed by vancomycin and clindamycin (78.9%), sulphamethoxazol and chloramphenicol (68.4%). These results were similar to that previously obtained in Egypt by AL-Ashmawy et al.**[31]** who found that MRSA isolates (n=414) which obtained from raw milk and dairy products were highly resistant to penicillin, clxacillin, amoxicillin (87.9%), (75.9%) and (55.6%) respectively. While showed low resistance to ciprofloxacin (15.5%) and sulphamethoxazol (14%).

In another study conducted by **Ismail et al.** the authors found that all MRSA isolates (n=20) which obtained from meat handlers' swabs were sensitive to ciprofloxacin (100%) and resistant to penicillin (100%) and sulphamethoxazol (100%) **[28]**.

4. Conclusion

The results reported in the present study confirmed that cows, buffaloes and their contact workers could play a significant role in transmission of MRSA, whereas detection of MRSA in the raw milk, teat swabs and feces of cows and buffaloes may create the opportunity for the transmission of such bacteria. MRSA could be transmitted to cattle and buffaloes through contaminated workers. On the other hand, unwise use of antibiotics in livestock could result in the development of antimicrobial resistance in MRSA, which is a growing problem in both developed and developing countries.

Table 4. Occurrence of *S. aureus* and MRSA in contact workers.

Type of Samples	*S. aureus*		MRSA Cefoxitin 2 µg		MRSA Cefoxitin 4 µg	
	No.	%	No.	%	No.	%
Nasal swabs (n=40)	34	85	12	35.3	7	20.6
Hand swabs (n=40)	33	82.5	11	33.3	7	21.2
Stool (n=40)	36	90	22	61.1	10	27.8
Total (n=120)	103	85.8	45	43.7	24	23.3

Table 5. Antibiotic susceptibility results of MRSA strains (n=19).

Antimicrobial agent	S		R	
	No.	%	No.	%
Cefoxitin (Fox)	-	-	19	100
Penicillin G (p)	-	-	19	100
Kanamycin (K)	2	10.5	17	89.5
Gentamycin (CN)	8	42.1	11	57.9
Clindamycin (DA)	15	78.9	4	21
Sulfamethxacin (SXT)	13	68.4	6	31.6
Ciprofloxacin (CIP)	19	100	-	-
Chloramphenicol (C)	13	68.4	6	31.6
Vancomycin (VA)	15	78.9	7	36.8
Fusidic acid (FA)	6	31.5	13	68.4

S: sensitive, R: resistant

Conflict of interest

No conflict of interest.

Author contributions

Thoraya Saad carried out the lab work, Mayada Giwda conceptualized the study, planned for the research activity, data analysis and wrote the manuscript. Adel El-Gohary and Amro Mohamed revised the final version. All authors have read and approved the final version of the manuscript for publication.

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