

Characterization and antimicrobial sensitivity of the pathogens isolated from bovine mastitis with special reference to *Escherichia coli* and *Staphylococcus* spp.

Z B DUBAL¹, H RAHMAN², PAPRI PAL³, A KUMAR⁴ and KALPANA PRADHAN⁵

ICAR Research Complex for NEH Region, Tadong, Gangtok, Sikkim 737 102 India

Received: 3 November 2009; Accepted: 15 September 2010

ABSTRACT

A study was conducted with the objective of isolation and molecular characterization of *E. coli* and *S. aureus* and identifying the efficacy of different antimicrobials against the bacteria associated with the mastitis. All the milk samples collected aseptically, tested for mastitis by California Mastitis Test (CMT), revealed 8 (27.58%) and 68 (24.63%) samples positive for subclinical mastitis from yak and cattle, respectively. *Escherichia coli* (18.18%), *Staphylococcus aureus* (42.14%), coagulase negative staphylococci (23.96%), *Streptococcus* spp. (3.30%), *Micrococcus* spp. (14.04%) and *Bacillus* spp. (5.78%) were isolated from 76 CMT positive milk samples and 45 samples from clinical cases of mastitis. *E. coli* were serotyped as O13, O132, O88, O26, O162, O88, O86 and O157. Sorbitol was not fermented by serotype O157 and it was isolated and reported for the first time from animal origin in Sikkim, India. *E. coli* O157 and O88 were positive for *stx 1* genes by Polymerase Chain Reaction (PCR). Twenty six (78.7%) isolates of *S. aureus* showed strong association of DNase and coagulase and all were untypable with the international sets of phages. It was observed by disc diffusion test that nitrofurantoin and tetracycline (97.77% each) to be most effective antimicrobial followed by chloramphenicol (95.55%) and cephotaxime (91.11%) against the pathogens associated with bovine mastitis.

Key words: Antimicrobial test, *Escherichia coli*, Molecular characterization, PCR, *Staphylococcus aureus*

Mastitis, a disease complex, is of great economic importance to the dairy industry throughout the world. The knowledge of microbial identification and susceptibility trends of bacterial species within a given herd is extremely important (Owens *et al.* 1997). Bacteria involved in bovine mastitis are broadly classified as contagious pathogens (*Staphylococcus aureus*, *Streptococcus agalactiae*, *Corynebacterium* spp. and *Mycoplasma* spp. (Fox and Gay 1993)), environmental pathogens (*Escherichia coli*, *Klebsiella* spp., *Streptococcus dysgalactiae*, *Streptococcus uberis*, *Enterococcus* spp. (Watts 1988)) and coagulase negative staphylococci or “environmental *Staphs*.” (Janus 2009). Contamination source of these organism includes hands of milking people, contaminated towels, water, dust, air, skin of animals, glandular tissues etc. Therefore, consumption of raw or under-pasteurized milk and their products from dairy cows constitutes potential microbiological hazards and affects the human health.

Even after following good managerial practices,

Present address: ^{1,2,3}Scientist, Animal Health Section (1st mail: drzunjar@yahoo.co.in, zunj@rediffmail.com); ⁵Disease Investigation Cell, State Animal Husbandry and Veterinary Science Department.

⁴Division of Veterinary Public Health, IVRI, Izatnagar, Uttar Pradesh 243 122.

mastitis is inevitable and calls for antibiotic therapy. Current mastitis control programmes are mainly targeted at contagious pathogens within the mammary gland. However, effectiveness of these programmes is questionable against environmental pathogens particularly *E. coli* (Brennan and Ergon 1995). Further, very limited work has been carried out about the organisms causing bovine mastitis, their characterization and control in Sikkim, India. Therefore, the present investigations were carried out to isolate and characterize *E. coli* and *Staphylococcus* spp. from bovine mastitis to evaluate the efficacy of different antimicrobials against different pathogens isolated from mastitis.

MATERIALS AND METHODS

Collection of samples: Milk samples (350), approximately 10 ml each from 32 yak and 318 cows, were collected aseptically from different locations covering all the 4 district of Sikkim. Some of the samples from clinical cases of mastitis were supplied by Disease Investigation Cell (DIC), State Veterinary and Animal Husbandry Department, Sikkim. All the milk samples excluding milk from clinical cases were tested by CMT for detection of subclinical cases of mastitis.

Bacterial isolation, identification and characterization: The CMT positive milk samples and milk from clinically affected quarters were diluted with 1% sterile peptone water

Table 1. Primers used in polymerase chain reactions for amplification of specific fragments from virulent genes of *E. coli*.

Primers	Oligonucleotide sequence (5'-3')	Product size (bp)	Reference
<i>stx1</i>	F-5' CAG TTA ATG TCG TGG CGA AGG-3' R-3' CAC CAG ACA ATG TAA CCG CTG-5'	348	Cebula <i>et al.</i> (1995)
<i>stx2</i>	F-5' ATC CTA TTC CCG GGA GTT TAC G-3' R-3' GCG TCA TCG TAT ACA CAG GAG C-5'	584	Cebula <i>et al.</i> (1995)
<i>est1</i>	F-5' TCT TTC CCC TCT TTT AGT CAG-3' R-3' ACA GGC AGG ATT ACA ACA AAG-5'	166	Moseley <i>et al.</i> (1983)
<i>elt1</i>	F-5' ATT TAC GGC GTT ACT ATC CTG-3' R-3' TTT TGG TCT CGG TCA GAT ATG-5'	280	Olive (1989)
<i>hlyA</i>	F-5' GGT GCA GCA GAA AAA GTT GTA G-3' R-3' TCT CGC CTG ATA GTG TTT GGT A-5'	1551	Schmidt <i>et al.</i> (1995)

and seeded on to a solid medium like blood agar, Mac Conkey's agar, Eosine Methylene Blue (EMB) agar, Vogel Johnson's agar for isolation and identification of *E. coli*, *S. aureus* and other organisms. *E. coli*, *Staphylococcus* spp., *Streptococcus* spp., *Micrococcus* spp. and *Bacillus* spp. were identified on the basis of morphological, cultural and biochemical characteristic (Cowan and Steel 1970, Edwards and Ewing 1972). Pathogens like *Streptococcus* spp., *Micrococcus* spp. and *Bacillus* spp. were not characterized further.

Characterization of *E. coli*: The confirmed *E. coli* isolates were serotyped at National Salmonella and Escherichia Centre, Central Research Institute, Kasauli, Himachal Pradesh, India. Different serotypes of *E. coli*, were then tested for sorbitol fermentation by using Sorbitol Mac Conkey agar and then 21 different strains of *E. coli* isolated from different sources including 12 strains from mastitis cases were subjected to PCR for their molecular characterization by using different primers such as *stx1*, *stx2*, *est1*, *elt1* and *hlyA* genes (Table 1).

Characterization of *S. aureus*: Isolates which were Gram (+) cocci, catalase (+), oxidase (–), oxidation-fermentation (O/F glucose, O/F mannitol) then named as *Staphylococcus*. Coagulase test were carried out to differentiate *S. aureus* from coagulase negative staphylococci. Thirty-three isolates of *S. aureus* were tested by DNase test for the determination of DNase production using DNase agar. Isolates were incubated on DNase agar at 35°C for 18 h. After incubation, 1 N HCl was poured on the plates and colonies with clear zone were considered as DNase positive. Twenty-six isolates were subjected for phage typing with international set of phages at ICMR staphylococcal phage typing centre, Maulana Azad Medical College, New Delhi, India.

Antibiogram of mastitis organism: Forty-five different isolates including 8 isolates of *E. coli*, 17 isolates of *S. aureus*, 12 coagulase negative staphylococci and 4 isolates each of *Micrococcus* spp. and *Streptococcus* spp. were subjected to *in-vitro* antimicrobial sensitivity test (Bauer *et al.* 1966) against 16 commercially available antimicrobial discs.

RESULTS AND DISCUSSION

Out of 350 samples collected aseptically 42 samples from cattle and 3 samples from yak were received from clinical cases of mastitis. All milk samples excluding milk from clinical cases were tested by CMT and detected 76 (24.9%) positive samples indicating of intramammary infection or subclinical mastitis. All the milk samples (clinical and subclinical) revealed *E. coli* (18.18%), *S. aureus* (42.14%), coagulase negative staphylococci (23.96%), *Streptococcus* spp. (3.30%), *Micrococcus* spp. (14.04%) and *Bacillus* spp. (5.78%). Thus, a total of 130 different bacteria were isolated from all milk samples (Table 2). The findings are in accordance with the findings of Grewal *et al.* (2005) who isolated *S. aureus* (54.54%), coagulase negative staphylococci (22.73%), *E. coli* (13.63%) and *Streptococcus* spp. (9.1%) from clinical cases of mastitis in Punjab, India.

E. coli isolates were serotyped as O13, O132, O88, O26, O162, O88, O86, O157 and untypable (Table 3). This indicates that serotype O157 may be involved in causation of mastitis. However, Turutoglu and Mudul (2002) indicated that *E. coli* O157:H7 does not cause bovine mastitis while testing the samples from Burdur province. Maximum number of serotypes is of enterotoxigenic and enterohemorrhagic which includes serotype O86, O88, O26 and O157 (Wolf 1997, Evans and Varnam 1991) which in turn may affect human health. All the isolates after inoculating on Sorbitol Mac Conkey agar revealed that it was not fermented by only O157 serotype. Different strains of *E. coli* including 12 isolates (from 7 different serogroups and 3 untypable strains) from mastitis cases were subjected to PCR for detection of virulent genes and found that, only one isolates each from serogroup O157 and O88 were positive for *stx1* (Fig.1). Ethelberg *et al.* (2004) also analysed the strain and patient factors associated with the development of bloody diarrhea and hemolytic uremic syndrome (HUS) among Shiga toxin producing *Escherichia coli* (STEC) patients registered in Denmark and found different serotypes which are *stx1* positive including O88 and O157.

S. aureus and coagulase negative staphylococci considered

Table 2. Different bacteria isolated from bovine mastitis

Isolates	Yak		Cattle		Total
	Clinical	Subclinical	Clinical	Subclinical	
No. of samples analysed	3	8/29	42	68/276	121/350
<i>E. coli</i>	—	7	6	9	22
<i>S. aureus</i>	2	5	18	26	51
Coagulase negative staphylococci	1	2	11	15	29
<i>Micrococcus</i> spp.	—	—	7	10	17
<i>Streptococcus</i> spp.	—	—	1	3	4
<i>Bacillus</i> spp.	—	—	1	6	7
Total	3	14	44	69	130

to be major pathogens associated with bovine mastitis and causes food borne intoxication, so identification of staphylococci causing intoxication is important for public health (Jablanski and Bohach 1997). In the present study, 51 *Staphylococcus* isolates were identified as *S. aureus* (coagulase positive) and 29 coagulase negative staphylococci (Table 3). DNase is as important as coagulase for pathogenesis (Pfaller and Herwaldt 1988). In our study, it was found that, there was a strong association of DNase and coagulase with *S. aureus* as 26 (78.7%) isolates showed activity for the production of DNase. The results are in accordance with the finding of Citak *et al.* (2003) where 93.6% coagulase positive staphylococci showed DNase activity whereas, Matsunaga *et al.* (1993) found all *S. aureus* isolates from bovine milk had DNase activity. All these 26 isolates after subjecting to phagotyping with the international sets of phages revealed that none of the isolates were typable indicated that the source of infection may be of animal origin. Phage typing of *S. aureus* isolated from poultry meat in Spain was carried out by Capita *et al.* (2001), who found only

Table 3. Antibiotic resistance pattern of different serotypes of *E. coli* isolated from bovine mastitis

Serotype	Serotype frequency	Antibiotic resistance pattern
O13	1	ACpMtPSz
O 26	1	AAMtP
O 86	1	AAMtPP
O 162	2	MtP
O 132	1	AAMCpMtP
O 157	1	MtSSmSzT
O 88	2	AAMtP
Untypable	3	AAM, P, Pb, Sm

A, Ampicillin; Am, amoxycillin; Cp, cephalixin; Mt, metronidazole; P, penicillin; G;Pb, polymyxin; B; Sm, sulfamethizole; Sz, sulfadiazine; S, streptomycin; T, tetracycline.

33.33% and 60% strains typable from legs and wings, respectively.

A total of 45 isolates of bovine mastitis including different serotypes of *E. coli* were subjected to antimicrobial sensitivity test and the results are depicted in Fig. 2. The highest sensitivity for *E. coli* was recorded against tetracycline, chlortetracycline and oxytetracycline (100.0% each) followed by nitrofurantoin and chloramphenicol (87.5% each). Whereas, for *S. aureus*, cephotaxime, nitrofurantoin and tetracycline showed hundred percent sensitivity followed by chloramphenicol, carbencillin and oxytetracycline (94.11% each) and streptomycin (88.23%). In case of coagulase negative staphylococci, highest sensitivity was recorded for chloramphenicol, nitrofurantoin, streptomycin and oxytetracycline (100% each) followed by cephotaxime and tetracycline (91.66% each) and oxytetracycline and carbencillin (83.33% each). *Micrococcus* spp. and *Streptococcus* spp. showed hundred percent sensitivity to nitrofurantoin followed by chlortetracycline and cephotaxime (75.0% each). Overall highest sensitivity was recorded against nitrofurantoin and tetracycline (97.77% each) followed by chloramphenicol (95.55%) and cephotaxime (91.11%) (Fig. 2). These findings were similar to Saluja *et*



Fig. 1. Results of characterization of *Escherichia coli* by PCR. Lane 1–8 & 10 - *E. coli* isolated from diff. clinical samples; lane 11–22 *E. coli* isolated from mastitis cases; lane 21 & 22 positive for *stx1*; lane 9 & 23 DNA marker.

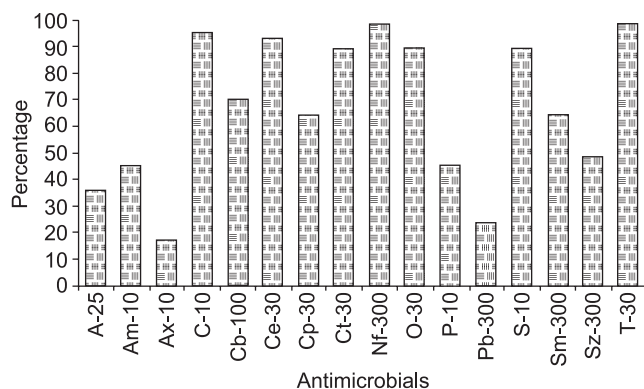


Fig. 2. Antimicrobial sensitivity pattern of bovine mastitis pathogens. A, ampicillin; Am, amoxycillin; Ax, ampicillin/cloxacillin; C, chloramphenicol; Cb, carbencillin; Ce, cephotaxime; Cp, cephalexin; Ct, chlortetracycline; Nf, nitrofurantoin; O, oxytetracycline; P, penicillin-G; Pb, polymyxin-B; S, streptomycin; Sm, sulphamethizole; Sz, sulphadiazine; T, tetracycline.

al. (2005) who recovered 266 isolates from subclinical mastitis and tested by antimicrobial sensitivity test which includes 64 strains of *S. aureus* showed 100% sensitivity to chloramphenicol, 98.43% to streptomycin and 96.87% to nitrofurantoin; 10 strains of *Str. agalactiae* showed 100% sensitivity to ampicillin and amoxycillin, 90% to chloramphenicol and nitrofurantoin and 15 strains of *Micrococcus* spp. showed 100% sensitivity to chloramphenicol, 93.3% to streptomycin and 86.6% to oxytetracycline and nitrofurantoin. Sikdar *et al.* (1994) also reported 78.90% and 67.89% sensitivity of *E. coli* strains to chloramphenicol and nitrofurantoin, respectively. Erskine *et al.* (2002) isolated *S. aureus* from bovine mastitis which were susceptible to cephalothin, gentamicin, pirlimycin and oxacillin (>95%). High sensitivity of the strains to these antimicrobial agents was attributed to the fact that these drugs were seldom used in the farm.

Our results indicated that *S. aureus*, coagulase negative staphylococci and *E. coli* were the major pathogens involved in causation of mastitis in Sikkim. Different serotypes of *E. coli* including serotype O157 may be one of the causes of mastitis, however clinical trials are required for its confirmation. There was a strong association of DNase and coagulase with *S. aureus* as maximum isolates showed activity for DNase production. Amongst different antimicrobial tested nitrofurantoin and tetracycline showed highest sensitivity followed by chloramphenicol and cephotaxime.

ACKNOWLEDGEMENTS

The authors are highly thankful to the Director, National Salmonella and Escherichia Centre, Central Research Institute, Kasauli, Himachal Pradesh, India for his kind help in serotyping of *E. coli* isolates and to the Head, ICMR Staphylococcal phage typing centre, Maulana Azad Medical

College, New Delhi, India for the phage typing of *S. aureus* isolates. We thank to the Director and Joint Director, ICAR Research Complex for NEH Region, India for providing necessary laboratory facilities. We also thank Disease Investigation Cell, State Animal Husbandry and Veterinary Science Department, Sikkim, India for collection and supplying of milk samples.

REFERENCES

- Bauer A W, Kirly W M M, Sherris J C and Truck M. 1966. Antibiotic susceptibility testing by standard single disc method. *American Journal of Clinical Pathology* **4**: 493–96.
- Brennan P and Ergon J. 1995. Minimum inhibitory concentrations for *Staphylococcus aureus* and *Escherichia coli* isolates from clinical bovine mastitis. *Irish Veterinary Journal* **48**: 185–86.
- Capita R, Alvarez-astorga M, Alonso-calleja C, Morena B and Garcia-fernandez M C. 2001. Phage typing of *Staphylococcus aureus* isolates from poultry meat in Spain. *Journal of Microbiology* **39** (3): 219–25.
- Cebula T A, Payne W L and Feng P. 1995. Simultaneous identification of strains of *Escherichia coli* serotype O157:H7 and their shiga-like toxin type by mismatch amplification mutation assay-multiplex PCR. *Journal of Clinical Microbiology* **33**: 248–50.
- Citak S, Varlik O and Gundogan N. 2003. Slime production and DNase activity of staphylococci isolated from raw milk. *Journal of Food Safety* **23**: 281–88.
- Cowan S T and Steel K J. 1970. *Manual for the Identification of Medical Bacteria*. 2nd edn. pp. 106–08. Cambridge University Press, London.
- Edwards P R and Ewing W H. 1972. *Identification of Enterobacteriaceae*. Burgess Publishing Company, Minneapolis, Minnesota, USA.
- Erskine R J, Walker R D, Bolin C A, Bartlett P C and White D G. 2002. Trends in antibacterial susceptibility of mastitis pathogens during a seven year period. *Journal of Dairy Science* **85**: 1111–18.
- Ethelberg S, Olsen K E P, Scheutz F, Jensen C, Schiellerup P, Engberg J, Petersen A M, Olesen B, Gerner-Smidt P and Molbak K. 2004. Virulence factors for hemolytic uremic syndrome, Denmark. *Emerging Infectious Disease* **10**: 842–47.
- Evans M G and Varnam A H. 1991. *Food-borne Pathogens- an illustrated text*. ISBN 0 7234 1521 8: pp 103. Wolfe publishing Ltd. BPCC Hazell Books, Aylesbury, England.
- Fox L K and Gay J M. 1993. Contagious mastitis. *Veterinary clinics of North America: Food Animal Practice*. Vol. **9**. pp. 475–87. (Ed.) Anderson K L. W B Saunders Company, Philadelphia, PA.
- Grewal K D, Gupta M P and Singh K B. 2005. Therapeutic efficacy of gentamicin in clinical cases of mastitis in buffaloes. *Indian Veterinary Journal* **82**: 123–25.
- Jablanski L M and Bohach G A. 1997. *Staphylococcus aureus*. *Food Microbiology: Fundamentals and Frontiers*. pp. 353–75. (Eds) Doyle M P, Beuchat L R and Montviell T J. ASM Press, Washington, D C.
- Janus A. 2009. Emerging mastitis pathogens. *Veterinary World* **2** (1): 38–39.

- Moseley S L, Hardy J W, Huq M I, Echeverria P and Falkow S. 1983. Isolation and nucleotide sequence determination of a gene encoding a heat-stable enterotoxin of *Escherichia coli*. *Infection and Immunity* **39**: 1167–74.
- Olive D M. 1989. Detection of enterotoxigenic *E. coli* after polymerase chain reaction amplification with thermostable DNA polymerase. *Journal of Clinical Microbiology* **27**: 261–65.
- Owens W E Ray C H, Watts J L and Yancey R J. 1997. Comparison of success of antibiotic therapy during lactation and results of antimicrobial susceptibility tests for bovine mastitis. *Journal of Dairy Science* **80**: 315.
- Pfaller M A and Herwaldt L A. 1988. Laboratory, clinical and epidemiological aspects of coagulase negative staphylococci. *Journal of Clinical Microbiology* **37**: 201–05.
- Saluja P S, Gupta S L, Kapur M P and Sharma A. 2005. Antibigram of bacterial isolates of bovine intramammary origin. *Indian Veterinary Journal* **82**: 323–24.
- Schmidt H, Beutin K and Karch H. 1995. Molecular analysis of the plasmid encoded hemolysin of *Escherichia coli* O157-H7 strain EDL 933. *Infection and Immunity* **63**: 1055–61.
- Sikdar D, Rahman H, Borah P and Boro B R. 1994. Occurrence of piglet diarrhoea in North-Eastern India: Isolation, serotyping and antibiogram of *Escherichia coli*. *Indian Journal of Animal Sciences* **64**: 728–30.
- Turutoglu H and Mudul S. 2002. Is *Escherichia coli* O157:H7 an etiological agent of bovine mastitis? *Israel Journal of Veterinary Medicine* **57** (2).
- Watts J L. 1988. Evaluation of the rapid strep system for identification of gram-positive, catalase-negative cocci isolated from bovine intramammary infections. *Journal of Dairy Science* **72**: 2728–32.
- Wolf M K. 1997. Occurrence, distribution and association of O and H serogroups, colonization factor antigens, and toxins of enterotoxigenic *Escherichia coli*. *Clinical Microbiological Review* **10** (4): 569–84.