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ANTIMICROBIAL RESISTANCE OF Β- LACTAMASE COAGULASE-NEGATIVE STAPHYLOCOCCI (CNS) PRODUCERS ISOLATED FROM DAIRY SHEEP AND GOATS

By

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ABSTRACT

Dairy goat and sheep farms suffer severe economic losses due to intramammary infections with *Staphylococcus* species representing the main cause of clinical mastitis in small ruminants. Antimicrobial resistance patterns were determined in 32 coagulase negative staphylococci (CNS) isolated from milk samples of goats (n=20) and sheep (n=12) with subclinical mastitis. The CNS isolates showed the highest resistance rate against cloxacillin and oxacillin (100 % each). All CNS isolates were sensitive to florfenicol, neomycin and gentamicin. All β- lactamase CNS producers detected from the examined sheep milk samples were resistant to penicillin (100%). While 66.7% of β- lactamase CNS producers detected from the examined goat were resistant to it. Antimicrobial susceptibility patterns should be identified for CNS; as current susceptibility data are necessary to select appropriate antibiotics for a successful treatment.

Key words:

Coagulase negative staphylococci (CNS), mastitis, antibiotic sensitivity test, β- lactamase, sheep, goat.

INTRODUCTION

The prevalence of *Staphylococcus* species in caprine and ovine bulk tank milk samples varies depending of the country .*S. aureus* was isolated from 30% from sheep milk and 37.5% from sheep milk and 15(37.5%) from goat`s milk samples in Sohag, Egypt (**Usama, 2014). Muehlherr *et al.* (2003)** detected *S. aureus* in 32% of the caprine and 33% of ovine bulk tank milk samples in Switzerland, while **Linage *et al.* (2012) and Álvarez-Suárez *et al.* (2015)** detected coagulase positive staphylococci in 66% of caprine and 15% of ovine milk samples in Spain. Coagulase negative staphylococci (CNS) have become increasingly recognized as important pathogen of nosocomial infection **(Stuart *et al*., 2011).** Severe local and systemic signs have been reported in animals with CNS intramammary infections **(Jarp, 1991).**

The role of CNS in the bovine mastitis syndrome has come under increased scrutiny since more CNS associated with clinical and subclinical mastitis are being reported **(Gentilini *et al*., 2002).** On farms that have successfully controlled mastitis caused by *S. aureus* and *S. agalactiae*, opportunistic bacteria such as CNS are frequently associated with bovine mastitis **(Ruegg, 2009).** The epidemiology of CNS mastitis is still unclear, although a number of studies have been conducted to identify the reservoirs of CNS. The widespread use of antibiotics on dairy farms, could lead to the selection and to the emergence of antibiotic-resistant bacterial strains **(Virdis *et al*., 2010).** The evaluation of the antimicrobial susceptibility of CNS isolated from dairy animals with subclinical mastitis (SCM) is of great interest for clinical purposes in order to decide which antibiotics should be administered, as well as, for monitoring the spread of multiple resistant strains on farms. There is little information on the CNS among dairy animals in Egypt. The current study seeks to investigate antibiotic resistance of CNS isolated from dairy sheep and goats.

MATERIAL AND METHODS

Bacterial Characterization:

A total of 32 CNS isolates collected from sheep (n=12) and goats (n=20) with subclinical mastitis were identified biochemically to be: *S. lugdunensis* (n=16), *S. saprophyticus* (n=4), *S. cohnii* (n=8), and *S. hominis* (n=4) using routinely microbiological procedures and using API-Staph Kit (bioMerieux) according to manufacturer's instructions. The CNS isolates were confirmed to be *Staphylococcus* species other than *S. aureus* using the following primers: *Staphylococcus* genus specific primers 16Sr RNA f (5' GTA GGT GGC AAG CGTTATCC 3') and 16Sr RNA r (5' CGC ACA TCA GCG TCA G 3') according to **Pereira *et al*. (2009)** as well as *S. aureus* specific primers Nuc 1 f (5'-GCGATTGATGGT GATACGGTT-3') and Nuc 2 r (5'-AGCCAAGCCTTGACGAACTAAAGC-3') as described by **Zhang *et al*. (2004).** Multiplex PCR assay was performed utilizing a both pair of primers.

B- Lactamase activity and antibiotic sensitivity test:

The β- lactamase activity among the CNS isolates was estimated using β-LACTA strip (Test-Line Ltd. Krizikova 68, 612 00 BRNO CZ.) according to **Livermore and Brown**

(2001). Also on each isolate the inhibition zones of 11 antibiotics used in veterinary medicines were determined by disk diffusion method **(Finegold and Martin, 1982**). The antibiotics (Oxoid) tested were amoxicillin (10µg/disk), amoxicillin + clavulanic acid (20+10 µg/disk), ampicillin (10 µg/disk), cloxacillin (5 µg/disk), erythromycin (15 µg/disk), florfenicol (30 µg/disk), gentamicin (10 µg/disk), neomycin (30 µg/disk), oxacillin (1 µg/disk), oxytetracycline (30 µg/disk) and penicillin G (10 units /disk).

RESULTS

CNS Isolates:

A total of 32 CNS isolates were selected for antimicrobial agent susceptibility test. All isolates were confirmed to be CNS using API test and PCR as shown in Fig. (1).

β- Lactamase activity among the CNS isolates:

B- Lacta strips; β- lactamase activity was investigated among the CNS isolates as shown in Table (1). It was clear that, 12 (60%) and 8 (66.7%) CNS isolated from goat and sheep respectively were β- lactamase positive.

Antibiotic sensitivity test of the CNS isolates

Table (2) shows that most of CNS isolates were sensitive to florfenicol, neomycin and gentamicin. Meanwhile the examined isolates were resistant to cloxacillin and oxacillin (100% each), amoxicillin and ampicillin (87.5 each %). The correlation between β- lactamase producer isolates and antibiotic sensitivity test was illustrated in (Tables 3 and 4).

DISCUSSION

Sheep and goats form the most important group of milk producing animals after dairy cattle in both temperate and tropical agriculture **(Devendra and Coop, 1982).** *Staphylococcus* spp. are the main etiological agents of small ruminant's intramammary infections (IMI), the more frequent isolates being *Staphylococcus aureus* in clinical cases and coagulase-negative staphylococci (CNS) in subclinical IMI **(Bergonier *et al.*, 2003). Recently Martins *et al.* (2017)** recorded that coagulase-negative staphylococci (CNS) are among the main responsible agents for mastitis in sheep. Increasing antimicrobial resistance has become a serious concern worldwide and antimicrobial use in animal agriculture is currently under scrutiny. Mastitis is the most common reason for antibiotic use in dairy herds and thus, antimicrobial resistance of mastitis pathogens has received recent attention. In the present study after identification 32 CNS isolated from sheep (12) and goats (20) using conventional, API test and PCR, the susceptibility-sensitivity profile was examined using the disk diffusion technique for 11 antimicrobial agents. Antimicrobial resistance in bacteria of animal origin and its impact on human health have drawn much attention worldwide **(Aarestrup, 2006).** Among various antimicrobial drugs, β-lactams represent one of the most significant classes of antimicrobials in providing striking therapeutic benefits for the treatment of bacterial infections. Indeed β-lactam resistance, including resistance to extended-spectrum β-lactams, has now been increasingly observed in bacteria of animal origin and of human health concern **(Li *et al*., 2007).** The presence of β-lactamases in CNS has been observed both in human and veterinary isolates. Using β-lactams strips, β- lactamase activity was investigated among the CNS isolates as shown in (Table 1). `It is clear that, 12 (60 %) and 8 (66.7 %) CNS isolated from goat and sheep respectively were β- lactamase positive. Resistance to β-lactams, macrolides and lincosamides have been reported in CNS isolated in milk from cows with subclinical and clinical mastitis **(Luthje and Schwarz, 2006).** It has also been hypothesized that CNS may serve as a reservoir for the transfer of antimicrobial resistance genes to *Staphylococcus aureus* **(Archer and Climo, 1994).** All penicillin-resistant isolates were β-lactamase producers **(Gentilini *et al*., 2002).** As shown in (Tables 3 and 4) all β- lactamase CNS producers (100%) detected from the examined sheep milk samples were resistant to penicillin, while 66.7 % of β-lactamase CNS producers detected from the examined goat were resistant to it. The reported percentage of penicillin resistance for CNS isolated in mastitis was 32 % in Finland **(Pitkala *et al*., 2004)** and 28 % in the Netherlands **(Maran, 2003).** The largest resistance percentage of CNS was found by **Martins *et al.* (2017)** for the penicillin (17.0%) and tetracycline (10.7%). Most of CNS isolates were sensitive to florfenicol, neomycin, gentamicin and erythromycin; meanwhile the examined isolates were resistant to cloxacillin, oxacillin, amoxicillin and ampicillin (Tables 3 and 4). **Sampimon *et al.* (2011)** concluded that CNS species isolated from bovine milk differ significantly in phenotypic and antimicrobial resistance profiles, 40.6% expressed resistance to a single compound or a single class of compounds, and 10.6% to multiple drug classes. (Tables 2 and 4) recorded that, 100 % of CNS isolated from the examined goat and sheep milk samples were resistant to oxacillin. CNS resistance to methicillin and other semi synthetic penicillins is now common **(Stuart *et al*., 2011).** The presence of *mecA* has been detected in various species of staphylococci including *S. intermedius*, *S. epidermidis*, *S. lentus*, *S. saprophyticus*, *S. xylosus*, *S. sciuri*, and *S. haemolyticus* **(Gortel *et al*., 1999; Yasuda *et al*., 2000).** Methicillin-resistant *S. aureus*

(MRSA) likely originated by acquisition of the staphylococcal cassette chromosome (SCC) from CNS **(Tulinski *et al.* 2011).** In the present study 66.7 % of CNS isolated from the examined sheep milk samples, respectively were resistant to oxytetracycline. Resistance to oxytetracycline of CNS isolated from mastitis was 9 % in Finland **(Pitkala *et al.*, 2004)** and 12 % in the Netherlands **(Maran, 2003).** It concluded that CNS strains are emerging as important minor mastitis pathogens and can be the cause of substantial economic losses. The present study revealed differences in antimicrobial susceptibility among the CNS species evaluated. Over 40 % of the tested CNS was resistant to at least one antimicrobial agent. The high resistance to penicillin plus the presence of methicillin-resistant isolates found in this study emphasize the importance of identification of CNS among mastitic animals.

REFERENCES

Aarestrup FM. (2006): Antimicrobia Resistance in Bacteria of Animal Origin. ASM Press. Washington. DC. USA.

Álvarez-Suárez M.-E., Otero A., García-López M.-L., Santos J. A. (2015): Microbiological examination of bulk tank goat's milk in the Castilla y León region in Northern Spain.J. Food Prot.78:2227-2232.

Archer GL., Climo. MW. (1994): Antimicrobial susceptibility of coagulase-negative staphylococci. Antimicrob. Agents Chemother. 38: 2231-2237.

Bergonier D., De Cremoux R., Rupp R., Lagriffoul G., Berthelot X. (2003): Mastitis of dairy small ruminants. Vet. Res. 34, 689 -716.

Devendra C., Coop I.E. (1982): Ecology and Distribution. In: I.E. Coop (Editor): World Animal Science C 1 Production System Approach: Sheep and Goat Production. Amsterdam: Elsevier. pp. 1-14.

Finegold SM., Martin, WJ. (1982): Bailey and Scott's Diagnostic Microbiology. 6th Ed., the C.V. Mosby Company, St. Louis, Torento, London.

Gentilini E., Denamiel G., Betancor A., Rebuelto M., Rodriguez Fermepin, M., De Torrest RA. (2002): Antimicrobial susceptibility of coagulase-negative staphylococci isolated from bovine mastitis in Argentina. J. Dairy .Sci. 85 (8):1913 - 1917.

Gortel K., Campbell KL., Kakoma, I., Whittem T., and Schaeffer DJ., Weisiger RM. (1999): Methicillin resistance among staphylococci isolated from dogs. Am. J. Vet. Res., 60:1526-1530.

Jarp J. (1991): Classification of coagulase-negative staphylococci isolated from bovine clinical and subclinical mastitis. Vet. Microbiol. 27: 151-158.

Li, Xian-Zhi., Mehrotra, Manisha, Ghimire, Shiva,and Adewoye, Lateef. (2007): B-Lactam resistance and B-Lactamases in bacteria of animal origin Vet. Microbiology, 121: 197-214.

Linage B., Rodriguez-Calleja JM., Otero A., Garcia-Lopez ML, Santos JA. (2012): Characterization of coagulase-positive staphylococci isolated from tank and silo ewe milk. J. Dairy Sci.951639 - 1644.

Livermore DM, Brown DF. (2001): Detection of beta-Lactamase mediated resistance. J. Antmicrob Chemother. 48 (1): 59 - 64.

Luthje P., Schwarz S. (2006): Antimicrobial resistance of coagulase-negative staphylococci from bovine subclinical mastitis with particular reference to macrolide-lincosamide resistance phenotypes and genotypes. J. Antimicrob. Chemo there. 57: 966 - 969.

Maran. (2003): Monitoring of antimicrobial resistance and antibiotic usage in animals in the Netherlands.

Martins KB, Faccioli PY, Bonesso MF, Fernandes S, Oliveira AA, Dantas A, Zafalon LF, Cunha ML. (2017): Characteristics of resistance and virulence factors in different species of coagulase-negative staphylococci isolated from milk of healthy sheep and animals with subclinical mastitis. J Dairy Sci. 2017 100 (3):2184 - 2195.

Muehlherr JE., Zweifel C., Corti S., Blanco JE., Stephan R. (2003): Microbiological quality of raw goat's and ewe's bulk-tank milk in Switzerland. J. Dairy Sci.86: 3849 -3856.

Pereira V., Lopes C., Castro A., silva J., Gibbs P., Teixeira P. (2009): Characterization for enterotoxin production, Virulence factors, and antibiotic susceptibility of Staphylococcus aureus isolates from various food in Portugal. Food Microbiology. 278-282.

Pitkala A., Haveri M., Pyorala S., Myllys V., Honkanen-Buzalski T. (2004): Bovine mastitis in Finland 2001 – Prevalence, distribution of bacteria, and antimicrobial resistance. J. Dairy Sci. 87: 2433-2441.

Sampimon OC., Lam TJ., Mevius DJ., Schukken YH., Zadoks RN. (2011): Antimicrobial susceptibility of coagulase-negative staphylococci isolated from bovine milk samples. Vet. Microbiol. 150 (1-2):173 - 179.

Stuart JI., John MA., Milburn S., Diagre D., Wilson B., Hussain Z. (2011): Susceptibility patterns of coagulase-negative staphylococci to several newer antimicrobial agents in comparison with vancomycin and oxacillin. Int. J. Antimicrob. Agents. 37 (3):248 -252.

Ruegg P. L. (2009): The quest for the perfect test: Phenotypic versus genotypic identification of coagulase-negative staphylococci associated with bovine mastitis Veterinary Microbiology. 134: 15-19.

Tulinski P., Fluit AC., Wagenaar JA., Mevius D., van de Vijver L., Duim B. (2011): Methicillin-resistant coagulase-negative staphylococci on pig farms act as a reservoir of heterogeneous SCCmec elements. Appl. Environ. Microbiol. Nov 11.

Usama H. Abo-Shama (2014): Prevalence and Antimicrobial Susceptibility of Staphylococcus Aureus Isolated From Cattle, Buffalo, Sheep and Goat`S Raws Milk In Sohag Governorate, Egypt. Assiut Vet. Med. J. Vol. 60 No. 141 63-72.

Virdis S., Scarano C., Cossu F., Spanu V, Spanu C., De Santis EP. (2010): Antibiotic Resistance in Staphylococcus aureus and Coagulase Negative Staphylococci Isolated from Goats with Subclinical Mastitis. Vet. Med. Int. 517060. 6 pages.

Yasuda R., Kawano J., On da H., Takagi M., Shimizu A., Anzai, and T. (2000): Methicillin-resistant coagulase-negative staphylococci isolated from healthy horses in Japan. Am. J. Vet. Res. 61: 1451–1455.

Zhang K., Sparling J., Chow BL., Elsayed S., Hussain Z., Church DL., Gregson DB., Louie T.,and Conly JM. (2004): New quadriplex PCR assay for detection of methicillin and mupirocin resistance and simultaneous discrimination of *Staphylococcus aureus* from coagulase-negative staphylococci. J. Clin. Microbiol. 42:4947 - 4955.

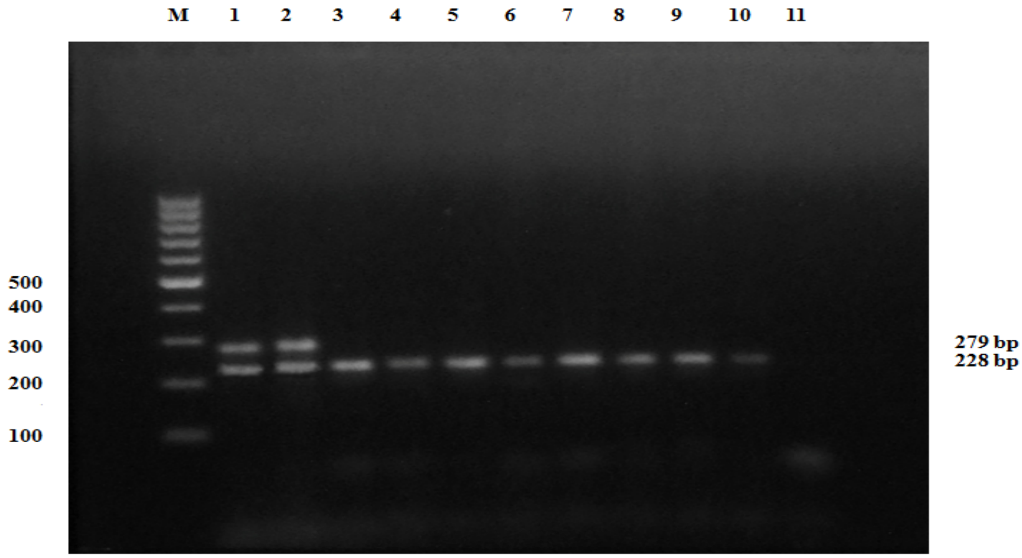

Fig. (1): Multiplex PCR assay to detect *Staphylococcus* isolates.

M: 100 bp DNA Ladder (Fermentas), Lanes 1 and 2: *S. aureus* ATCC 25923 reference strain (228 bp & 279 bp amplified bands), Lanes 3-10 CNS isolates (228 bp only) and Lane 11: negative control.

Table (1): β- lactamase activity of CNS isolates.

CNS	Sheep			Goat		
	n	No.	%	n	No.	%
***S. lugdunensis* (n=16)**	4	4	100	12	8	66.7
***S. saprophyticus* (n=4)**	-	-	0	4	4	100
***S. cohnii* (n=8)**	8	4	50	-	-	0
***S. hominis* (n=4)**	-	-	0	4	-	0
Total (n=32)	12	8	66.7	20	12	60

N= number of examined CNS isolates No. = Number of positive β- lactamase isolates.

% was calculated according to the number of examined CNS isolates.

Table (2): Prevalence of antibacterial resistance among the CNS isolated from milk samples.

Source of CNS isolates	AMC		AMP		AML		OT		P		CX		FFC		N		G		O		E	
	NO.	%	NO.	%	NO.		NO.	%	NO.	%	NO.	%	NO.	%	NO.	%	NO.	%	NO.	%	NO.	%
Goat milk (n=20)	16	80	16	80	16	80	0	0	12	60	20	100	0	0	0	0	0	0	20	100	0	0
Sheep milk (n=12)	0	0	12	100	12	100	8	66.7	12	100	12	100	0	0	0	0	0	0	12	100	0	0
Total (n=32)	16	50	28	87.5	28	87.5	8	25	24	75	32	100	0	0	0	0	0	0	32	100	0	0

Table (3): Correlation between β lactamase CNS producers and resistant to antibacterial agents among goat isolates.

CNS isolates	AMC		AMP		AML		OT		P		CX		FFC		N		G		O		E	
	NO.	%	NO.	%	NO.	%	NO.	%	NO.	%	NO.	%	NO.	%	NO.	%	NO.	%	NO.	%	NO.	%
S. lugdunensis (n=8)	8	100	8	100	8	100	0	0	8	100	8	100	0	0	0	0	0	0	8	100	0	0
S. saprophyticus (n=4)	0	0	0	0	0	0	0	0	0	0	4	100	0	0	0	0	0	0	4	100	0	0
Total (n=12)	8	66.7	8	66.7	8	66.7	0	0	8	66.7	12	100	0	0	0	0	0	0	12	100	0	0

N: number of examined CNS isolates, No.: number of resistant isolates, % was calculated according to the number of examined CNS isolates, AML: amoxicillin (10 μg/disk), AMC: amoxicillin + clavulanic acid (20+10 μg/disk), AMP: ampicillin (10 μg/disk), CX: cloxacillin (5 μg/disk), E: erythromycin (15 μg/disk), FFC: florfenicol (30 μg/disk), G: gentamicin (10 μg/disk), N: neomycin (30 μg/disk), O: oxacillin (1 μg/disk), OT: oxytetracycline (30 μg/disk) and P: penicillin G (10units/disk).

Table (4): Correlation between β lactamase CNS producers and resistant to antibacterial agents among sheep isolates.

CNS isolates	AMC		AMP		AML		O		P		CX		FFC		N		G		O		E	
	NO.	%	NO.	%	NO.	%	NO.	%	NO.	%	NO.	%	NO.	%	NO.	%	NO.	%	NO.	%	NO.	%
S. lugdunensis (n=4)	0	0	4	100	4	100	0	0	4	100	4	100	0	0	0	0	0	0	4	100	0	0
S. cohnii (n=4)	0	0	4	100	4	100	4	100	4	100	4	100	0	0	0	0	0	0	4	100	0	0
Total (n=8)	0	0	8	100	8	100	4	50	8	100	8	100	0	0	0	0	0	0	8	100	0	0

N: number of positive β lactamase CNS isolates, No.: number of resistant isolates, % was calculated according to the number of positive β lactamase CNS isolates, AML: amoxicillin (10 µg/disk), AMC: amoxicillin + clavulanic acid (20+10 µg/disk), AMP: ampicillin (10 µg/disk), CX: cloxacillin (5 µg/disk), E: erythromycin (15 µg/disk), FFC: florfenicol (30 µg/disk), G: gentamicin (10 µg/disk), N: neomycin (30 µg/disk), O: oxacillin (1 µg/disk), OT: oxytetracycline (30 µg/disk) and P: penicillin G (10units/disk).