Isolation and Antibiogram Profiles of *Staphylococcus aureus* Isolates from Cow milk and Dog samples

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ABSTRACT

Staphylococcus aureus (*S. aureus*) is a commensal bacterium associated with serious infections in men and animals. Recently, multidrug-resistant (MDR) strains of *S. aureus* especially the so-called methicillin-resistant *S. aureus* (MRSA), represent a serious challenge that hinders the control of infections in man and animals. This study aimed to monitor milk samples from mastitic cows and vaginal and nasal swabs from dogs for the incidence of *S. aureus*. In addition, the isolates' antibiogram profiles were assessed to determine the extent of MDR and MRSA existence among the recovered isolates. Out of 260 samples, 29 (11%) *S. aureus* isolates were recovered with the highest incidence in milk samples (15/90, 17%), followed by vaginal swabs (8/90, 9%) and nasal swabs (6/80, 7%). Identification of the isolates was confirmed by PCR amplification of 16S rRNA gene sequence. Twenty *S. aureus* isolates were tested against seven antibacterial agents. Surprisingly, all the twenty isolates were MRSA and three bitch vaginal isolates were MDR. The findings of this study call for more research and cooperation between authors interested in assessing the MRSA and MRD bacterial incidence in both medical and veterinary fields. The cooperation will augment the challenge of disseminating MRSA and MDR staphylococci from animals to humans and vice versa.

INTRODUCTION

The emergence of antibiotic-resistant bacteria in livestock and companion animals poses an emerging challenge for disease prevention and control and a potential public health concern. The use of antimicrobial agents on dairy farms as well as in pet animals is a major concern in the emergence of resistant bacterial pathogens. Although different antibiotic classes of chemotherapeutics are used in animal health management and human medicine, the selection of resistance to one drug class may lead to cross-resistance to another. Monitoring the emergence of resistant pathogens in animal reservoirs is important, particularly for those with zoonotic potential (**Haran *et al.,* 2012**).

Staphylococcus aureus (*S. aureus*), a Gram-positive catalase-positive bacterium, is a common inhabitant of the skin and mucosa including the anterior nares of humans and animals. Although classified as a commensal bacterium, *S. aureus* is a facultative pathogen known as a major potential cause

of infections ranging from mild skin lesions to severe and probable fatal conditions (**Crespo-Piazuelo and Lawlor, 2021**).

In animals, *S. aureus* has been known to cause mastitis in dairy farms of cattle and small ruminants, bumble feet in chickens, and many pyogenic infections in different livestock. Animal-associated *S. aureus* strains have been reported to differ from those affecting humans (**Smith, 2015**).

Livestock-associated strains are commonly distinct due to antibiotics in animal management as feed additives to enhance growth in livestock and poultry projects. Antibiotics are also used for animal disease prevention and control (**Silbergeld *et al.,* 2008**). Unfortunately, antibiotics utilized in the veterinary field include many classes prescribed for humans such as tetracyclines, macrolides, penicillins, sulfonamides and other classes. Therefore, antimicrobial resistance generated during animal husbandry, as a result of the misuse of antibacterial therapeutics, may be transmitted to the human public.

This transmission could be through different ways such as contact with contaminated meat products and occupational contact. In turn, secondary spread to the wider community from occupationally-exposed persons is possible (**Smith, 2015**).

The first cases of penicillin-resistant *S. aureus* (the so-called *S. pyrogens* then) were reported less than a decade after the discovery of penicillin in the 1940s. Such resistance was controlled by plasmids containing the so-called ß-lactamase gene (*bla*Z) that produces an enzyme able to destroy the β-lactam ring of penicillin and other antibiotics (**Barber, 1949; Novick, 1963; Novick and Bouanchaud, 1971; Lowy, 2003**). Methicillin, a β-lactamase resistant antibiotic, was developed two decades after penicillin. Unfortunately, methicillin-resistant *S. aureus* (MRSA) emerged within two years. Methicillin resistance was found to be driven by the *mec*A gene, which encodes a protein named penicillin-binding protein 2a (PBP2a) to be distinguished from the low-affinity PBP (**Jevons, 1961; Barber, 1961; Hartman and Tomasz, 1984**).

While methicillin resistance has been the main research, the resistance of *S. aureus* to any antibacterial therapeutic agent is possible. It can potentially threaten the successful treatment of *S. aureus* infections in animals and the human population (**Crespo-Piazuelo and Lawlor, 2021**). Hence, animals can act as a reservoir for antimicrobial-resistant *S. aureus* that can be disseminated to other animals and human consumers in contact. Monitoring the emergence of resistant pathogens in animal reservoirs is important for better control of Staphylococcal infection in animals and man.

Therefore, this study targeted monitoring samples of clinically fit cattle and dogs for their potential reservoirs for multidrug-resistant *S. aureus.*

MATERIALS AND METHODS

Samples and bacterial isolation
Milk samples
Milk samples (no. = 90) were collected from mastitic cows belonging to small flocks at Giza Governorate. After cleaning the teat orifice using cotton soaked in 70% ethanol, the first streams of milk were discarded and about 15 ml were taken into a sterile 50 ml falcon tube. Samples were taken on ice to the laboratory with minimum delay (**Matyi *et al.,* 2013**).

The bacteriological investigation was initiated by streaking 100 µl of milk onto 5% sheep blood agar plate. After aerobic incubation at 37°C for 18–24 hours, colonies suspected of being staphylococci were processed for further identification.

Nasal swabs
Nasal specimens (No. = 80 were collected from the anterior nares of dogs admitted to veterinary clinics at Giza using sterile cotton swabs dipped in physiological saline. The swab was introduced into the external nostril for 1-2 cm and rotated 2-3 times with gentle pressure for a few seconds. The swabs were taken into sterile test tubes and transported to the laboratory on ice with minimum delay. Upon arrival at the laboratory, the collected swab samples were streaked onto mannitol salt agar (MSA) plates to isolate *S. aureus*. The inoculated MSA plates were incubated at 37°C for 24 hours. The suspected *S. aureus* colonies were further processed for identification and characterization. (**Thulunga, *et al.,* 2015**).

Vaginal swabs
A total of 90 samples were obtained from the anterior vagina of bitches recruited to private veterinary clinics in Cairo and Giza. Each bitch was secured in a standing position with a deflected tail and the external peri-vulvar area was disinfected with 5% povidone iodine antiseptic. A sterile disposable swab was carefully introduced, softly rolled against the vaginal wall and put back into its sterile tube. A sterile disposable swab was carefully introduced, softly rolled against the vaginal wall and put back into a sterile tube then transported to the laboratory. For bacterial isolation, swabs were dealt with as described above with the nasal swabs (**Mamman *et al.,* 2022**).

Identification of *S. aureus* isolates
S. aureus isolates were identified based on colony characteristics, Gram staining and biochemical tests. A catalase test was performed and the catalase-positive *Staphylococcus* spp isolates were tested using the tube coagulase method. Coagulase-positive strains were confirmed as *S. aureus* using PCR (**Dai *et al.,* 2019**).

DNA extraction and PCR assay
DNA was extracted from each coagulase-positive staphylococcus isolate using the QIAamp DNA extraction kit (QIAGEN Ltd., Manchester, United Kingdom) following the manufacturer's instructions. PCR Master Mix was prepared using Emerald Amp GT PCR master mix (Takara, Japan, Code No. RR310A) according to kit manufacturer instructions. PCR was carried out in a 25 µl final volume using the primers and the thermocycling program as shown in tables 1 and 2 (**Mason *et al.,* 2001**). The PCR products were separated by electrophoreses in 1% agarose gel in TBE buffer followed by visualization on a UV transilluminator (**Sambrook *et al.,* 1989**).

Table 1: Oligonucleotide primer sequences specific for the staphylococcus 16s rRNA gene.

Gene	Primer sequences (5'-3')	Length of amplified product
16S rRNA	Forward: CCTATAAGACTGGGATAACTTCGGG	791 bp
	Reverse: CTTTGAGTTTCAACCTTGCGGTCG	

Table 2: PCR thermocycler conditions

Primary denaturation	Secondary denaturation	Annealing	Extension	No. of cycles	Final extension
94˚C (5 min)	94˚C (30 sec)	55˚C (40 sec)	72˚C (45 sec)	35	72˚C (10 min)

Antimicrobial Susceptibility Testing (Dai *et al.,* 2019)

The Kirby–Bauer disk diffusion method was used to test *S. aureus* antibiotic susceptibility. Zone diameter interpretations were based on the protocol specified in the guidelines of the Clinical and Laboratory Standards Institute [CLSI] (2015). Twenty *S. aureus* isolates were assessed for antimicrobial susceptibility to 7 antibiotics (Oxoid, United Kingdom): methicillin (MET, 5µg/disc), cefoxitin (CX, 30µg/disc), penicillin G (P, 10 µg/disc), colistin (CL, 10µg/disc), vancomycin (VA, 30µg/disc), imipenem (IPM, 10µg/disc) and levofloxacin (LE, 5µg/disc).

RESULTS

The results in table (3) show that, out of 90 cow milk samples, 15 *s. aureus* isolates were recovered (17 *%*). Concerning dog samples, 8 and 6 *s. aureus* isolates were obtained out of 90 vaginal swabs (9%),6 out of and 80 nasal swabs (9% and 7%), respectively. The overall incidence of *S. aureus* in the examined samples was (11%) where 29 isolates were recovered from 260 samples.

Table 3: Incidence of *S. aureus* in cow milk samples and dog nasal and vaginal swabs

Samples	No. of samples	No. of *S. aureus* isolates no.	Percentages of *S. aureus* isolates
Milk samples	90	15	17
Vaginal swabs	90	8	9
Nasal swabs	80	6	7
Total	260	29	11

Genotypically, the staphylococcal-specific product of the 16S rRNA gene (791 bp) was detected in all 29 isolates (Fig. 1).
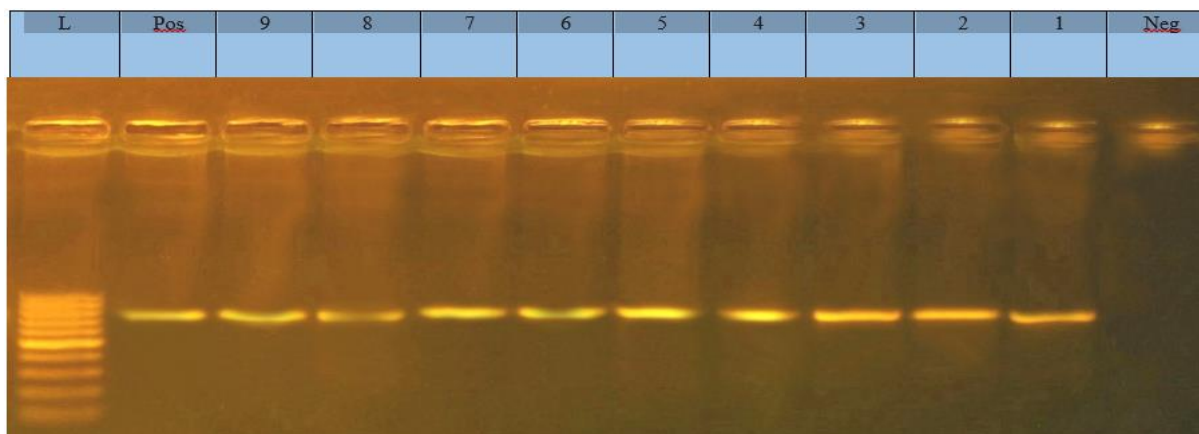

Fig. 1: Agarose gel electrophoresis of 16 S rRNA gene PCR products from *S. aureus* isolates. Lane L: 100 bp DNA size marker, Lane pos: *S. aureus* positive control product, lanes 1-9: PCR products of 10 *S. aureus* test isolates (791 bp) and lane Neg.: negative control (master mix without DNA template).

Concerning the antibiogram profile (table 4), 19 *S. aureus* isolates (95%) were sensitive to imipenem and 13 were sensitive to levofloxacin (65%). On the other hand, all isolates were resistant to methicillin and penicillin (100%) and 11 isolates were resistant to cefoxitin (55%).

Table 4: The antibiogram profile of 20 *S. aureus* isolates against 7 antibacterial agents

Isolate No. and source	MET.		CX.		P.		CL.		VA.		IPM.		LE.	
1 (milk sample)	12	R	26	S	14	R	14	NI	16	NI	28	S	21	S
2 (milk sample)	11	R	28	S	15	R	12	NI	18	NI	30	S	22	S
3 (milk sample)	9	R	28	S	18	R	12	NI	19	NI	44	S	32	S
4 (milk sample)	8	R	22	I	12	R	9	NI	15	NI	34	S	24	S
5 (milk sample)	9	R	21	I	14	R	12	NI	16	NI	35	S	22	S
6 (milk sample)	0	R	0	R	12	R	0	NI	15	NI	20	S	18	I
7 (milk sample)	9	R	24	I	18	R	12	NI	19	NI	40	S	28	S
8 (Nasal swab)	0	R	0	R	16	R	0	NI	18	NI	26	S	21	S
9 (Nasal swab)	0	R	0	R	12	R	0	NI	17	NI	27	S	21	S
10 (Nasal swab)	0	R	0	R	13	R	0	NI	15	NI	26	S	19	S
11 (Nasal swab)	8	R	22	I	0	R	0	NI	12	NI	36	S	26	S
12 (Nasal swab)	0	R	0	R	9	R	0	NI	18	NI	28	S	22	S
13 (Nasal swab)	11	R	20	I	18	R	0	NI	13	NI	28	S	17	S
14 (Nasal swab)	14	R	18	R	13	R	0	NI	14	NI	27	S	16	S
15 (vaginal swab)	16	R	20	I	13	R	0	NI	15	NI	30	S	15	R
16 (vaginal swab)	0	R	0	R	0	R	0	NI	16	NI	26	S	17	I
17 (vaginal swab)	0	R	0	R	0	R	0	NI	15	NI	0	R	16	I
18 (vaginal swab)	0	R	0	R	0	R	0	NI	14	NI	23	S	13	R
19 (vaginal swab)	0	R	0	R	9	R	0	NI	15	NI	24	S	17	I
20 (vaginal swab)	0	R	0	R	0	R	0	NI	15	NI	26	S	18	I
(S%)	0%		15%		0%		0%				95%		65%	
(I%)	0%		30%		0%		30%				0%		25%	
(R%)	100%		55%		100%		70%				5%		10%	

Methicillin (MET), Cefoxitin (CX), Penicillin G (P), Colistin (CL), Vancomycin (VA), Imipenem (IPM) and Levofloxacin (LE). S: sensitive, I: intermediate sensitive and R: resistant NI: not interpreted according to CLSI as no zone diameter standards.

DISCUSSION

S. aureus is a commensal pathogen that causes severe diseases in animals with a negative economic impact on the farm industry and affects the human population. Although *S. aureus* has been susceptible to every developed antibiotic, the organism also demonstrated the ability to develop antibiotic resistance mechanisms to compete and survive against antibiotics (**Chai** *et al.,* **2021**). In the current study, bovine milk samples and dog nasal and vaginal swabs were monitored for the existence of *S. aureus* in general and the multidrug-resistant (MDR) strains. The obtained results indicate the high prevalence of *S. aureus* in milk samples followed by vaginal samples and the least incidence was recorded in the nasal samples (17%, 9% and 7%, respectively). The overall incidence of *S. aureus* in the examined samples was 11% (29 isolates out of 260 samples).

In contrast,**Thomson** *et al.,* **(2022)** reported a high incidence of staphylococci in the nares of dogs and cats (56.6% and 46.6%, respectively). However, in their results, coagulase-negative staphylococci (CoNS) were the most prevalent, while *S. aureus* was recovered from only two out of 34 nasal swabs of dogs. Therefore, when considering *S. aureus*, our results coincide with theirs. The presence of CoNs may hinder the colonization of the commensal *S. aureus* leading to its low prevalence. Similar results were recorded by **Nemeghaire** *et al.,* **(2014)** who reported a low prevalence of *S. aureus* in nasal swab samples of cattle.

Concerning milk samples, a relatively high prevalence of *S. aureus* was reported in this study (17%). **Tibebu** *et al.,* **(2021)** reported an even higher incidence rate of *S. aureus* in milk samples of a cattle herd (25.53%). Therefore, they called for effective mastitis control programs and restriction of antibiotics application in dairy projects. *S. aureus* is a common finding in bovine clinical and subclinical mastitis. The consequence of both is persistent and recurrent infections and a low cure rate after antibiotic therapy (**Haag** *et al.,* **2019).**

In our study, the incidence of *S. aureus* in the vaginal samples of bitches was 9%. **Bukar-kolo** *et al.,* **(2016)** documented a higher incidence as they obtained 10 *S. aureus* isolates from 15 anterior vaginal swabs of bitches (66.66%). This big difference can be attributed to the health status of the tested animals as our samples were collected from bitches with no reproductive complaints.

Identification of all *S. aureus* isolates was made on morphological and biochemical bases. In addition, PCR assay targeting the staphylococcal 16S rRNA gene confirmed the identification. The PCR results align with **Al–Alak and Qassim (2016)** who recommended applying PCR to identify *S. aureus* isolates using 16S rRNA gene-specific primers. Variable results were obtained with the antibiogram assays performed on 20 *S. aureus* isolates in this study (table 4). However, the very interesting similarity shared by all isolates is their methicillin resistance (MRSA). This alarming finding must be considered seriously as these MRSA strains can be disseminated not only to other animals but to humans in contact. In addition, all isolates conferred resistance to penicillin.

Our results contradict those obtained by **Mourabit** *et al.***, (2020)** who reported the absence of MRSA among *S. aureus* isolates recovered from the anterior nares of farm animals. Multidrug resistance (MDR) has been defined as acquired non-susceptibility to at least one agent in three or more antimicrobial categories (**Magiorakos** *et al.,* **2012**). This definition is applied to three isolates out of six vaginal swabs of bitches (50%). Two MDR isolates were similar as they conferred resistance to methicillin, cefoxitin, Penicillin G and levofloxacin. The third MDR isolate was slightly different and conferred resistance to methicillin, cefoxitin, Penicillin G and imipenem. The promising antibacterial agent found in this study is imipenem, which was effective against 19 of 20 isolates tested.

The high incidence of MRSA and the existing MDR strains of *S. aureus* in the current study highlight the need for improved efforts and programs to face such a challenge in the study area. The control of antibiotic use is a highly recommended intervention to reduce the spread of MRSA between animals and humans (**Pantosti, 2012**).

CONCLUSION

S. aureus surveillance is often done within human clinics and hospitals versus little research on animals. In turn, the dissemination of clues of *S. aureus* from livestock and companion animals to humans or vice versa needs to be included. Therefore, considering the event as one health aspect, the relationship between public health and the agricultural and food industry should be assistance rather than antagonism. In other words, working together will undoubtedly ensure safer food products and well-protected consumers, owners and occupational personnel. Lastly, more attention to this type of research is needed to improve the effectiveness of antimicrobial stewardship in medical and veterinary practices.

Conflict of interest

The authors declare that they are not involved in any potential conflicts of interest.

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