

Antibiogram of Coagulase-Negative Staphylococci (CNS) associated with subclinical mastitis in dairy buffaloes (*Bubalus Bubalis*)

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Abstract

The milk samples of subclinical mastitis collected during routine screening of dairy buffaloes were subjected to indirect mastitis tests and microbial culture in Mastitis Laboratory, Guru Angad Dev Veterinary and Animal Sciences University, Ludhiana. Of the 297 quarters foremilk samples collected from 77 apparently healthy buffaloes, 91 bacterial isolates of different microorganisms were recovered. Coagulase-negative staphylococci (CNS) were the most frequently recovered bacterial species accounting for 59.34% of all isolates followed by *Staphylococcus aureus* (18.68%), *Corynebacterium spp.* (15.38 %), *Streptococcus spp.* (5.49 %), and *Klebsiella spp.* (1.11 %). CNS were confirmed by standard biochemical characterization and further subjected to antimicrobial sensitivity testing. A total of 30 antibiotics belonging to 11 groups were used to study the drug resistance pattern. Out of 54 CNS isolates, antibiotic sensitivity testing was done only in 48 isolates, which revealed highest susceptibility to chloramphenicol (97.9%), ciprofloxacin (95.8%), gentamicin (93.8%), linezolid (91.7%), oxacillin (91.7%) and ceftiozime (89.6%), while resistance was observed against penicillin (58.3%), amoxicillin (56.3%), ampicillin (54.2%), and cefaperazone (33.3%).

Keywords: Buffaloes, Mastitis, Coagulase-negative staphylococci, Culture, Antibiotic

India has approximately 94.13 million buffaloes of the world and contributes approximately 53 percent of the total milk produced. Mastitis, the inflammation of mammary glands, is one of the most costly diseases of dairy animals resulting in the reduction of milk yield and quality (Denis *et al.*, 2011). Mastitis can occur either in subclinical or clinical form and aetiology has been classified into contagious and environmental pathogens. Mastitis caused by coagulase-negative staphylococci (CNS) has been primarily considered subclinical, with a slight increase in milk somatic cell count (SCC) (Honkanen-Buzalski *et al.*, 1994). However, in the recent studies, CNS species have been recovered from cases of sub-clinical as well as clinical mastitis (Taponen *et al.*, 2006; Supré *et al.*, 2011). CNS species were initially considered as minor pathogens, but their importance as the predominant pathogens of mastitis has been established in the recent years (Taponen *et al.*, 2007; Waller *et al.*, 2011). Though, CNS species are less virulent than *Staphylococcus aureus*, but these generally exhibit higher antimicrobial resistance, and more often show multi-antimicrobial resistance (Pyorala and Taponen, 2009). Mastitis is the single greatest cause of antibacterial use on dairy farms, and emergence of antibacterial resistance

among pathogens that impact animal health has been a growing concern in veterinary medicine and have also been incriminated as a potential health risk for humans (Sears and McCarthy, 2003).

The present study was therefore, planned to determine the *in vitro* antibiotic susceptibility of coagulase-negative staphylococci (CNS) strains isolated from subclinical cases of mastitis in dairy buffaloes. Antibiotic sensitivity test is widely used clinical investigation being followed worldwide in cases of bovine mastitis with the sole purpose to select most appropriate antimicrobial agent for therapeutic use. Antibiotic susceptibility profile of CNS isolates will be helpful to recommend early therapy at the field level prior to availability of CST results.

Materials and Methods

Collection of milk samples

The milk samples of subclinical mastitis collected during routine screening of dairy buffaloes were subjected to indirect mastitis tests and microbial culture in Mastitis Laboratory, Guru Angad Dev Veterinary and Animal Sciences University, Ludhiana. A total of 297 quarter foremilk samples collected from 77 apparently healthy buffaloes, were included in the

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study.

Isolation and biochemical characterization of coagulase-negative staphylococci

Procedures described by National Mastitis Council USA (1987) were followed for culture of milk samples and identification of mastitis pathogens. The milk samples were mixed thoroughly and 0.05 ml of quarter milk sample was streaked with sterilized platinum loop on blood agar plates. The plates were incubated aerobically at 37°C and examined after 18-24 hours for the presence of any bacterial growth. The organisms were identified on the basis of colony characteristics on blood agar, Gram staining, clumping factor, growth characteristics on manitol salt agar, DNase agar and Baird parker agar and tube coagulase test.

Culture sensitivity testing

Susceptibility of bacterial isolates from mastitic milk to anti-microbials was subjected using the disc diffusion susceptibility test (Kirby Bauer method) (Quinn *et al.*, 2000). Briefly, fresh inoculums in 5 ml nutrient broth were incubated at 37°C until light visible turbidity appeared, comparable to 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. Commercially available antibiotics discs (Hi-Media) 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 millimeters and reported as, susceptible, intermediate, and resistant, as per CSLI guidelines. Since the CSLI guidelines does not provide interpretive criteria for some of the mastitis pathogens/ antimicrobial combinations, whenever guidelines for testing and breakpoints were not available for a specific pathogen, guidelines for other pathogens/ antimicrobial combinations of the same group were used. Such a method is commonly used by commercial laboratories and has been reported in literature (Gentilini *et al.*, 2002, and Pitkala *et al.*, 2004). Thus, the breakpoints for ampicillin were used for amoxicillin and those for oxacillin were used for cloxacillin and for streptomycin and neomycin, the breakpoints established by Bauer *et al.* (1966) were used.

Results and Discussion

The present study was envisaged to identify the

CNS in the mastitic milk samples of dairy buffaloes and to reveal their antibiotic resistant pattern and to carry out rationale treatment. Of the 297 quarters foremilk samples collected from 77 apparently healthy buffaloes, 91 bacterial isolates of different microorganisms were recovered. Coagulase-negative staphylococci were the most frequently recovered bacterial species accounting for 59.34 per cent of all isolates followed by *Staphylococcus aureus* (18.68%), *Corynebacterium* species (15.38 %), *Streptococcus spp.* (5.49 %), and *Klebsiella* species (1.11 %). In the present study, CNS were found to be the most prevalent mastitis-causing agent, which agrees with previous studies reporting high prevalence of CNS in India (Javed and Siddique, 1999; Kaya *et al.*, 2000; Sharma *et al.*, 2007, Pankaj *et al.*, 2013) and abroad (Hawari and Dabas, 2008; Byarugaba *et al.*, 2008, Tenhagen *et al.*, 2009, Nam *et al.*, 2010, Abrahmsén *et al.*, 2012). In many modern dairy herds, opportunistic bacteria such as CNS are frequently associated with bovine mastitis, and hence, CNS could be described as emerging pathogens (Pyarala and Taponen, 2009). Coagulase-negative staphylococci possesses traits of both environmental and contagious pathogens. Although, they are not normal inhabitants of the mammary gland, they commonly colonize the skin of the teat, the teat end, teat injuries, and the hands of milkers. Therefore, they can spread mechanically and present major risks for cows with teat-end injuries (Piessens *et al.*, 2011).

The increasing role of CNS as a causative agent of subclinical mastitis and their antimicrobial resistance is progressively becoming a matter of concern for the dairy industry (Botrel *et al.*, 2010). In the present study, a total of 30 antibiotics belonging to 11 groups were used to study the drug resistance pattern. Sensitivity of the CNS isolates to antibiotic agents (along with their concentration) is presented in Table 1. In the present study, CNS revealed highest susceptibility to chloramphenicol (97.9%), ciprofloxacin (95.8%), gentamicin (93.8%), lenezolid (91.7%), oxacillin (91.7%) and ceftiozime (89.6%), while resistance was shown against penicillin (58.3%), amoxicillin (56.3%), ampicillin (54.2%), and cefaperazone (33.3%).

Among β -Lactams, cloxacillin and potentiated penicillins were highly sensitive while resistance was shown against penicillin, ampicillin and amoxicillin. The rate of penicillin resistance (58.3%) observed in this study is much higher than reported by other

Table 1: Antibiotic sensitivity test of CNS isolated from buffalo milk samples against different antibiotics

Group	Antibiotic	Intr.	N	%	Group	Antibiotic	Intr.	N	%		
β-Lactams	Penicillin-G	S	20	41.7	Aminoglycosides	Gentamicin	S	45	93.8		
		I	NA	NA			I	3	6.2		
		R	28	58.3			R	0	0.0		
	Amoxycillin+ Clavulanate	S	31	64.6		Neomycin	S	40	83.3		
		I	NA	NA			I	4	8.3		
		R	17	35.4			R	4	8.3		
	Ampicillin	S	22	45.8		Streptomycin	S	41	85.4		
		I	NA	NA			I	NA	NA		
		R	26	54.2			R	7	14.6		
	Amoxycillin+ Sulbactam	S	38	79.2		Amikacin	S	46	95.8		
		I	3	6.3			I	1	2.1		
		R	7	14.6			R	1	2.1		
	Amoxycillin	S	21	43.8		Floroquinolones	Enrofloxacin	S	41	85.4	
		I	NA	NA				I	4	8.3	
		R	27	56.3				R	3	6.3	
	Cloxacillin	S	40	83.3			Ciprofloxacin	S	46	95.8	
		I	3	6.3				I	1	2.1	
		R	5	10.4				R	1	2.1	
	Oxacillin	S	44	91.7			Moxifloxacin	S	41	85.4	
		I	0	0.0				I	4	8.3	
		R	4	8.3				R	3	6.3	
	Ceftriaxone + sulbactam	S	35	72.9			Sulphona-mide	Cotrimazole	S	41	85.4
		I	8	16.7					I	0	0.0
		R	5	10.4					R	7	14.6
	Ceftriaxone + tazobactam	S	37	77.1			Macrolide	Erythromycin	S	27	56.3
		I	7	14.6					I	18	37.5
		R	4	8.3					R	3	6.3
	Ceftriaxone	S	32	66.7			Tetra-cycline	Tetracycline	S	34	70.8
		I	10	20.8					I	4	8.3
		R	6	12.5					R	10	20.8
Ceftazidime	S	37	77.1	Lincosa-mide	Clindamycin		S	35	72.9		
	I	9	18.8				I	10	20.8		
	R	2	4.2				R	3	6.3		
Cefaperazone	S	32	66.7	Oxazolidinone	Lenezolid		S	44	91.7		
	I	NA	NA				I	NA	NA		
	R	16	33.3				R	4	8.3		
Ceftixozime	S	43	89.6	Phenicols	Chloramphenicol		S	47	97.9		
	I	2	4.2				I	1	2.1		
	R	3	6.3				R	0	0.0		
Ceforoxime	S	43	89.6	Aminocoumarine	Novobiocin		S	42	87.5		
	I	3	6.3				I	NA	NA		
	R	2	4.2				R	6	12.5		
Glyco-peptide	Teicoplanin	S	43	89.6	Miscellaneous	Bacitracin	S	41	85.4		
		I	3	6.3			I	2	4.2		
		R	2	4.2			R	5	10.4		

S: Sensitive, I: Intermediate, R: Resistant, Intr.: Interpretation, NA: Not Applicable, A: Ampicillin (10 mcg), AM: Amoxycillin (10 mcg), AS: Amoxycillin+sulbactam (30/15 mcg), AC: Amoxycillin+clavulanate (30 mcg), P: Penicillin (2 units), OX: Oxacillin (1 mcg) COX: Cloxacillin (10 mcg), CI: Ceftriaxone (10 mcg), CIS: Ceftriaxone+sulbactam (30/15 mcg), CIT: Ceftriaxone+tazobactam (30/10 mcg), CAZ: Ceftazidime (30 mcg), CPZ: Cefaperazone (75 mcg), CXM: Ceforoxime(30 mcg), CZX: Ceftixozime (30 mcg), EX: Enrofloxacin (10 mcg), CIP: Ciproflaxacin (5 mcg), MO: Moxifloxacin (5 mcg), G: Gentamicin (10 mcg), AK: Amikacin (30 mcg), S: Streptomycin (25 mcg), N: Neomycin (30 mcg), COT: Cotrimazole (25 mcg), E: Erythromycin (10 mcg), T: tetracycline (10 mcg), CD: Clindamycin (2 mcg), Lz: Lenezolid (30 mcg), C: Chloramphenicol (30 mcg), Tei: Teicoplabnin (30 mcg), B: Bacitracin (10 mcg), NV: Novobiocin (30 mcg)

workers (Rajala-Schultz *et al.*, 2009 Pitkala *et al.*, 2004). Resistance in staphylococci has been associated with the production of β -lactamases, which has been reported in 19–84 per cent of CNS isolated from dairy cows with mastitis (Myllys *et al.*, 1998; Gentilini *et al.*, 2002; Taponen *et al.*, 2006).

High sensitivity of CNS to aminoglycosides and fluoroquinolones observed in this study is in agreement with results of earlier workers (Dhakal *et al.*, 2007, Sumathi *et al.*, 2008 Anakalo *et al.*, 2009 and Alekish *et al.*, 2013). Moderate resistance observed in tetracyclines (20.8%) during the present study, is comparable with results of Pitkala *et al.* (2004) and Maran (2004), who observed a resistance of 9 per cent and 16 per cent, respectively. In contrast, Alekish *et al.* (2013) and Mahami *et al.* (2011) observed 100 per cent resistance to tetracycline.

In the present study, resistance was observed only in few routinely used antibiotics such as penicillin (58.3%), amoxicillin (56.3%), ampicillin (54.2%), and cefaperazone (33.3%), while as moderate to high sensitivity was shown by newer and older antibiotics, showing rational use of these antibiotics at farms under study. Antibiotic resistance patterns change from different geographical regions and also from farm to farm depending upon the prevalence of different organisms and usage of antibiotics; therefore antibiotic sensitivity testing is suggested before the institution of antimicrobial therapy. This approach enhances the efficacy of treatment and reduces the chances of emergence of antimicrobial resistance by bacterial pathogens. Also antibiotic sensitivity of CNS isolates will be helpful to recommend early therapy at the field level prior to availability of CST results and in prioritizing mastitis control efforts.

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