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Original Research Article

Coagulase Negative Staphylococci as an emerging cause of bovine mastitis: prevalence, antimicrobial resistance and biofilm formation

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Abstract

Coagulase negative *Staphylococci* are the most prevalent cause of bovine subclinical mastitis. The current study were designed to study their occurrence, antibiogram and their ability to form biofilms. A total number of 95 CNS isolates were recovered from 400 lactating. *S. xylosus* (36.84%), *S. chromogenes* (12.63%), *S. epidermidis* (10.53%), *S. saprophyticus* (8.42%), *S. haemolyticus* (7.38%) were the most common recovered species. Disk diffusion method against 14 antimicrobials discs was used to detect their antibiogram. 100% were sensitive to Imipenem, 96.84% were sensitive to Enrofloxacin, 85.26% to Chlramphenicol and 84.21% to Vancomycin. But, 95.79% were resistant to Ampicillin, 77.9% resistant to Cefoxitin, 35.8% resistant to Cefuroxime, 32.63% resistant to Amoxycillin and 18.95% resistant to Clindamycin. Cultivation on Congo Red Agar (CRA) was carried out to detect biofilm formation. 47.37% were positive and *S. epidermidis* was the most biofilm positive species on CRA by the percentage of 70%. Haemolysins were studied by cultivating CNS on sheep blood agar. 25.26% were β-haemolytic, 71.57% (n=68) were γ-haemolytic and 3.15% were α- haemolytic.

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1. Introduction

Staphylococci are one of the most significant causative mastitis pathogens in both clinical and subclinical especially *S. aureus*. For a long time, *S. aureus* was considered the only important pathogen among *Staphylococcus* species. However, recently in both that subclinical and clinical mastitis cases throughout the world, increased attention has been paid to Coagulase-Negative Staphylococci (CNS) (Bal *et al.*, 2010). CNS have become the predominant pathogens associated with mastitis (Tenhagen *et al.*, 2006). They are currently considered emerging pathogens of bovine mastitis (Soares *et al*. 2012). Mastitis caused by CNS usually remains subclinical or mildly clinical, however CNS have been shown to cause persistent infections, which result in increased somatic cell count (SCC) in milk and cause milk production loss and mammary tissue damage (Soares *et al*., 2012). Severe local and systemic signs have been reported in animals with CNS intramammary infections (IMI) (Jarp, 1991). Other researches also indicated that CNS are capable of persisting in udders for longer periods of time (Taponen *et al.*, 2006). Prevalent CNS species vary according to the geographical region under scrutiny (Huxley *et al.*, 2002). It is important to monitor antimicrobial susceptibility of mastitis pathogens including CNS, as antimicrobials play a major role in the control of mastitis (Sawant *et al*., 2009). Although some CNS based mastitis infections respond well to most antimicrobial agents, many other show increasing rates of resistance to beta lactams, macrolides, lincosamides and other groups of antimicrobials. The results of in vitro susceptibility testing are an important tool to guide the veterinarian in selecting the most efficacious antimicrobial agent(s) for both therapeutic and prophylactic interventions . (Lüthje and Schwarz, 2006). Carriage of antimicrobial resistance genes by CNS species in cattle may also be relevant because it potentially poses a human health hazard. It can happens both through lateral transfer of resistance genes between staphylococcal species and through direct transmission of resistant pathogens (Walther and Perreten, 2007). Biofilm is one of the important microbial virulence factors found in staphylococci, consisting of multilayered cell clusters embedded in a matrix of extracellular polysaccharide, which facilitate the adherence of microorganism (Jain and Agarwal, 2009). Biofilm may play a role in CNS persistence in the intramammary environment. Additionally, CNS isolates growing within biofilms are less susceptible to antimicrobials commonly used on farms (Tremblay *et al.*, 2013). Therefore, biofilm formation by CNS species could possibly impede antimicrobial therapy.

2. Material and methods

2.1. Samples

A total number of 364 quarter milk samples were collected aseptically from 400 lactating cows from different size dairy herds located in El-Fayoum and Beni-Suef Governorates during the period from October 2015 to June 2016. Lactating cows were examined then tested for subclinical mastitis by California Mastitis Test (CMT), positive quarters were sampled in clean sterile labelled containers and sent to lab for isolation and identification.

2.2. CNS Isolation and identification

Preliminary incubation of each milk sample for 18–24 h at 37^0 C. A loopfull from each sample was cultured on mannitol salt agar (Oxoid) and incubated at 37 C for 18–24 h and examined for bacterial growth, bacterial films were made from suspected colonies and stained with Gram's stain to confirm being Staphylococci. Coagulase test was carried out using citrated rabbit plasma to differentiate between coagulase-positive and negative Staphylococci after incubation of tested isolates on Trypton Soy Broth (Oxoid). For further identification selected colonies were streaked on 5% sheep blood agar and Baird Parker medium (Oxoid), supplemented with egg yolk tellurite emulsion. All plates were incubated at 37 C for 18–24 h and analyzed for: hemolytic pattern on sheep blood agar, lecithinase activity on Baird Parker medium. API-Staph Kit (bioMe´rieux) was used for identification of CNS isolates following the instructions of kit's insert and then the strips were read by the mini API instrument and associated software.

2.3. CNS Antimicrobial susceptibility testing

Susceptibility to antibiotics was examined according to the guidelines of the National Reference Centre for Antimicrobial Susceptibility and internationally recognized standards of the Clinical and Laboratory Standards Institute (CLSI, 2015). Determinations were carried out using the diffusion disk method on Müller-Hinton agar (Oxoid). The following discs (Oxoid) were used: Ampicillin (AM, 10 µg), Amoxycillin (AML, 10 µg), Amoxycillin-clavulunic acid (AMC, 30 µg), Cefoxitin (FOX, 30 µg) was used for detection of methicillin resistance, Cefuroxime (CXM, 30 µg), Vancomycin (VA, 30 µg), Imipenem (IMP, 10 µg), Enrofloxacin (ENR, 5 µg), Tetracycline (T, 30 µg), Clindamycin (DA, 2 µg), Kanamycin (K, 30 µg), Chloramphenicol (C, 30 µg) and Sulfamethoxazole-trimethoprim (SXT, 1.25/23.75 µg). Briefly, a fresh colony of the isolates was transferred to a tube containing 5 ml Müller-Hinton broth (Oxoid). The mixture was incubated at 37°C until light visible turbidity appeared; this was compared with the McFarland 0.5 turbidity standard. The suspension of test organism was streaked over the surface of Muller Hinton agar plates using a sterile disposable cotton swab. Antibiotics discs were firmly placed on plates by means of sterile forceps and plates were incubated for 24 h at 37°C. The diameters of growth-inhibition were measured in mm and reported as, susceptible, intermediate, and resistant, as per CSLI guidelines.

2.4. CNS Biofilm detection

Biofilm production was assessed qualitatively by the Congo red agar method, as described by Arciola *et al.*, (2015). Isolates were streaked onto CRA, incubated at 37°C for 24 hours, and then kept at room temperature for 48 hours. Colony color was determined using a four-color reference scale varying from red to black. Black colonies were considered to be biofilm-producing isolates, while almost-black colonies were considered weak biofilm producers. Red and purple colonies were considered non-biofilm producers.

3. Results

3.1. CNS prevalence

A total number of 95 coagulase negative Staphylococci isolates were isolated from 364 milk samples out from 400 lactating cows by the ratio of 26.09%.

Table 1. Occurrence of each CNS recovered species

Species	S. xylosus	S. chromogenes	S. epidermidis	S. aprophyticus	S. haemolyticus	S. lentus	S. auricularis	S. hominis	S. sicuri	S. warneri	S. simulans	S. caprae	S. cohnii	Total
N	35	12	10	8	7	4	4	3	3	3	2	2	2	95
%	36.8	12.6	10.5	8.4	7.4	4.2	4.2	3.2	3.2	3.2	2.1	2.1	2.1	100

Table 2. CNS antimicrobial susceptibility results

Class	Agent	Disk conc. (µg)	Resistant		Intermediate		Sensitive	
			n=	%	n=	%	n=	%
Penicillins	Ampicillin	10	91	95.79	0	0	4	4.21
	Amoxycillin	10	31	32.63	47	49.47	17	17.89
B-lactamase stable penicillins	Amoxy-Clavulinic acid	30	17	17.9	0	0	78	82.1
Cephalosporines	Cefoxitin	30	74	77.9	0	0	21	22.1
	Cefuroxime	30	34	35.79	13	13.68	48	50.53
Glycopeptides	Vancomycin	30	9	9.47	6	6.32	80	84.21
Carbapenems	Imipenem	10	0	0	0	0	95	100
Fluoroquinolones	Enrofloxacin	5	0	0	3	3.16	92	96.84
Tetracyclines	Tetracycline	30	15	15.79	9	9.47	71	74.74
Lincosamides	Clindamycin	2	18	18.95	26	27.37	51	53.68
Aminoglycosoides	Kanamycin	30	11	11.58	12	12.63	72	75.79
Chloramephenicol	Chloramphenicol	30	9	9.47	5	5.26	81	85.26
Potentiated Sulphonamides	Sulphametoxazole-Trimethoprim	1.25/ 23.75	10	10.53	9	9.47	76	80

Table 3. CNS biofilm formation on CRA results

Species	S. xylosus	S. chromogenes	S. epidermidis	S. aprophyticus	S. haemolyticus	S. lentus	S. auricularis	S. hominis	S. sicuri	S. warneri	S. simulans	S. caprae	S. cohnii	Total
N	35	12	10	8	7	4	4	3	3	3	2	2	2	95
n=	21	7	7	2	4	0	1	1	1	0	0	1	0	45
%	60	58.3	70	25	57.1	0	25	33.3	33.3	0	0	50	0	47.4

3.2. CNS species occurrence

Recovered 95 CNS isolates were identified by API® Staph and the results revealed that *S. xylosus* was the predominant as a causative agent of subclinical mastitis at the percentage of 36.48%, then *S. chromogenes* (12.63%), *S. epidermidis* (10.53%), *S. saprophyticus* (8.42%) and *S. haemolyticus* (7.38%). Other results are demonstrated in table no. (1).

3.3. CNS haemolysis on blood agar

Recovered CNS isolates were streaked on sheep blood agar to detect their haemolytic characters. It was found that 28.42% (n=27) of CNS isolates showed haemolytic activity in general; 3.15% (n=3) were α–haemolytic and 25.26% (n=24) were β–haemolytic. But, 71.57% (n=68) were non (γ) – haemolytic. Also, 100% of S.*saprophyticus, S. lentus*, S. *auricularis,* S. *hominis*, S. *sicuri*, *S. simulans* and *S. cohnii* were γ- haemolytic. While 100% of *S. haemolyticus* and S. *caprae* were β-haemolytic. 34.29% of *S. xylosus, 33.33*% of *S. warneri*, 25% of *S.chromogenes* and 20% of *S. epidermidis* were α–haemolytic.

3.4. CNS antimicrobial susceptibility

The results showed that 100% of recovered CNS were sensitive to imipenem, 96.84% were sensitive to enrofloxacin, 85.26% were sensitive to chloramphenicol and 84.21% were sensitive to vancomycin. While 95.79% were resistant to Ampicillin, 77.9% were resistant to Cefoxitin, 35.79% were resistant to Cefuroxime and 31.58% were resistant to Amoxycillin. Detailed results of each antimicrobial are demonstrated in table (2).

3.5. CNS biofilm formation on CRA

The results showed that 100% of recovered CNS were sensitive to imipenem, 96.84% were sensitive to enrofloxacin, 85.26% were sensitive to chloramphenicol and 84.21% were sensitive to vancomycin. While 95.79% were resistant to Ampicillin, 77.9% were resistant to Cefoxitin, 35.79% were resistant to Cefuroxime and 31.58% were resistant to Amoxycillin. Detailed results of each antimicrobial are demonstrated in tables no. (2).

4. Discussion

Coagulase-negative staphylococci (CNS) have become the predominant pathogens isolated from bovine mastitis in several countries, even could be described as emerging mastitis pathogens (El-Jakee *et al.*, 2013) not only in subclinical mastitis but also clinical mastitis (Brinda *et al.,* 2010). Intramammary infection with CNS has been proved to cause elevated Somatic Cell Count (SCC), change in milk quality, decrease in milk production and even damage in udder tissue (Soares *et al*. 2012).

CNS can represent as public health hazard due to capability to carry antimicrobial resistance genes between cattle and humans. That happens through lateral transfer of resistance genes between staphylococcal species and through direct transmission of resistant pathogens Walther & Perreten (2007).

Infection with CNS is also interfering with manufacturing of some dairy products because of using antibiotics in prophylaxis or treatment.

The impact behind CNS mastitis is due to the subclinically infected cows are reservoirs invisibly spreading the infection to other cows in the herd, that made some researchers consider control measures to subclinical mastitis is more important than clinical mastitis cases El-Jakee et al., (2013).

In the present study, the prevalence, species variation, antibiogram and biofilm formation of CNS were studied among 364 quarter milk samples collected from 400 lactating animals. A total number of 95 CNS isolates were recovered at a percentage of 26.09%. The prevalence of CNS causing mastitis has been worldwide investigated. In Germany, CNS were isolated from 9% of the quarter milk samples in a total of 80 dairy herds by Tenhagen *et al.* (2006). In Belgium, Piepers *et al.* (2007) reported that more than 50% of all IMI were caused by CNS. In a similar Turkish study, Bal *et al*. (2010) isolated 100 CNS species from 221 quarter milk samples in a percentage of 45.25%.

From Egypt El-Jakee et al. (2013) reported a prevalence of 16.6% as they isolated 76 CNS isolates out of 459 subclinical mastitis samples. In china Xu *et al.* (2015) identified 76 CNS isolates out of 209 subclinical mastitis milk samples from a single Chinese dairy herd in a prevalence of 36.36%. While in Brazil Tomazi *et al.* (2015) reported a prevalence of 9.47%.

The present study illustrated that *S. xylosus* was the predominant isolate recovered from the examined cow by the ratio of 36.84%, followed by *S. chromogenes* (12.36%), *S. epidermidis* (10.53%), *S. saprophyticus* (8.42%) and *S. haemolyticus* (7.83%). The predominance of S. *xylosus* among the mastitis causing CNS was recorded earlier in cattle by Brinda *et al.*, (2010), Bochniarz and Wawron (2012), Soares *et al.*, (2012) and Frey *et al.* (2013). However, despite variations between herds and countries, others CNS i.e. *S. chromogenes S. epidermidis,* and *S. simulans,* in general, appear to be the most frequently isolated CNS from mastitis milk samples worldwide Klimiene *et al.* (2016), Moser *et al.* (2013), Raspanti *et al.* (2016), Sheikh and Mehdinejad (2012) and Taponen *et al.* (2006).

CNS are capable of producing various enzymes facilitating the invasion of host tissues and spread of the inflammatory process (e.g. lipase, fibrinolysin, urease). Moreover, they were found capable of producing proteolytic enzymes and haemolysins, which facilitate the uptake of the important iron Bochniarz and Wawron (2012).To date, the role of CNS as a cause of bovine mastitis and human infections and their hemolysin factors is not completely clear Moraveji et al. (2014). So, in this study, distribution of hemolysins in CNS isolates from subclinical mastitis were phenotypically studied. The results showed that 71.6% (n=68) of isolated CNS were γ-haemolytic (non-haemolytic), unlike data reported by Moraveji et al. (2014) that found 25% of CNS strains were non hemolytic. . While, 25.26% (n= 24) of isolated CNS were β-haemolytic. And 100% of *S. haemolyticus* (n=7) and S. *caprae* (n=2), 34.3% of S. *xylosus* (n=12) and 33.3% of S. warneri (n=1) were β- haemolytic. But, 3.15% (n=3) of isolated CNS were α-haemolytic as 2 isolates of S. *xylosus* and one isolate of *S. chromogenes.* So *S. haemolyticus* was the highest producer of β- haemolysin and S. *xylosus* was the highest producer of α – haemolysins. Previous results were in agreement with results of Bochniarz and Wawron (2012).

Investigating susceptibility and resistance patterns of CNS, disk diffusion method against 13 antimicrobial of 11 different classes. The result in table no. (2) showed the given results of CNS susceptibility. Methicillin Resistant *Staphylococci* (MRS) can be phenotypically detected by using disks of Cefoxitin Jain *et al.* (2008).

Ampicillin was the most antimicrobial agent showing resistance against CNS by the percentage of 95.8%, followed by Cefoxitin (77.9%), Cefuroxime (35.8%) and Amoxycillin (32.63%). The highest percentages of resistance of CNS were focused on members of β-lactam group. Different percentages of resistance against β-lactam antibiotics are worldwidly reported. In Germany, Lüthje and Schwarz *et al.* (2006) reported a percentage of 18.1% against Ampicillin. In Turkey, Bal *et al.* (2010) reported a percentage of 48% against Ampicillin. Bansal *et al.* (2015) in India reported a percentage of 52.9 % against both Ampicillin and Amoxycillin. In South Africa Schmidt et al. (2015) reported a percentage of 37.3% of CNS from subclinical mastitis were resistant to Ampicillin. The present study reported that 77.9% of isolated CNS were MRS. Bal *et al.* (2010) reported a lower percentage of MRS among isolated CNS by 21.95%. Silva *et al.* (2014) reported a 20% of MRS among isolated CNS.

Elevated β-lactam resistance in CNS can be attributed to two well documented mechanisms. One is accomplished by the presence of β-lactamase activity and another one is presence of *mecA* and *blaZ* genes products in the case of penicillinase – resistant (Bansal *et al.* 2015).

More than half of the intramammary preparations available for use comprise penicillin or ampicillin. Furthermore, many of the preparations are available to farmers over the world, making it difficult to monitor and control antimicrobial usage Schmidt e*t al.* (2015).

On the other hand, CNS were found to be 100% sensitive to Imipenem, 96.84% to Enrofloxacin, 85.26% to Chloramphenicol and 84.21% to Vancomycin.

Results of Bal *et al.* (2010) were dissimilar as Chloramphenicol was the most antimicrobial recovered CNS were sensitive to by the percentage of 96%, then Tetracycline and clindamycin by the percentage of 86%. Other results by Bansal *et al.* (2015) were also in disagreement as they found recovered CNS isolates were highly susceptible to Chloramphenicol (98.3%), Gentamicin (93.1%), Streptomycin (91.4%), Linezolid (91.4%), Ceftixozime (87.9%), Cloxacillin (86.2%), Clotrimazole (86.2%), bacitracin (86.2%) and Enrofloxacin (84.5%). Different percentages of susceptibility of CNS to different antimicrobials can be attributed to geographical differences besides change in type of used antimicrobials in either prophylaxis or treatment.

The importance of detecting antibiogram of CNS is due to use of these information to select the most efficacious antimicrobial agent(s) for both therapeutic and prophylactic interventions Lüthje and Schwarz (2006). Another importance appears as the given data can be an early sign of a public health hazard as the high percentages of resistance against many antimicrobials and the possible carriage of CNS with resistance genes that can be laterally transferred between cattle and Walther and Perreten (2007).

Slime production and the ability to adhere to surfaces, facilitating the formation of a biofilm, is one of the important factors responsible for the CNS pathogenicity. It's also one of the most important elements in the intramammary survival of such organisms (Bochniarz *et al.*, 2014).

Additionally, Biofilms help directly in lowering the susceptibility of used antimicrobials and impeding the antimicrobial therapy Tremblay *et al.*, (2013).

For detection of biofilm in CNS Osman *et al.* (2015) found that CRA tests provide reliable results for biofilm detection in CNS and are adequate for routine use when compared to PCR results.

The recent study reported that 47.4% of recovered CNS isolates were phenotypically positive for biofilm formation. At the species level; 70% of *S. epidermidis*, 60% of *S. xylosus*, 58.3% of S.chromogenes, 57.1% of *S. haemolyticus*, 50% of *S. caprae* and 33.3% of both *S. hominis* and *S. sicuri* were positive for phenotypic biofilm formation on CRA. Slime-producing ability was observed by in 54% of recovered CNS in the study by Bochniarz *et al.,* (2014). Osman *et al.* (2015) reported that 70.2 % of recovered CNS species were positive for their ability to produce slime and those with slime-positive strains which appeared as black colonies.

S. *epidermidis* was the most species forming biofilms compared to other CNS species. The same was reported by Oliveira and Cunha (2008) and Simojoki et al. (2012). While, Rumi et al. (2013) disagreed as S. chromogenes was the most predominant in their work. Tremblay et al. (2013) entirely disagreed as they reported that S. epidermidis was the species with the lowest ability to form biofilm. While, S. xylosus was the species with the highest ability to form biofilm. Srednik et al. (2017) differently reported that S. chromogenes and S. sciuri were the most species with biofilm formation incidence. S. haemolyticus and S. devriesei isolates formed significantly more strong biofilms than other CNS.

5. Conclusions

The current study highlights the emerging of CNS in bovine mastitis and their virulence constituents helping in complicating their infection besides their decreasing susceptibility and increasing resistance to antibiotics as well CNS acquisition of resistance genes that can be easily transmitted to other pathogenic bacteria co-existing in udder or even to human pathogens which represents a public health hazard. Country-cross CNS epidemiology studies and investigation are recommended, also more simplified methods either biochemical or genetic to detect them rapidly and accurately are much required. Besides, other advanced mastitis control measures are necessary.

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