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Short Paper

A report of coagulase-negative *Staphylococci* from clinically incurable cases of bovine mastitis: prevalence, biofilm formation, and resistance profile

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Abstract

Background: Given the abuse of broad-spectrum agents in the treatment of clinical bovine mastitis, coagulase-negative *Staphylococci* (CNS) have emerged to be of clinical and epidemiological significance. **Aims:** The study aimed to identify CNS and *Staphylococcus aureus* in incurable clinical mastitis in 50 cattle and 90 buffaloes, determine antibiotic resistance profile, and biofilm-forming ability of CNS and *S. aureus* isolates. **Methods:** 140 milk samples were collected from four villages in Sharkia, Egypt, for bacteriological isolation and molecular investigations. **Results:** Forty-nine *Staphylococcus* isolates were identified, including 11 CNS and 38 coagulase-positive *S. aureus*. The most recorded CNS strains were *S. epidemidis* (3), *S. simulans* (2), *S. hominis* (2), *S. chromogen* (2), *S. xylosus* (1), and *S. warneri* (1). A 63.2% of *S. aureus* and 27.3% of CNS isolates showed the ability to form biofilm, which was confirmed by *ica* PCR. *S. epidemidis* and *S. chromogen* were extensively drug-resistant, and most *S. aureus* isolates showed multidrug resistance (MDR). The proportion of methicillin-resistant was lower among *S. aureus* (84.2%), compared with CNS (90.9%). **Conclusion:** CNS present a challenge due to their uprising resistance compared with *S. aureus*. The appearance of CNS-MDR strains carrying *ica* gene leads to treatment protocol failure on bovine farms and improper control of bovine mastitis.

Key words: Antibiotics resistance, Biofilm formation, Clinical mastitis, CNS

Introduction

Mastitis, an infectious disease affecting dairy animals, leads to significant economic losses due to reduced milk quality, treatment costs, and unresponsive cases (Wyder *et al*., 2011; Asli *et al*., 2017).

Staphylococci are the most common cause of bovine mastitis; where *Staphylococcus aureus* is associated with a more severe illness than coagulase-negative *Staphylococci* (CNS) (Bradley *et al*., 2007).

CNS is a global cause of bovine mastitis, with increasing frequency in dairy cattle (Klibi *et al*., 2018). Many CNS species, including *S. xylosus*, *S. sciuri*, *S. saprophyticus*, *S. chromogenes*, *S. warneri*, and *S. epidermidis*, have incriminated in causing mastitis (El-Ashker *et al*., 2020; Lee and Lee, 2022), and showed antibiotic resistance and biofilm production (Raspanti *et al*., 2016; Lee and Lee, 2022).

Methicillin-resistant (MR) *Staphylococci* are mostly caused by spread of *mec*A gene, which hampers treatment of MR-CNS and *S. aureus* infections and poses a public health hazard (Elhaig and Selim, 2015; Wang *et al*., 2015).

In recent years, multidrug-resistance has been observed in buffaloes and cattle infected with coagulase positive *Staphylococci* and CNS (Dorgham *et al*., 2013). Proper use of antibiotics is an essential strategy to control bovine mastitis (Klibi *et al*., 2018).

In Egypt, the prevalence of CNS in dairy bovine is limited, with few studies reporting CNS from milk (El-Jakee *et al*., 2013; El-Ashker *et al*., 2020; Ibrahim *et al*., 2022) and no reports about CNS and their biofilm-forming ability in the studied regions, so far. Therefore, the present study investigated the presence of CNS in cattle and buffalo milk with clinical mastitis in Minya al-Qamh, Sharkia, Egypt, to determine antibiotic resistance and methicillin-resistant (MR) among isolated *S. aureus* and CNS strains.

Materials and Methods

Ethics statement

The study protocol was approved by the Ethics

Committee of Faculty of Veterinary Medicine, Suez Canal University (approval No.: 2023022).

Sampling and study area

A total of 140 milk samples were randomly taken from 50 cows and 90 buffaloes from four villages, Minya al-Qamh, Sharkia governorate (30.7°N 31.8°E), Egypt, during 2021-2022. The samples were taken from animals with clinical mastitis, showing udder redness, swelling, change in milk characteristics, and unresponsive antimicrobial treatments. Milk samples (15 ml) were collected in sterile tubes after washing the teat ends with water and rubbing with 70% ethanol and discarding the first milk streams. Then, samples were transported in an ice tank and processed in the bacteriological laboratory, Bacteriology Department, Faculty of Veterinary Medicine, Suez Canal University.

Isolation and characterization of *Staphylococcus* spp.

A heavy loopful of the specimen was inoculated onto nutrient agar, blood agar, *Staphylococcus* agar, and mannitol salt agar plates, incubated at 37°C for 24-48 h, and examined for bacterial growth and an *in-vitro* antimicrobial susceptibility. Isolates were identified according to previous protocols (Kalorey *et al*., 2007). Identified isolates were subjected to Gram staining and biochemical confirmation by the VITEK 2 system. The VITEK 2 compact instrument (bioMérieux, France) was used according to the method of Spanu *et al*. for accurate biochemical identification of the obtained colonies (Spanu *et al*., 2003; Wahdan *et al*., 2022).

PCR experiments

DNA was extracted using QIAamp DNA mini kits (QIAGEN, Hilden, Germany). Genus-specific PCR was used to detect *Staphylococci* by amplifying 570 bp of the *16S rRNA* gene using 16S rRNA-F: GCA AGC GTT ATC CGG ATT T and 16S rRNA-R: CTT AAT GAT GGC AAC TAA GC (Al-Talib *et al*., 2009). Species-specific PCR for detection of *S. aureus* was performed to amplify *nuc* gene (270 bp) using the following primers: nuc-F: 5´-GCG ATT GAT GGT GAT ACG GTT-3´ and *nuc*-R: 5´-AGC CAA GCC TTG ACG AAC TAA AGC-3´ (Brakstad *et al*., 1992). A positive control (confirmed *S. aureus* from Animal Health Research Institute, Dokki, Egypt) and a negative control (sterile DNA-free water) were included. PCR amplicons were visualized on 1.5% agarose gel stained with ethidium bromide (0.5 µg/ml) and examined using gel documentation system (Biospectrum UVP, UK).

Detection of MR strains by PCR

Detection was performed by PCR targeting *mecA* gene (553 bp) using *mec*A1: 5´-AAA ATC GAT GGT AAA GGT TGG C-3´ and *mec*A2: 5´-AGT TCT GCA GTA CCG GAT TTG C-3´ (Murakami *et al*., 1991).

Detection of biofilm formation by PCR

Detection was performed by PCR targeting *ica* gene (1315 bp) using forward primer 5´-CCT AAC TAA CGA AAG GTA G-3´ and reverse primer 5´-AAG ATA TAG CGA TAA GTG C-3´ (Ciftci *et al*., 2009).

Antimicrobial susceptibility test

The antimicrobial susceptibility of recovered isolates was assessed through disc diffusion method, according to the guidelines of Clinical Laboratory Standards Institute (CLSI). Ciprofloxacin (CIP, 5 µg), imipenem (IMP, 10 µg), doxycycline (DOX, 10 µg), erythromycin (E, 15 µg), gentamicin (CN, 10 µg), ampicillin (AMP, 10 µg), tetracycline (TE, 30 µg), cefoxitin (FOX, 30 µg), vancomycin (VA, 30 µg), and clindamycin (DA, 2 µg) (Oxoid, Basingstoke, UK) were used. Results (resistant, intermediate resistance, or sensitive to antimicrobials) were given in accordance with CLSI guidelines (CLSI, 2015). Cefoxitin-resistant *Staphylococci* classified as MR.

Statistical analysis

The study used PAST statistical analysis 4.03 to identify genetic similarities and relatedness among confirmed isolates (analysis was based on presence of *16S rRNA*, *nuc*, *mec*A, and *ica* genes), with Chi-square (http://vassarstats.net) calculated to evaluate significant differences between cattle and buffaloes, and MedCalc Software Ltd. used for 95% confidence intervals.

Results

Phenotypic and genotypic characterization of recovered isolates

Forty-nine (49/140, 35%) *Staphylococci* isolates were isolated and identified, with 38 (38/49, 77.6%) identified *S. aureus*, based on *nuc*-PCR, and 11 (11/49, 22.4%) *as* CNS (Table 1). Six species of CNS were classified, and their distribution is shown in Table 2, with *S. epidermidis* being the most prevalent. The detection rate was not-significantly higher in cattle (38%) than buffaloes (33.3%). The study found that 24 (63.2%) of *S. aureus* and 3 (27.3%) of CNS strains carried *ica* gene (Table 2). Thirty-two (84.2%) *S. aureus* and 10 (90.9%) CNS isolates harbored *mec*A gene.

Table 1: Results of *Staphylococci* isolation by bacterial culture from 140 clinical mastitic milk samples

Animals	Bacterial culture		P-value	Distribution of *Staphylococci* in animal samples (%)	
	Pos., n (%)	95% CI		*S. aureus*	CNS
Cows (n=50)	19 (38)	0.3-0.6	0.6^{NS}	15 (30)	4 (8)
Buffaloes (n=90)	30 (33.3)	0.22-0.5		23 (25.6)	7 (7.8)
Total (n=140)	49 (35)	0.3-0.5		38 (27.1)	11 (7.6)

^{NS} The result is non-significant at P>0.05

Table 2: Prevalence of *ica* gene, and resistance profile in CNS

CNS spp.	Number	*ica* gene (%)	Antibiotic resistance profile
S. epidemidis	3	1 (33.3)	DA, TE, AMP, FOX, DOX, CN, VA
S. simulans	2	1 (50)	DA, TE, AMP, CN
S. hominis	2	0	DA, TE, AMP, E
S. chromogen	2	1 (50)	DA, TE, AMP, FOX, E, CN
S. xylosus	1	0	AMP, TE, DA
S. warneri	1	0	Sensitive
Total	11	3 (27.3)	

Table 3: *In-vitro* comparison of antimicrobial susceptibilities between coagulase-positive and coagulase-negative *Staphylococcus*

Classes	Antimicrobials	*S. aureus* (n=38)			CNS (n=11)		
		S (%)	I (%)	R (%)	S (%)	I (%)	R (%)
Quinolones	CIP	29 (76.3)	5 (13.1)	4 (10.5)	11 (100)	0 (0)	0 (0)
Carbapenem	IMP	31 (81.5)	2 (5.2)	5 (13.1)	9 (81.8)	2 (18.1)	0 (0)
Tetracyclines	DOX	28 (73.6)	8 (21)	2 (5.2)	7 (63.6)	3 (27.3)	1 (9.1)
	TE	5 (13.1)	0 (0)	33 (86.9)	2 (18.1)	0 (0)	8 (72.7)
Macrolides	E	30 (78.9)	3 (7.8)	5 (13.1)	7 (63.3)	2 (18.1)	2 (18.1)
Aminoglycosides	CN	31 (81.5)	4 (10.5)	3 (7.8)	5 (45.4)	1 (9.1)	5 (45.5)
Lincosamides	DA	6 (15.7)	2 (5.2)	30 (78.9)	3 (27.3)	1 (9.1)	7 (63.6)
Glycopeptides	VA	10 (26.3)	3 (7.8)	25 (65.7)	9 (81.8)	1 (9.1)	1 (9.1)
Penicillins	AMP	9 (23.6)	10 (26.3)	19 (50)	1 (9.1)	1 (9.1)	9 (81.8)
Cephalosporins	FOX	7 (18.4)	3 (7.8)	28 (73.6)	5 (45.5)	3 (27.3)	3 (27.3)

S: Sensitive, I: Intermediate resistance, and R: Resistance

Antimicrobial susceptibility testing and MR distribution

Table 3 shows the distribution of *S. aureus* and CNS isolates against ten antimicrobial agents. Over 50% of *S. aureus* isolates showed resistance to tetracycline, clindamycin, cefoxitin, vancomycin, and ampicillin. CNS isolates showed high resistance to ampicillin, tetracycline, clindamycin, and gentamycin. Over 50% of *S. aureus* strains showed multidrug resistance (MDR), with an increase in resistance among CNS strains. *S. epidemidis* and *S. chromogen* showed extensive drug-resistance (XDR). *S. simulans* and *S. hominis* showed resistance to clindamycin, tetracycline, ampicillin, gentamycin, and erythromycin. *S. xylosus* showed some resistance to clindamycin, tetracycline, and ampicillin. In contrast, *S. warneri* remains sensitive to all tested antibiotics.

Distribution of *16S rRNA*, *nuc*, *mecA*, and *ica* genes among *staphylococcal* isolates

The cluster (Fig. 1) showed 21 *S. aureus* isolates (S1, S2, S3, S6, S7, S8, S9, S11, S16, S17, S19, S21, S22, S23, S25, S26, S28, S29, S31, S33, and S35) carrying *16S rRNA*, *nuc*, *mecA* and *ica* genes. *S. aureus* isolates (S4, S13, S18, S30, S37, and S38) did not have *mecA* gene. *S. aureus* isolates (S1, S2, S3, S6, S7, S8, S9, S11, S13, S16, S17, S19, S21, S22, S23, S25, S26, S28, S29, S31, S33, S35, S37, and S38) carried *ica* gene. Three CNS isolates (S39, S44, and S46) carried *16S rRNA*, *mecA*, and *ica* genes. The tested CNS did not carry *nuc* gene. All CNS carried the *mecA* gene, but the isolate S40 did not carry *mecA* gene.

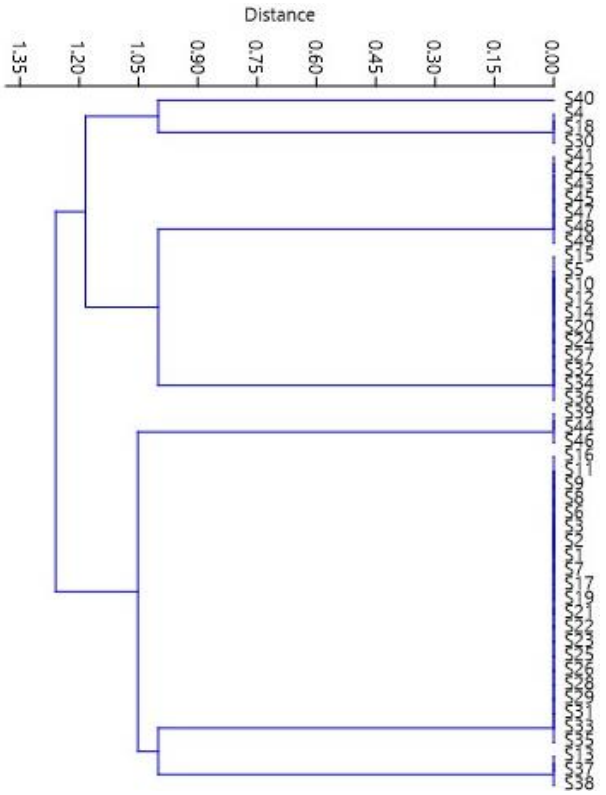

Fig. 1: Classical cluster showing distribution of *16S rRNA*, *nuc*, *mecA*, and *ica* genes among 49 Staphylococcal isolates

Discussion

Staphylococcal mastitis is a major threat among dairy animals all over the world and causes health concerns for humans (Hoque *et al*., 2018). In Egypt, data about CNS

involved in clinical bovine mastitis is limited. The study found that 22.4% of *Staphylococci* isolates were CNS, with the highest resistance to ampicillin (81.8%), tetracycline (72.7%), and clindamycin (63.6%). 91% of CNS isolates were methicillin-resistant by *mec*A PCR.

Bacterial culture results confirmed presence of *Staphylococcus* sp. in 35% of milk samples from bovine clinical mastitis, indicating a considerable association between *Staphylococcus* infection and clinical mastitis in livestock.

Current results showed that CNS could cause bovine clinical mastitis in 7.6% of milk samples, with lower than 11.3% reported in the USA (Gillespie *et al*., 2009) and 50% in Finland (Pitkälä *et al*., 2004).

In Egypt, CNS was found in 16.6% and 59.4% of cattle and buffaloes with subclinical mastitis, while CNS was not isolated from clinical mastitis, and *S. aureus* was the predominant isolate in cows, buffaloes, sheep, and goats (El-Jakee *et al*., 2013). Other studies reported variable rates of CNS occurrence in dairy animals with clinical or subclinical mastitis, with rates of 11.76% in Damietta (Hussein *et al*., 2018), 12.4% in Dakahlia (El-Ashker *et al*., 2020), and 44.12% in Giza (Ibrahim *et al*., 2022).

Table 2 shows six species of CNS with varying prevalence rates. Previous reports have indicated discrepancies in CNS species patterns. In Egypt, *S. sciuri* being the most prevalent, followed by *S. chromogenes*, *S. haemolyticus*, *S. xylosus*, *S. hyicus*, and *S. warneri* in bovine mastitis (El-Ashker *et al*., 2020). In Argentina, *S. chromogenes* and *S. haemolyticus* were the predominant species (Raspanti *et al*., 2016). This may be due to the ability of some species to adapt to udder tissue or due to differences in control strategies (Raspanti *et al*., 2016).

In this study, 27.3% of CNS isolates showed the ability to form biofilms. Lee and Lee (2022) found 78.4% of CNS isolates from tank milk in Korea formed biofilms. While Tremblay *et al*. (2013) in Canada and Srednik *et al*. (2017) in Argentina reported >44% of CNS isolates from bovine mastitis formed a moderate to strong biofilm.

Interestingly, the capability of *S. aureus* to produce biofilm was higher than CNS isolates, a finding like that found in Egypt (Raheel *et al*., 2023).

The dominance of *S. aureus* among the causative agents of mastitis in cows and sheep has been reported earlier (El-Jakee *et al*., 2013), supporting our findings. This possibly due to its presence inside or outside the udder or a misclassification bias related to CMT (Dingwell *et al*., 2003). Clinical mastitis due to *S. aureus* has a significant local or global prevalence, with higher rates in Egypt reaching 38.3% in Ismailia (Elhaig and Selim, 2015) and 42% in Dakahliya and Damietta (Awad *et al*., 2017), and lower rates in Sadat, Egypt; 11.2% (Elsayed *et al*., 2015) and India; 50% (Nigam, 2015). These variations may be attributable to geographical and management differences, as well as the use of different methodologies (Li *et al*., 2017).

The infection rate of both CNS and *S. aureus* was higher in cattle (38%) than in buffalo (33.3%), which is similar to a previous study from Dakahlia, Egypt (El-Ashker *et al*., 2020). Conversely, in two studies on mastitis from Egypt, the prevalence of *S. aureus* was higher in buffalo (50%) than cattle (39.29%) (Elsayed *et al*., 2015) and CNS was lower in cattle (16.6%) than buffalo (59.4%) (El-Jakee *et al*., 2013). Such cattle-related findings are possibly due to the use of suboptimal hygiene practices during the milking process in the study area. The study findings present a challenge when it comes to comparing them with other studies due to variations in *S. aureus*'s role in mastitis, influenced by factors such as animal handling practices and hygiene levels (Jayarao *et al*., 2004).

Treatment and control of bovine mastitis caused by *Staphylococci* often fail, due to the complex nature of the organism and its increasing resistance to drugs (Wang *et al*., 2015). In this study, MDR *S. aureus* and XDR CNS were reported to have uprising resistance against tested antimicrobial groups, posing concerns in the veterinary field and highlighting potential risks.

El-Ashker *et al*. (2020) found that the majority of CNS showed low rates of resistance genes, while *S. haemolyticus* and *S. warneri* showed resistance to more than three antibiotics; however, in our study, *S. epidermidis* and *S. chromogen* showed resistance to more than four antibiotics, and *S. warneri* did not show resistance to any of the antibiotics examined. Klibi *et al*. (2018) found high antimicrobial resistance in CNS, primarily to beta-lactams and tetracycline in cows with mastitis, possibly due to the use of these antibiotics in Tunisia. In China and Bangladesh, *S. aureus* causes bovine mastitis was resistant to various antimicrobials; erythromycin, clindamycin, penicillin, trimethoprim/ sulfamethoxazole, oxytetracycline, and doxycycline (Wang *et al*., 2015; Li *et al*., 2017; Hoque *et al*., 2018). Moreover, several CNS strains were resistant to DA, TE, AMP, FOX, DOX, CN, and VA; possibly due to frequent and improper use of antibiotics, which may explain the failure of treatment in this study.

The *mecA* gene was detected in 42 isolates, confirming MR in 90.9% of CNS and 84.2% of *S. aureus* isolates, a higher percentage than in Korea (Lim *et al*., 2012) and China (Li *et al*., 2017). These variations may be attributable to the effects of animal populations, methodologies, and geography (Li *et al*., 2017).

Wide resistance to antimicrobials among *S. aureus* and CNS strains from clinical bovine mastitis, and the identification of most *S. aureus* and CNS strains as MR, poses a significant challenge to treatment (Wang *et al*., 2015; Shah *et al*., 2019). Continuous observation of antimicrobial resistance of CNS and *S. aureus* is required since our study reported a significant positive correlation between resistance to cefoxitin and the presence of *mecA* and *nuc* genes among recovered isolates.

The study reported a high prevalence of *S. aureus* and CNS in incurable mastitis cases in the Sharkia governorate, Egypt. Poor hygienic conditions lead to the detection of CNS and *S. aureus*, which are more resistant to several antimicrobials, indicating treatment failure. Some CNS were encoded for a specific gene responsible

for adhesion intramammary. The latter finding has potential consequences regarding vets' decisions to treat animals for mastitis, particularly if no bacterial culture analysis is performed for comparison. The restricted locality is a limitation of this study. Further studies with a larger sample size and early diagnosis of mastitis etiology could improve outcomes, treatment, and control measures.

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Conflict of interest

The authors declare that they have no conflicts of interest.

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