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Antibacterial Sensitivity and Resistance Pattern of Yak Intramammary Infection from Arunachal Pradesh, India

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ABSTRACT

Mastitis is an economically important bacterial disease of the dairy animals. During the study period (i.e., June to October, 2013) 200 animals, including lactating yak, mithun and local cattle were screened for Intramammary Infection (IMI) caused by major pathogens in organized and unorganized farms in North Eastern Hill (NEH) region, Dirang, Arunachal Pradesh. Out of 200 animals screened for the status of IMI, 19 yaks were found positive for mastitis. The major pathogens isolated and characterized from positive milk samples categorized in four types of bacteria and amongst them majority were coagulase negative *Staphylococcus aureus* (47.36%) which was the most prevalent pathogen in the region, followed by *E. coli* (26.3%), coagulase positive *Staphylococcus aureus* (15.78%) and *Streptococcus agalactiae* (10.5%). The antibacterial sensitivity pattern revealed maximum sensitivity for ceftriaxone (94.74%) and minimum for ampicillin (15.79%). The resistance pattern showed coagulase negative *Staphylococcus aureus* (CNS) was most resistant bacteria against ampicillin and amoxicillin, where the antibacterial zone depicted to be 7 and 9 mm, respectively. The present study reported, the general sensitivity and resistance pattern of the antibiotic against the major pathogens isolated from yak mastitis milk samples from NEH region of Arunachal Pradesh.

Key words: Yak, mastitis, antibacterial sensitivity, resistance pattern, Arunachal Pradesh

INTRODUCTION

Mastitis is a complex multifactorial disease of dairy animals caused by wide range of pathogens which is often associated with intensive dairy farming (Zadoks *et al*., 2011; Bradley, 2002). Mastitis is a serious concern to Indian economy as reported by different authors (Singh and Singh, 1994; NAAS, 2013) and it also cause diseases in man like staphylococcal toxemia, sore throat, scarlet fever (Kadariya *et al*., 2014) and gastroenteritis in young calves (Radostits *et al*., 2007). Mastitis is generally associated with high producing dairy animals although intramammary infection in yak from yak rearing countries has been reported (Wiener *et al*., 2003; Joshi *et al*., 1997). Though the yak is not a true dairy type animal like cattle and buffalo but it provides milk and milk products to the resident of the difficult and high mountainous region. Milk and milk products of yak are

highly nutritive and source of livelihood for the natives, but sometimes it is a possible source of shiga-toxin producing coli bacilli to the tribal highlanders and nomadic yak herdsmen as they prefer consuming raw milk many times (Bandyopadhyay *et al*., 2012). The occurrence of sub clinical mastitis is generally higher compared to clinical mastitis, which makes it difficult to identify the stale milk; as such there are no physical changes in the milk when the animal is infected sub-clinically. Consuming such milk leads to serious health hazards to highlanders. Further, very limited work has been carried out about the organisms causing bovine mastitis in and around North Eastern Hill (NEH) region of Dirang, Arunachal Pradesh, India. Therefore, the present investigations were carried out to isolate and characterize major mastitis causing pathogens from the cases of bovine mastitis with special reference to yak and antibacterial sensitivity of major antibiotic groups and resistance to antibiotic caused by the pathogens.

MATERIALS AND METHODS

Screening of lactating bovines and milk collection: Milk samples were collected from free ranging yaks and also from organized dairy farm in and around Dirang valley of West Kameng district (91°30'-92°40' East longitudes and 26°54'-28°01' North latitudes) of Arunachal Pradesh, during June to October, 2013. A total of 200 animals, including lactating yak, local cattle and mithun were screened for mastitis by California Mastitis Test (CMT), (Schalm and Noorlander, 1957). In brief, the milk from each quarter were directly stripped onto the CMT paddle, to it equal amount of CMT reagent was poured, swirled and immediately viewed for any changes in the milk. The reaction is graded by intensity of gel formation and color changes and scored as trace to plus ++ for sub clinical mastitis and plus +++, ++++ for clinical mastitis. Milk samples depicted, positive CMT point scores were collected aseptically (30 mL). The samples were transported to the laboratory on ice in isothermal boxes.

Isolation and identification of bacterial pathogens: Milk samples were collected in sterile vials after cleaning the teat orifice with 70% ethyl alcohol and after discarding few streams of milk. With the help of a sterile tip 10 μL of milk is taken on 5% sheep blood agar plate and spread it evenly with a sterile 'L' shape glass rod. The inoculated plates were incubated at 37°C for 24 h. The causative organism of the milk samples were identified initially on the basis of colony morphology and smell on 5% blood agar as per Cruickshank (1962). Further the colonies from blood agar plates were transferred to selective media. The organisms were identified on the basis of colony morphology, characteristic hemolytic pattern and gram's staining as per the method described elsewhere (Watts, 1988).

Isolation of *Staphylococcus aureus*: Large, round colonies with zone of hemolysis on 5% sheep blood agar were subcultured on Mannitol Salt Agar (HiMedia, Mumbai, India) and Baird Parker Media with egg yolk and Sodium telluride supplementation to confirm the presence of pathogenic *Staphylococcus aureus* (*S. aureus*) and coagulase positive *S. aureus*, respectively.

Isolation of *Streptococcus agalactiae* on selective media and hotis reagent: Small creamy white colonies with zone of hemolysis of 5% sheep blood agar were subcultured in Edwards medium (HiMedia, Mumbai, India) with 5% sheep blood supplementation. In brief, 9.5 mL of the fresh milk is taken in to a sterile test tube and shaken thoroughly then 0.5 mL Hotis reagent is poured and mixed in a vortex mixture. The tube is incubated at 37°C for 24 h.

Isolation of *E. coli*: Suspected colonies were subcultured into MacConkey and Eosin-Methylene blue agar plates for further identification of gram negative pathogens.

Biochemical characterization of the isolates: Isolates were further subjected to biochemical test using HiStaph™ Identification Kit, Hi *E. coli*™ Identification Kit and HiStrep™ Identification Kit (HiMedia, Mumbai) for identification of *S. aureus*, *Streptococcus agalactiae* and *E. coli*, respectively as per method described by Balows *et al.* (1991). Further screening for the presence of coagulase activity in *S. aureus* by Tube coagulation test was done according to the procedure recommended by Quinn *et al.* (1994).

Antibacterial sensitivity test (ABST): The pathogenic organisms which were grown on 5% blood agar were taken (3-4 colonies) and it was dipped in sterile normal saline solution. The organism was thoroughly mixed in the solution, thereafter the turbidity of the solution was matched with the Mcferlen tubes ($1\text{-}4\times10^7$ CFU mL^{-1}). A sterile swab was dipped in this solution and was smeared over the Mueller-Hinton Agar. The standard antimicrobial discs i.e., Ceftriaxone (CTR), Cefotaxime (CTX), Co-Trimoxazole (COT), Erythromycin (E), Enrofloxacin (ENR), Ampicillin (AMP), Chloramphenicol (C), Amikacin (AK), Amoxicillin (AMX) and Tetracycline (TE) discs were inoculated over the plates. The plates were placed at 4°C for 2 h and thereafter the plates were incubated at 37°C for 24 h. The diameter of the zone of inhibition was measured in millimeters. All the tests were performed in duplicate. The resistance pattern of isolates was determined using an agar disk diffusion method according to standards of Clinical and Laboratory Standards Institute (CLSI, 2013).

Statistial analysis: Results were analysed using one way analysis of variance (ANOVA).

RESULTS

A total of 200 animals (yak, local cattle and mithun) were screened for mastitis by CMT. Out of 200 animals screened for the status of intramammary infection, 16 yaks were found positive for Sub Clinical Mastitis (SCM) and 3 yaks were positive for clinical mastitis. The CMT point scores was 0 in normal healthy animals, where it ranged between trace to 2 + + point score in sub clinical mastitis and +++ to ++++ in clinical mastitis.

Milk samples were grown primarily on blood agar plates followed by on selective media. Coagulase positive *S. aureus* (CPS) was 15.78% (3/19) as identified on the basis of characteristic color changes on Mannitol salt agar plate (yellow discoloration), black colonies in Baird Parker Media and viscous clotting of rabbit plasma by tube coagulase test. Nine isolates did not fermented MSA and no black colonies on Baird Parker Media and no clotting of rabbit plasma, identified to be coagulase negative *S. aureus* (CNS) (47.36%). Culture grown on Hotis reagent revealed 2 isolates of other *Streptococcus agalactiae* (10.5%) on the basis of small yellow balls appearing on the wall of the test tube or the milk changes its color from purple to blue gray to yellowish shades. Cultures grown on EMB and MacConkey agar revealed 5 isolates (26.3%) of Gram negative coliform bacilli on the basis of characteristic growth pattern and color changes. The colonies grown as purple green with metallic sheen on EMB agar and pink colonies on MacConkey agar were presumptively identified as *E. coli*. *Escherichia coli* were further confirmed by Hi *E. coli*™ Identification Kit.

Table 1: Resistance patterns of *Staphylococcus aureus, Streptococcus agalactiae, E. coli* isolates isolated from yak mastitic milk

	Antimicrobials										Isolate wise S-I-R patterns (%)		
Isolates	AMX	AK	AMP	CTX	CTR	C	COT	ENR	E	TE	S	I	R
CPS-1	R	S	R	I	S	S	S	S	S	S	70.00	10.0	20.0
CPS-2	R	S	R	R	S	S	S	R	S	S	60.00	0.0	40.0
CPS-3	S	S	R	S	S	S	S	I	S	S	80.00	10.0	10.0
CNS-1	S	R	R	S	S	S	S	R	S	S	70.00	0.0	30.0
CNS-2	R	I	R	S	S	S	I	S	S	S	60.00	20.0	20.0
CNS-3	S	S	R	S	S	S	R	S	R	S	70.00	0.0	30.0
CNS-4	S	S	R	I	S	S	R	R	R	S	50.00	10.0	40.0
CNS-5	S	R	S	S	S	S	S	R	I	S	70.00	10.0	20.0
CNS-6	R	R	R	S	S	S	S	S	S	S	70.00	0.0	30.0
CNS-7	R	I	R	S	S	S	S	S	S	I	60.00	20.0	20.0
CNS-8	S	S	R	I	I	S	S	R	S	S	60.00	20.0	20.0
CNS-9	R	S	R	I	S	S	S	S	S	I	60.00	20.0	20.0
EC-1	S	S	R	I	S	S	S	S	S	S	80.00	10.0	10.0
EC-2	S	S	R	S	S	S	S	S	S	S	90.00	0.0	10.0
EC-3	S	S	S	S	S	S	S	S	S	S	100.00	0.0	0.0
EC-4	S	S	S	S	S	I	R	S	S	S	80.00	10.0	10.0
EC-5	R	S	I	S	S	S	S	S	S	I	70.00	20.0	10.0
STA-1	R	NP	R	S	S	S	I	S	S	S	66.67	11.1	22.2
STA-2	R	NP	R	S	S	S	S	S	S	S	77.78	0.0	22.2

CPS: Coagulase positive *Staphylococcus aureus*, CNS: Coagulase negative *Staphylococcus aureus*, EC: *Escherichia coli*, STA: *Streptococcus agalactiae*, CTR: Ceftriaxone, CTX: Cefotaxime, COT: Co-trimoxazole, E: Erythromycin, ENR: Enrofloxacin, AMP: Ampicillin, C: Chloramphenicol, AK: Amikacin, AMX: Amoxicillin, TE: Tetracycline, R: Resistant, I: Intermediate, S: Susceptible, NP: Not preformed

In the present study, ten different types of antimicrobials were selected to know the resistance and sensitive pattern among isolates. The antibacterial resistance and sensitive outline of these isolates were depicted in Table 1.

Three isolates of CPS1 to CPS3 were sensitive (100%) to CTR and C, resistance to AMX (66%). Maximum number of CNS (47.36%) were isolated from the yak milk samples, 9 isolates were sensitive to C (100%) followed by CTR (88%) and resistant to AMP (88%). Five isolates of *E. coli* were sensitive to CTR and AK (100%) and resistant to AMP (60%). While two isolates of *Streptococcus* sp. were sensitive to CTR and CTX (100%) and resistant to AMX and AMP (100%). All the isolates were sensitive to CTR and resistant to AMP followed by AMX (Table 1).

The resistance patterns among the CPS revealed that CPS-2 was resistant to ampicillin, amoxicillin, cefotaxime and enrofloxacin. It was observed that CPS-1 was resistant to both ampicillin and amoxicillin, whereas CPS-1 only to ampicillin. All the CPS were sensitive (100%) to AK, CTR, C, COT, E and TE. Resistance of CPS-1, CPS-2 and CPS-3 to different drugs was 20, 40 and 10%, respectively (Table 1). Among the CNS, the resistance profile was more and less similar to CPS but CNS-4 was least sensitive (50%) to all the antimicrobials. Interestingly, CNS-1, CNS-3 and CNS-6 were shown the similar resistance (30%) and sensitive (70%) pattern to all the drugs whereas CNS-2, CNS-7, CNS-8 and CNS-9 were also found the same picture (R = 20% and S = 60%). The EC-3 was the only isolate which had shown 100% sensitivity to all the antimicrobials followed by EC-2, EC-1, EC-4 and EC-5. The resistance pattern among the two isolates of *Streptococcus agalactiae* was identical (22.2%) but sensitive prototype was different.

Table 2: Drugwise resistance pattern of the isolates

Drug wise pattern (%)	AMX	AK	AMP	CTX	CTR	C	COT	ENR	E	TE
S	52.63	70.59	15.79	68.42	94.74	94.74	73.68	68.42	84.21	84.21
I	0.00	11.76	5.26	26.32	5.26	5.26	10.52	5.26	5.26	15.78
R	47.36	17.64	78.94	5.26	0.00	0.00	15.78	26.31	10.52	0.00

CTR: Ceftriaxone, CTX: Cefotaxime, COT: Co-trimoxazole, E: Erythromycin, ENR: Enrofloxacin, AMP: Ampicillin, C: Chloramphenicol, AK: Amikacin, AMX: Amoxicillin, TE: Tetracycline

Drug wise resistance pattern revealed that ceftriaxone and chloramphenicol was most effective (94.74%) antimicrobials to all the isolated strains followed by tetracycline (84.21%), erythromycin (84.21%) and cefotaxime (68.42%), respectively (Table 2). Most of the isolates were resistant to ampicillin (78.94%) followed by amoxicillin (47.36%) and enrofloxacin (26.31%), respectively.

DISCUSSION

Out of 200 animals screened from Dirang valley for the status of udder health status, 19 yaks were found positive for IMI. The overall prevalence of clinical mastitis was 1.5% and subclinical mastitis was 8%. Dubal *et al.* (2010) reported 0.85% as clinical and 2.28% for sub clinical mastitis from NEH Sikkim region from yak milk samples, the prevalence reported was lower as compared to our study. From the yak milk samples pathogens like CPS, CNS, *Streptococcus* and *Colibacilli* were isolated, similarly Dubal opcit isolated the major pathogens from yak milk samples. In this study, CNS was the principal organism causing clinical and subclinical intramammary infection (IMI); the results are in accordance with the finding of Pantoja *et al.* (2009) and Cook *et al.* (2005) from Wisconsin dairy farms. The prevalence of *Streptococcus agalactiae* was 10.5% and this was the lowest amongst the pathogens which we could isolate from the yak milk samples in respect to other causative organism, similar results were observed by other workers in ewes and cattle (Albenzio *et al.*, 2002; Ferguson *et al.*, 2007).

Antibiotics used for the treatment of bovine mastitis and it the only proven method of therapy. We studied the sensitivity and resistance pattern against the isolates from yak milk; in the present study most of the isolates were sensitive to CTR and resistant to AMP and AMX. Similar findings were also reported by Moges *et al.* (2011) in cattle from Ethiopian dairy farm. The susceptibility and resistance pattern of the bacterial isolates in our study was comparable to previous reports (National Mastitis Council, 1996). However, in other study, ciprofloxacin and enrofloxacin were found most effective drugs against major bacterial pathogens in Indian dairy farms (Awandkar *et al.*, 2009; Muhammad *et al.*, 1995). Moreover Bandyopadhyay *et al.* (2012) isolated *E. coli* from raw milk and chhurpi, a yak milk product and observed that the isolates were resistant against all types of commonly used antimicrobial agents in Dirang valley. In the present study maximum resistance profile was depicted by the penicillin group of antibiotic which could be due to frequent and indiscriminate use of the antibiotics for the treatment of diseases against the infectious conditions in those localities (Calderon-Jaimes *et al.*, 2002). Sumathi *et al.* (2008) opined that the resistance against the penicillin group is due to plasmid mediated beta-lactamase enzyme. Resistance mechanism in CNS bacteria was also studied by Lindsay (2014), where the authors explained that bacterial resistance had acquired by horizontal gene transfer. On the contrary, Pantosti *et al.* (2007) detailed that the resistance could be due to spontaneous mutations and positive selection.

CONCLUSION

CPS, CNS, Streptococcus and Colibacilli were isolated from yak milk samples in Dirang valley of Arunachal Pradesh. The CNS was the most frequently isolated organism causing IMI in yak. Most of the isolates were sensitive to CTR and resistant to AMP followed by AMX. Though, the study was done in small geographic location of the NEH region of Arunachal Pradesh. The results revealed presence of microorganism and antibacterial sensitivity pattern in this zone and some significant point about the present yak husbandry and udder hygiene status to this part and other yak tracks of this country. Further such types of research may be taken up in other part of the yak rearing states to know the insight of the disease pattern.

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REFERENCES

Albenzio, M., L. Taibi, A. Muscio and A. Sevi, 2002. Prevalence and etiology of subclinical mastitis in intensively managed flocks and related changes in the yield and quality of ewe milk. Small Ruminant Res., 43: 219-226.

Awandkar, S.P., N.V. Khode, V.M. Sardar and M.S. Mendhe, 2009. Prevalence and current antibiogram trend of mastitic agents in Udgir and its vicinity, Maharashtra State, India. Int. J. Dairy Sci., 4: 117-122.

Balows, A., H. Truper, M. Dvorkin, W. Harder and K. Schleifer, 1991. The Prokaryotes. 2nd Edn., Vol. 2, Springer-Verlag, New York, pp: 1468, 1485, 1564.

Bandyopadhyay, S., C. Lodh, H. Rahaman, D. Bhattacharya and A.K. Bera *et al*., 2012. Characterization of shiga toxin producing (STEC) and enteropathogenic *Escherichia coli* (EPEC) in raw yak (*Poephagus grunniens*) milk and milk products. Res. Vet. Sci., 93: 604-610.

Bradley, A.J., 2002. Bovine mastitis: An evolving disease. Vet. J., 164: 116-128.

CLSI, 2013. Performance standards for antimicrobial susceptibility testing: Twenty-first informational supplement. CLSI Document M100-S23, Vol. 33, No. 1, Clinical and Laboratory Standards Institute, Wayne, PA., USA., January 2013.

Calderon-Jaimes, E., L.E.E. de los Monteros and R. Avila-Beltran, 2002. Epidemiology of drug resistance: The case of *Staphylococcus aureus* and coagulase-negative staphylococci infections. Salud Publica Mexico, 44: 108-112.

Cook, N.B., D.A. Pionek and P. Sharp, 2005. An assessment of the benefits of Orbeseal® when used in combination with dry cow antibiotic therapy in three commercial dairy herds. Bovine Pract., 39: 83-94.

Cruickshank, R., 1962. Mackie and Mc Cartney's Handbook of Bacteriology. 10th Edn., E and S Livingstone Ltd., London.

Dubal, Z.B., H. Rahman, P. Pal, A. Kumar and K. Pradhan, 2010. Characterization and antimicrobial sensitivity of the pathogens isolated from bovine mastitis with special reference to *Escherichia coli* and *Staphylococcus* spp. Indian J. Anim. Sci., 80: 1163-1167.

Ferguson, J.D., G. Azzaro, M. Gambina and G. Licitra, 2007. Prevalence of mastitis pathogens in Ragusa, Sicily, from 2000 to 2006. J. Dairy Sci., 90: 5798-5813.

Joshi, D.D., J. Lensch, M. Sasaki and G. Hentsch, 1997. Epidemiological aspects of yak diseases in Nepal. Proceedings of the 2nd International Congress on Yak, September 1-6, 1997, Xining, China, pp: 229-233.

Kadariya, J., T.C. Smith and D. Thapaliya, 2014. *Staphylococcus aureus* and staphylococcal food-borne disease: An ongoing challenge in public health. BioMed Res. Int., 10.1155/2014/827965

Lindsay, J.A., 2014. Staphylococcus aureus genomics and the impact of horizontal gene transfer. Int. J. Med. Microbiol., 304: 103-109.

Moges, N., Y. Asfaw, K. Belihu and A. Tadesse, 2011. Aantimicrobial susceptibility of mastitis pathogens from smallholder dairy herds in and around Gondar, Ethiopia. J. Anim. Vet. Adv., 10: 1616-1622.

Muhammad, G., M. Athar, A. Shakoor, M.Z. Khan, F. Rehman and M.T. Ahmad, 1995. Surf field mastitis test: An inexpensive new tool for evaluation of wholesomeness of fresh milk. Pak. J. Food Sci., 3: 91-93.

NAAS, 2013. Mastitis management in dairy animals. Policy Paper No. 61, National Academy of Agricultural Sciences, New Delhi, India, September 2013.

National Mastitis Council, 1996. Current Concepts of Bovine Mastitis. 4th Edn., National Mastitis Council, Madison, WI., USA.

Pantoja, J.C.F., C. Hulland and P.L. Ruegg, 2009. Somatic cell count status across the dry period as a risk factor for the development of clinical mastitis in the subsequent lactation. J. Dairy Sci., 92: 139-148.

Pantosti, A., A. Sanchini and M. Monaco, 2007. Mechanisms of antibiotic resistance in *Staphylococcus aureus*. Future Microbiol., 2: 323-334.

Quinn, P.J., M.E. Carter, B. Markey and G.R. Carter, 1994. Clinical Veterinary Microbiology. 1st Edn., Mosby-Yearbook Europe Ltd., UK..

Radostits, O.M., C.C. Gay, K.W. Hinchcliff and P.D. Constable, 2007. Veterinary Medicine: A Textbook of the Diseases of Cattle, Sheep, Pigs, Goats and Horses. 10th Edn., WB Saunders Co., London, Toronto, ISBN-10: 0702027774.

Schalm, O.W. and D.O. Noorlander, 1957. Experiments and observations leading to development of the California mastitis test. J. Am. Vet. Med. Assoc., 130: 199-204.

Singh, P.J. and P.B. Singh, 1994. A study of economic losses due to mastitis in India. Ind. J. Dairy Sci., 47: 265-272.

Sumathi, B.R., B.M. Veeregowda and A.R. Gomes, 2008. Prevalence and antimicrobial profile of bacterial isolates from clinical bovine mastitis. Vet. World, 1: 237-238.

Watts, J.L., 1988. Etiological agents of bovine mastitis. Vet. Microbiol., 16: 41-66.

Wiener, G., H. Jianlin and L. Ruijum, 2003. The Yak. 2nd Edn., Food and Agricultural Organization, Bangkok, Pages: 460.

Zadoks, R.N., J.R. Middleton, S. McDougall, J. Katholm and Y.H. Schukken, 2011. Molecular epidemiology of mastitis pathogens of dairy cattle and comparative relevance to humans. J. Mammary Gland Biol. Neopl., 16: 357-372.