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Antibiogram of CoPS and CoNS Isolated from Bovine Mastitis in Banaskantha District of Gujarat, India

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A B S T R A C T

Bovine mastitis poses great economic threat to dairy industry affecting livestock farmers across the globe. Various species of staphylococci bacteria are one of the major pathogen responsible for the bovine mastitis. The present investigation was carried out to assess *in vitro* antibiogram of field isolates of coagulase positive and coagulase negative staphylococci (CoPS and CoNS, respectively), isolated from 95 bovine mastitis cases in Banaskantha district of Gujarat state. Milk samples were screened for the presence of *Staphylococcus* species by cultural, colonial, microscopic and biochemical characteristics and finally, 29 samples (30.53 %) were confirmed for the same. Coagulase test was performed and 12 CoPS (41.38%) and 17 CoNS (58.62%) strains were found. All 12 CoPS were *S. aureus* as confirmed by PCR. Antibiogram trend against nine commonly used antimicrobial drugs for treatment of bovine mastitis in the region showed that both CoPS and CoNS groups were highly resistant to penicillin-G (83.33 and 88.24 %, respectively) and tetracycline (83.33 and 76.47%, respectively).

Introduction

Bovine mastitis is one of the major ill-health of cattle and buffaloes, responsible for great economic loss to dairy industry affecting livestock farmers worldwide. The major economic consequences of clinical bovine mastitis are due to treatment, loss in milk production, culling, changes in product quality and the risk of other diseases (Halasa *et al.,* 2007). For etiological diagnosis of bovine mastitis, staphylococci are divided into coagulase-positive staphylococci (CoPS) and coagulase-negative staphylococci (CoNS) based on the ability to coagulate rabbit plasma. CoPS are almost solely represented by *Staphylococcus aureus*, an important cause of udder infections in dairy herds (Barkema *et al.*, 2006). Although, pathogenic *S. aureus* are coagulase positive, but some strains of *S. aureus* are also reported to be coagulase negative which causes mastitis in dairy

animals (Fox *et al.,* 1996). CoNS group of bacteria which comprises of more than 40 species (important are *S. chromogenes, S. epidermidis, S. intermedius, S. warneri, S. haemolyticus, S. sciuri* and *S. xylosus*), are often responsible for causing sub-clinical mastitis but many reports now reveals that CoNS are also causing clinical mastitis, mostly with mild form. CoNS are normal inhabitant of udder skin, teat canal and milkers' hand; thus, they are regarded as opportunistic, nosocomial and emerging pathogen (Pyorala and Taponen, 2009; Taponen and Pyorala, 2009; El-Jakee *et al.,* 2013). Effectiveness of antibiotic therapy to treat bovine mastitis has been demonstrated to be compromised due to induction of antibiotic resistance and formation of biofilms which can be produced by both CoPS and CoNS (Babra *et al.,* 2013; Darwish and Asfour, 2013: Tiwari *et al.,* 2013; Al-Rubaye *et al.,* 2016). Among many reasons behind failure of successful treatment of bovine mastitis, one is the failure of appraisal of the antimicrobial resistant pattern of pathogens involved (Du Preez, 2000). Thus, appraising the emerging antimicrobial resistant strains of pathogens is an important facet in effective mastitis therapy. Hence, the present investigation was carried out to isolate, identify and assess *in vitro* antibiogram of field isolates of CoPS and CoNS derived from clinical bovine mastitis cases of Banaskantha district of Gujarat, India. For generating antibiogram trend, nine commonly used antimicrobials were selected decisively based on their usage pattern for the treatment of bovine mastitis in the same region.

Materials and Methods

Study area and sample details

The present study was carried in Banaskantha district of North Gujarat State, home to Banas Dairy which ranks first in Asia in terms of milk procurement. Milk samples of 95 bovine mastitis cases were collected aseptically from all quarters of infected animals presented at Government Veterinary Dispensaries, Animal health division of Banas Dairy Co-operative Ltd., and Teaching Veterinary Clinical Complex, Deesa, Veterinary College, SDAU, Sardarkrushinagar, from October 2015 to March 2017.

Cultural and biochemical characteristics

The bacteria were isolated in BHI agar by spreading heavy inoculums of thoroughly mixed milk after incubated at 37°C for 24 hours. Bacteria on BHI medium were identified tentatively according to conventional methods including Gram stain staining, colony morphology and catalase test as per standard methods described by Buchanan and Gibson (1974) and Barrow and Feltham (1993). Thereafter, colonies showing Gram positive, cocci in bunches indicative of presumptive *Staphylococcus spp*. were transferred to its selective medium Mannitol Salt Agar (MSA).

The growth on MSA was assumed to be of Staphylococcal colonies. Finally, differentiation of coagulase positive and coagulase negative staphylococci was done by tube coagulase method. The colonies forming yellow colouration on MSA indicative of mannitol fermentation and coagulase positive isolates were assumed to be *S. aureus* and subjected to PCR confirmation.

PCR confirmation of *Staphylococcus spp.* and *Staphylococcus aureus*

The DNA extractions of isolated *Staphylococcus spp.* were done using the protocol of QIAGEN DNeasy blood and tissue kit. A loop full of culture grown overnight in 4 ml BHI broth at 37°C was used to isolate the DNA. Molecular confirmation of *Staphylococcal spp.* was done by genus specific 16S-rDNA gene amplification

(Lovseth *et al.,* 2004) while specific genotypic confirmation of *Staphylococcus aureus* were carried out using species specific sa442 gene amplification (Martineau *et al.,* 1998; Bhagat *et al.,* 2017). Detail of primers used for PCR reaction is presented in Table 1.

The PCR cycling condition for 16S-rDNA gene was subjected to 30 cycles consisting at 94°C for 5 min followed by denaturation 94°C for 30 sec followed by annealing 60°C for 30 sec followed by extension at 72°C for 1 min and final extension at 72°C for 5 min. while cycling condition for *Sa442* gene was same as 16S-rDNA gene with slight change in annealing temperature at 55°C for 30 sec and final extension at 72°C for 7 min. The PCR product was visualized on a 1.5% agarose gel using ethidium bromide and a UV transilluminator (Bhagat *et al.,* 2017).

Method for antibiogram of *Staphylococcus* isolates

All the isolates obtained were subjected to *in vitro* antibiotic sensitivity test, as per the method described by Bauer *et al.,* (1966) with different antibiotic discs *i.e.* Amoxycillin-Clavulanate (30 mcg), Ampicillin-Sulbactam (10/10 mcg), Ciprofloxacin (30 mcg), Enrofloxacin (10 mcg), Gentamicin (10 mcg) and Penicillin G (10 Units).

The sensitivity patterns of isolates to different antibiotic disc was read by measuring the diameter of zone of inhibition as per chart provided by manufactures and classified as sensitive, intermediate and resistant.

Quantitative determination of susceptibility of *Staphylococcus* spp. against remaining three antimicrobials *i.e.* ceftizoxime, ceftriaxone and tetracycline were performed by e-test using Ezy MIC^{TM} strips (HiMedia Lab. Pvt. Ltd., Mumbai) as per guidelines of manufacturers (CSLI, 2012).

Results and Discussion

Out of total 95 bovine mastitis milk samples collected, 52 samples were from cattle and 43 were from buffaloes. Out of 52 cow mastitis cases, 19 were of mild to severe clinical cases whereas 33 cases were of sub-clinical nature; whereas among 43 buffalo cases, 32 were clinical and 11 were of sub-clinical mastitis.

After screening of all milk samples with cultural and biochemical examination, 29 samples (30.53 %) were found positive for *Staphylococcus* species. Then coagulase test was performed on all 29 samples, out of which 12 samples were found positive (CoPS; 41.38%) whereas 17 samples were found negative (CoNS; 58.62%). DNA samples extracted from bacterial growth were also subjected to PCR and were confirmed as *Staphylococcus* species. Then, all 12 samples showing coagulase positive reaction were subjected to PCR for species confirmation using *Sa442* gene amplification, and all 12 CoPS isolates were confirmed as *Staphylococcus aureus*. In present study, 30.53% of bovine mastitis cases were found to be associated with *Staphylococcus species* whereas higher prevalence (45%) for same pathogen was reported in a meta-analysis of major mastitis pathogens in dairy cattle in India from year 2005 to 2016 (Krishnamoorthy *et al.,* 2017).

Antibiogram trend against nine commonly used antimicrobial drugs for treatment of bovine mastitis in the Banaskantha district is presented in Table 2. Antibiogram study showed that, among the 29 isolates, three isolates were susceptible and five were resistant to all the antimicrobials used in this study.

Aminopenicillins in combination with beta-lactamase inhibitors *i.e.* amoxycillin-clavulanate and ampicillin-sulbactam were the most effective antimicrobial agents among

the tested drugs *in vitro* against CoPS group. The use of beta-lactamase inhibitors could be the possible reasons for more effectiveness of these combinations by bacterial escaping of drug resistance to some extent.

Table.1 Detail of primers for amplification of 16S-rDNA and *sa442* genes employed in Polymerase Chain Reaction

Gene designated	Primer sequence (5' - 3')		Size of amplified products (bp)
16S - rDNA	Forward	GTAGGTGGCAAGCGTTATCC	228
	Reverse	CGCACATCAGCGTCAG	
Sa442	Forward	AATCTTTGTCGGTACACGATATTCTTCACG	108
	Reverse	CGTAATGAGATTTCAGTAGATAATACAACA	

Table.2 Antibiogram trend of coagulase positive and negative *Staphylococcus* isolates against nine antimicrobial agents

Sr. No	Antimicrobial agent	No. of isolates in CoPS group (n=12)			No. of isolates in CoNS group (n=17)		
		Sensitive	Intermediate	Resistant	Sensitive	Intermediate	Resistant
1	Amoxy-clav*	8 (66.67 %)	1 (8.33 %)	3 (25.00 %)	11 (64.71 %)	1 (5.88 %)	5 (29.41 %)
2	Ampi-sulbactam**	8 (66.67 %)	0	4 (33.33 %)	9 (52.94 %)	1 (5.88 %)	7 (41.18 %)
3	Ceftizoxime	6 (50.00 %)	2 (16.67 %)	4 (33.33 %)	11 (64.71 %)	2 (11.76 %)	4 (23.53%)
4	Ceftriaxone	6 (50.00 %)	1 (8.33 %)	5 (41.67 %)	8 (47.06 %)	0	9 (52.94 %)
5	Ciprofloxacin	4 (33.33 %)	1 (8.33 %)	7 (58.33 %)	9 (52.94 %)	1 (5.88 %)	7 (41.18 %)
6	Enrofloxacin	6 (50.00 %)	0	6 (50.00 %)	10 (58.82 %)	1 (5.88 %)	6 (35.29 %)
7	Gentamicin	7 (58.33 %)	0	5 (41.67 %)	11 (64.71 %)	0	6 (35.29 %)
8	Penicillin-G	2 (16.67 %)	0	10 (83.33 %)	2 (11.76 %)	0	15 (88.24 %)
9	Tetracycline	1 (8.33 %)	1 (8.33 %)	10 (83.33 %)	4 (23.53 %)	0	13 (76.47 %)

*Amoxycillin-clavulanate; **Ampicillin-sulbactam

Combination of amoxycillin-clavulanate was also most effective drug against CoNS group of mastitis pathogens along with ceftizoxime and gentamicin. Likewise, based on MIC studies, Moroni *et al.,* (2006) also concluded that only certain β-lactamase–resistant penicillins (specifically cloxacillin) or penicillin combinations (amoxycillin plus clavulanate) were consistently effective against *S. aureus* isolated from subclinical bovine mastitis in Italy. In addition, the MIC_{90} for amoxycillin plus clavulanate against the *S. aureus* strains isolated from bovine mastitis was substantially lower than the MIC_{90} observed for ampicillin alone (De Oliveira *et al.,* 2000). Pharmacoepidemiological study on pattern of clinical usage of veterinary antimicrobials in Northern Gujarat (India) revealed that practitioners prefer beta-lactamase inhibitors (salbactam) or β-lactamase resistant penicillins (cloxacillin) in combination of penicillins or cephalosporins to treat cases of mastitis (Patel *et al.,* 2014). Kumar *et al.,* (2011) reported higher susceptible to amoxicillin-clavulanate (81.3%) of *S. aureus* isolates obtained from mastitic Sahiwal cattle. In present study, it was observed that both groups (CoPS and CoNS) were most resistant to penicillin-G (83.33 and 88.24 %, respectively) followed by tetracycline (83.33 and 76.47%, respectively). Ranjan *et al.,* (2010) also found penicillin-G with streptomycin as most resisted drugs based on antibiogram study of various isolates from

clinical bovine mastitis from Ranchi region (Jharkhand, India). Similarly, Coagulase-negative staphylococci isolates from bovine mastitis in Argentina also exhibited the highest degree of resistance to penicillin of all antimicrobial agents tested (Gentilini *et al.*, 2002). In present study, fluoroquinolones (ciprofloxacin and enrofloxacin) were demonstrated to be more effective in terms of susceptibility against CoNS group (52.94 and 58.82%, respectively) than CoPS group (33.33 and 50.00%, respectively). However, Jahan *et al.*, (2015) from Bangladesh reported higher susceptibility (83.33%) of raw cow milk *S. aureus* isolates to ciprofloxacin.

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