Journal of Animal Research: v.7 n.1, p. 77-84. February 2017

DOI: 10.5958/2277-940X.2017.00011.0

Prevalence and Characterization of Methicillin-Resistant *Staphylococcus aureus* (MRSA) Mastitis in Dairy Cattle in Jabalpur, Madhya Pradesh

Neeraj Shrivastava[1*], Varsha Sharma[1], Anju Nayak[1], A.B. Shrivastava[2], B.C. Sarkhel[3], P.C. Shukla[4] and Arpita Shrivastava[5]

[1]Department of Veterinary Microbiology, C.V. Sc & A. H., Jabalpur, INDIA
[2]Centre for Wildlife Forensic and Health, N.D.V.S.U., Jabalpur, INDIA
[3]Animal Biotechnology Centre, N.D.V.S.U., Jabalpur, INDIA
[4]Department of Veterinary Medicine, C.V. Sc & A. H., Jabalpur, INDIA
[5]Department of Veterinary Pharmacology and Toxicology, C.V. Sc & A. H., Jabalpur, INDIA

***** *Corresponding author: N Shrivastava; Email: neerajvet32@gmail.com*

Received: 26 Aug., 2016 **Revised:** 04 Sep., 2016 **Accepted:** 16 Sep., 2016

ABSTRACT

Methicillin-resistant *Staphylococcus aureus* (MRSA) is a dreaded pathogen in human and veterinary medicine. MRSA as a cause of mastitis in dairy cattle has got profound economic and serious public health significance. A total of 111 dairy cattle were screened for mastitis by CMT from six dairy farms of Jabalpur. The 85 mastitis milk samples were screened for MRSA by bacterial culture method using cefoxitin for enrichment. The MRSA strains were identified and characterized by phenotypic and genotypic methods for virulence determinants and methicillin resistance. The MRSA strains showed β-haemolysis as a predominant haemolysis pattern. The strains were positive for *mecA* gene which is considered as a gold standard for the confirmation of methicillin resistance. The prevalence of MRSA mastitis in dairy cattle was 16.47%. The MRSA strains were positive for the virulence factors associated with pathogenicity. The economic and public health implication of the finding is discussed.

Keywords: MRSA, Prevalence, mastitis, dairy cattle

Mastitis is a global problem as it adversely affects animal welfare, quality of milk and the economies of milk production, affecting every country involved in dairying. (FAO, 2014). The economic losses due to mastitis in India have increased about 115 folds in the last five decades and presently the loss due to mastitis is to the tune of ₹ 7165.51 crores per annum (Bansal and Gupta, 2009). In India the prevalence of bovine mastitis due to *Staphylococcus aureus* (*S. aureus*) is around 30-40% (Patel *et al.,* 2012; Sharma *et al*., 2012).

S. aureus is a contagious mastitis pathogen and the infected udder is the main source of infection to the herd. Transmission is thought to occur from cow to cow primarily via the milkers' hands, udder cloths or milking machine (Radostits *et al*., 2006). Although recently it has been recognized that its epidemiological behaviour is not clear cut, with strains demonstrating contagious and/or environmental transmission patterns (Fernandez *et al*., 2013).

S. aureus is important in veterinary medicine as it causes mastitis in bovines which is difficult to treat, control and prevent. The efforts to control and prevent *S. aureus* mastitis are often disappointing due to false negative results from bacteriological culture as the organism is shed intermittently, resulting in undetected cases that may re-infect the rest of the herd, and the poor response of *S. aureus* mastitis to treatment (Barkema *et al*., 2006). The poor cure rate is attributed to a range of surface-exposed (protein A, fibrinogen- and fibronectin-binding proteins) and secreted (enterotoxins, haemolysins and coagulase) virulence factors that allow it to colonize, invade and multiply in bovine mammary epithelial cells which in turn

make antimicrobial agents poorly effective (Le Marechal *et al.,* 2011; Spoor *et al*., 2013).

S. aureus is known to acquire antimicrobial resistance very quickly. Methicillin resistance is of particular relevance because it imparts resistance to all β-lactam anti-microbial agents- penicillins, cephalosporins, carbapenems (Weese and van Duijkeren, 2010). Historically, Methicillin-resistant *S. aureus* (MRSA) was considered as human pathogen. The earlier reports of MRSA as a cause of mastitis in bovines were shown to be of human origin (Devriese and Hommez, 1975; Juhasz-Kaszanyitzky *et al*., 2007) but through the past decade MRSA reported as cause of mastitis are bovine adapted and has also been isolated world-wide from other food producing animals like pigs and poultry (Fluit, 2012). Additionally several molecular epidemiological studies have demonstrated host specificity is a lineage specific trait that can rapidly evolve and various host shift between human and bovine has occurred in recent and distant past. (Sakwinska *et al*., 2011; Resch *et al*., 2013). This has raised public health concerns. It is therefore important that bacterial populations at the animal-human interface should be monitored routinely.

The WHO Global Strategy for Containment of Anti-Microbial Resistance (AMR) 2001(WHO, 2012) identifies effective surveillance as a corner stone of national and international efforts to control AMR. Tracking antibiotic usage and the emergence and spread of resistant strains of bacteria provides information, insights and tools needed to guide and evaluate policy and measures which has to be taken to promote appropriate anti-microbial use at all levels- local, national and international. The detection of AMR and monitoring its spread requires laboratory based surveillance.

The prevalence of MRSA in human hospitals of Madhya Pradesh is reported to be around 30 – 40% (Joshi *et al*., 2013; Tripathi, 2015). The prevalence of MRSA in veterinary setting in the state of Madhya Pradesh had been lacking. This study was designed to study the prevalence scenario of MRSA in dairy cattle of the Jabalpur.

MATERIALS AND METHODS

A total of 111 cows were screened from six dairy farms at Jabalpur for the presence of mastitis by clinical examination and California mastitis test (Ruegg and Reinemann, 2002; Dhakal, 2006). Milk from infected quarter or udder with CMT score of ≥ 1 was aseptically collected in sterile tubes transported in ice and stored at -20 °C until further use.

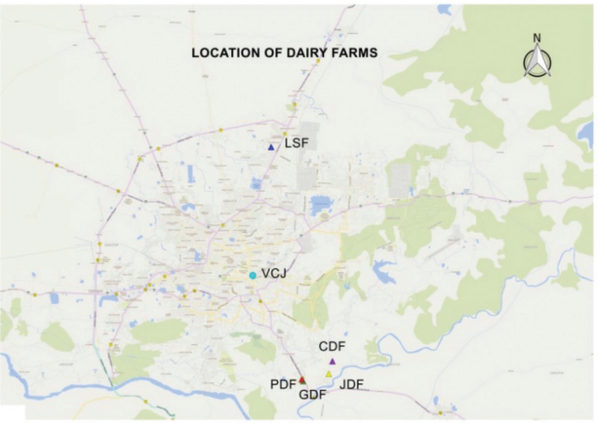

Fig. 1: Location of Dairy farms in Jabalpur, Madhya Pradesh used in the study. CDF, GDF, JDF, PDF, LSF represent the dairy farms and VCJ is the laboratory. (QGIS, 2015)

Isolation of Methicillin-resistant *Staphylococcus aureus was* carried out according to the protocol published by EFSA, 2012 with some modifications. Briefly the milk samples were subjected to one freeze-thaw cycle and were mixed thoroughly before pre-enrichment in Mueller-Hinton (MH) broth (HiMedia) supplemented with 6.5 % NaCl at 35 °C for 16-20 hours; followed by enrichment in Tryptone Soya Broth (HiMedia) with 3.5 mg/L cefoxitin (Sigma) and 75 mg/L aztreonam (Sigma) at 35 °C for 16-20 hours. The HiChrome MeReSa agar plates with cefoxitin and methicillin supplement (HiMedia) were used as selective agar and were incubated at 35 °C for 16-20 hours. Up to five blue-green colonies indicative of being MRSA were chosen and were streaked in Tryptone soy agar and incubated at 35 °C for 16-20 hours.

The presumptive isolates were phenotypically characterized using Catalase test, Gram's reaction, Voges-Proskauer test, Clumping factor test, Tube coagulase test, Haemolysis in Sheep blood agar, Novobiocin and Polymyxin B resistance, Mannitol fermentation in Mannitol salt agar and Maltose fermentation in Purple agar (National Mastitis Council, 2004; Tille, 2014). The isolates were further confirmed using Staphytect Plus Latex agglutination test kit (Oxoid, United Kingdom) performed as per the manufacturers instruction.

The Methicillin-resistance was phenotypically characterized using cefoxitin (30 µg) disk by disc

diffusion assay (CLSI, 2012) and by Penicillin Binding Protein-2a (PBP2a) Latex Agglutination Test (LAT) for the detection of PBP-2a (Oxoid, United Kingdom) as per the manufacturer's instruction.

The genotypic characterization of the Methicillin- resistant *S. aureus* (MRSA) was done using the multiplex –PCR for the detection of *spa* and *mecA* genes (EFSA, 2012). *S. aureus* ATCC 43300 was used as positive control for the *spa* and *mecA* gene.

The PCR-amplified samples were analysed by agarose gel electrophoresis by using a horizontal 1.5% (w/v) agarose gel in 1X Tris Borate EDTA (TBE) buffer (pH 8.3; 89.0 mM Tris, 89.0 mM boric acid, 2.0 mM EDTA) at 80 Volts for 2 hours. The PCR products were visualized and photographed on a Gel documentation unit (Alpha Innotech). The 100 base pair (bp) ladder molecular weight marker was run in parallel with the samples. The *spa* fragment resulting from the amplification was variable in size and ranges from 180-600 bp depending on the *spa* type present and this fragment should be amplified from all *S. aureus* strains. It is the method of identification and an important virulence determinant. The amplification of the *mecA* gene with a size of 162 bp confirms methicillin resistance.

RESULTS AND DISCUSSION

Prevalence of clinical and subclinical mastitis in bovines

A total of 111 dairy cows from six dairy farms were screened for mastitis by California Mastitis test (CMT). A total of 85 quarter or composite samples of milk from 111 dairy cows with CMT score ≥ 1 were collected.

Out of the 85 milk samples collected from dairy cattle, a total of 32 samples were of clinical mastitis and 53 samples were of subclinical mastitis. A prevalence of 28.83% and 47.75% of clinical and subclinical mastitis respectively was found in 111 dairy cattle. Mir *et al*. (2014) found a higher prevalence of subclinical mastitis (57.8%) when following the criteria of both CMT and culturally positive samples. However, these results were obtained from farms with machine milked cows. Sharma *et al.* (2012) also reported the prevalence of 39.8% and Bangar *et al*. (2015) determined the pooled prevalence of subclinical mastitis in cattle in India to be 46.4%. In another study of 263 cows in Karnataka, the prevalence of clinical mastitis was 4.7 – 8 % depending on the diagnositic tests (Kurjogi and Kaliwal, 2014). The variation in prevalence of mastitis between the different studies might be partly due to different types of diagnostic tests, sampling procedures and criteria for mastitis and to factors such as stage of lactation, parity number and breed of the animals included in the studies.

A total of 21 (24.70 %) samples were found to be culture negative, which indicates that these quarters did not shed bacteria in a sufficient amount or that the infection had been eliminated. However, mastitis causing bacteria can occur in substantial quantities also in growth-negative milk samples (Taponen *et al*., 2009a; Kuehn *et al*., 2013) meaning that an infection cannot be excluded even with a negative sample.

Prevalence and characterization of Methicillin-resistant *S. aureus* (MRSA) from milk

In human medicine *S. aureus* is considered as a major and common pathogen of genus *Staphylococcus* and is often considered synonymous with coagulase positive staphylococci (CoPS). The case in veterinary medicine is not very straightforward where although *S. aureus* is considered as a major coagulase positive staphylococci, other coagulase positive staphylococci like *Staphylococcus intermedius* (Robertson *et al.,* 1992; Krithiga *et. al.,* 2011) and *Staphylococcus pseudintermedius* (Pilla *et. al.,* 2013) have also been reported to be cause of mastitis in bovines. Additionally the coagulase-negative staphylococci (CoNS) like *S. epidermidis*, *S. chromogenes* and *S. simulans* which are considered as emerging cause of subclinical mastitis in bovines are present as normal microflora in bovine skin and commonly carry the gene for methicillin resistance (Taponen *et al*., 2009b; Feβler *et al*., 2010). In this context the accurate identification of the *Staphylococcus* species and the demonstration of virulence factors in it cannot be over-emphasized.

A total of 85 milk samples were screened for the Methicillin-resistant *S. aureus* (MRSA) following the two step enrichment in Mueller-Hinton broth with 6.5% sodium chloride (NaCl) and growth in Tryptone Soy broth (TSB) with cefoxitin (CX = 3.5 mg/L) and aztreonam (AT = 75mg/L). Out of 85 samples, 25 samples showed typical bluish green coloured colony of MRSA in HiCrome

MeReSa agar with both the MeReSa selective supplement and cefoxitin supplement.

Using phenotypic tests – catalase, Gram's reaction, clumping factor, tube coagulase and Voges-Proskauer tests, a total of 14 of these strains were presumptively identified as *S. aureus* as per the recommended criteria of NMC, 2004 (Table 1).

Table 1: Screening of milk samples for Methicillin-resistant *S. aureus*

Sl. No.	Particulars	No. of isolates positive for the test	Per cent positive
1	MH broth + 6.5% NaCl	85	100
2	Growth in TSB + CX + AT	85	100
3	Growth in HiCrome MeReSa Agar	25	29.41
4	Catalase	25	29.41
5	Gram positive cocci	25	29.41
6	Clumping Factor	17	20
7	Voges-Proskauer test	14	16.47
8	Tube coagulase	14	16.47

The 14 isolates were phenotypically characterized by mannitol and maltose fermentation, susceptibility to Novobiocin (5 µg disc), resistance to Polymyxin B (300 µg disc) and haemolysis in Sheep blood agar (SBA).

The 14 isolates (100%) were positive for mannitol fermentation in mannitol salt agar and for maltose in Purple agar (P agar). Similarly all the 14 isolates (100%) were susceptible to Novobiocin (zone diameter ≥ 17 mm) and resistant to Polymyxin B (zone diameter ≤ 10 mm) in Kirby Bauer disk diffusion assay.

The alpha (α) and beta (β) haemolysis was studied in 5 per cent Sheep blood agar (HiMedia). While 3 isolates (21.42 %) and 8 isolates (57.14 %) showed alpha (α) and beta (β) haemolysis respectively, a total of 3 isolates (24.56%) showed both alpha (α) and beta (β) haemolysis (Table 02).

Table 2: Phenotypic characterization of Methicillin- resistant *Staphylococcus aureus*

Sl. No.	Particulars	No. of isolates positive for the test	Per cent positive
1	Coagulase	14	100
2	Mannitol fermentation	14	100
3	Maltose fermentation	14	100
4	Novobiocin susceptibility	14	100
5	Polymyxin B resistance	14	100
6	α haemolysis	3	21.42
7	β haemolysis	8	57.14
8	α and β haemolysis	3	21.42
9	Staphytect Plus test	14	100

All the 14 isolates (100%) were positive for *S. aureus* in the Staphytect Plus latex agglutination test which detects the clumping factor, Protein A and capsular polysaccharide present in the cell wall of *S. aureus*.

The surface exposed (fibrinogen and fibronectin-binding proteins, the staphylococcal protein A or spa) and secreted (coagulase, haemolysins -alpha-toxins, Luk *MF',* Luk *ED*; thermo-nuclease, enterotoxins) are some of the virulence factors commonly studied in *S. aureus* mastitis in bovines (Le Thanh *et al*., 2008; Singh *et al*., 2011)

In this study the virulence determinants phenotypically studied included the coagulase production, haemolysin in sheep blood agar and the ability to ferment mannitol. The protein A and capsular polysaccharides were detected immunologically by Staphytect Plus Latex agglutination test kit (Oxoid, UK). Additionally the staphylococcal protein A or *spa* gene was also genotypically detected using PCR. The strains which produced all these virulence factors were only included in the study. Haemolysis in blood agar represents an important criterion for rapid presumptive identification of *S. aureus* isolates of bovine origin (Boerlin *et al*., 2003). The phenotypic alpha- and beta-haemolysis shows geographical variation. (Le Thanh *et al*., 2008). Among the MRSA strains in this study the predominant pattern was β haemolysis. Virtually

all mastitis strains produce toxins that are active on neutrophils. All contain at least one leukotoxin gene: a γ-haemolysin (*hlg*) variant is present in all strains, *LukED* is present in most strains and *lukMF'-PV* in 15–86% of strains (Monecke *et al*., 2007; Yamada *et al*., 2005). The α-haemolysin (*hla*) gene is present in most strains, and β-haemolysin (*hlb*) is more common in strains of bovine origin than those of human origin, and both toxins may play a role in the pathogenesis of mastitis (Bramley *et al*., 1989).

The methicillin resistance of the isolates was characterized by phenotypic methods – Resistance to cefoxitin (30 µg) disc in disk diffusion assay, production of Penicillin binding protein – 2a (PBP2a). The 14 (100%) MRSA isolates were resistant to cefoxitin. All the isolates (100%) produced PBP-2a as determined by the latex agglutination assay. This is in agreement with the study of Lee *et al*. (2004) which showed the test had sensitivity and specificity of 100%. The study found this test was a reliable and rapid method of detecting MRSA in the veterinary field.

After phenotypic characterization the 14 isolates were genotypically characterized for *spa* and *mecA* gene by multiplex PCR. All the 14 isolates (100%) were positive for *spa* and for *mecA* gene (Fig. 2). So the prevalence of 16.47 % MRSA mastitis in dairy cattle was found in this study.

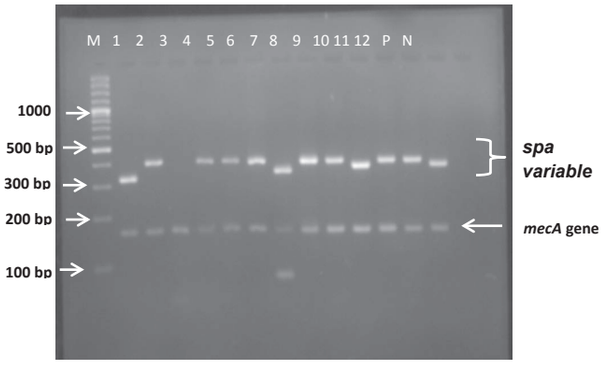

Fig. 2: Agarose gel electrophoresis analysis showing multiplex PCR amplification products for the detection of the spa and mecA gene (168 bp) in the MRSA isolates. The Lanes 1, 2, 4-12 shows the mecA gene (168 bp) in the MRSA isolates. P = positive control S. aureus ATCC 43300, N = negative control

Methicillin-resistant *S. aureus* (MRSA) includes those strains that have acquired genes (*mecA* or *mecC*) conferring resistance to methicillin and essentially all other β-lactam antibiotics. MRSA was initially reported as a nosocomial pathogen in human hospitals (or hospital-associated MRSA, HA-MRSA). Since the 1990s, community-acquired or community-associated MRSA (CA-MRSA) infections have also been reported to affect people having no epidemiological connection with hospitals (Deurenberg and Stobberingh, 2008). In 1972 a cow in Belgium with mastitis was the first reported MRSA infection in an animal (Devriese *et al.,* 1972).

More recently, MRSA has been isolated from most food-producing animals and foods of animal origin, raising public health concerns. MRSA strains have been isolated from cows' or small ruminants' milk and various dairy products in many countries. Since early 2000s we are witnessing the epidemic wave 6 of MRSA which is for the first time related to livestock (Chatterjee and Otto, 2013). The MRSA prevalence in milk and dairy products recorded in different countries or even regions of the same country differs significantly. High MRSA prevalence have been recorded in milk produced in most African countries, for instance as high as 60.3% in Ethiopia. The MRSA prevalence in Asian countries varies from high e.g. 28.3% in Iran to less than 1% in Japan (Pexara *et al.,* 2013). The prevalence has been reported to be 13.9 % in South Korea (Park *et al.,* 2016). In most European countries, the MRSA prevalence in milk and dairy products has been generally found to be low. In the US and Canada, zero to low MRSA prevalence estimates have been reported (Pexara *et al.*, 2013). The investigation of MRSA prevalence in milk may serve as a tool for assessing both the sanitary conditions employed in dairy herds and the health risks that humans may encounter when infected with antibiotic-resistant strains.

In India the prevalence of MRSA in bovine milk as detected by *mecA* gene has been reported to vary widely between 5.11 to 27% (Prashanth *et al*., 2011; Singh *et al*., 2011; Chandrasekhran *et al*., 2014; Kutar *et al*., 2015).

The methicillin resistance in the mastitis causing pathogen incurs greater economic loss to the farmer as it leads to decreased treatment options as the β-lactam antibiotics are not effective, increased cost of treatment due to increased therapeutic failures, poor prognosis and occupational hazard (Barkema *et al*., 2006; Bardiau *et al*., 2013).

Staphylococcus aureus is a well-known commensal and opportunistic pathogen of both bovines and humans. *S. aureus* is a clonal organism. However while *S. aureus* are

quite ubiquitous in terms of host species, different animals tend to harbor different genetic lineages. The genetic lineages of *S. aureus* in general and MRSA in particular has been extensively defined by various typing methods like staphylococcal protein A or *spa* typing, Multi Locus Sequence Typing (MLST) and Staphylococcal Cassette Chromosome (SCC) *mec* typing. (Deurenberg and Stobberingh, 2008). The molecular typing of MRSA of both human and bovine origin has demonstrated that some of the clones are host specific while others have wider host specificity (Fournier *et. al*., 2008; Piccinini *et. al*., 2010). The prevalence of MRSA as a cause of mastitis is of great concern as it has got wider implications on animal welfare and public health. The predominance of β-haemolysis in the MRSA strains in this study is suggestive of human origin of the isolates. However this needs to be validated using spa, MLST and SCC*mec* typing which will provide the true picture in terms of epidemiology, zoonotic risk and antimicrobial resistance.

ACKNOWLEDGEMENTS

The authors are thankful to the Dean, College of Veterinary Science and Animal Husbandry, Jabalpur for providing necessary facilities and funding during the entire research work.

REFERENCES

Bangar, Y.C., Singh, B., Dohare, A.K. and Verma, M.R. 2015. A systematic review and meta-analysis of prevalence of subclinical mastitis in dairy cows in India. *Trop. Anim. Health Prod.*, **47**: 291–297.

Bansal, B.K. and Gupta, D.K. 2009. Economic analysis of bovine mastitis in India and Punjab – A Review. *Indian J. Dairy Sci.*, **62**: 337-345.

Bardiau, M., Yamazak,i K., Duprez, J.N., Taminiau, B., Mainil, J.G. and Ote, I. 2013. Genotypic and phenotypic characterization of Methicillin-resistant *Staphylococcus aureus* (MRSA) isolated from milk of bovine mastitis. *Lett. Appl. Microbiol.,* **57**:181-186.

Barkema, H.W., Schukken, Y.H. and Zadoks, R.N. 2006. Invited review: the role of cow, pathogen, and treatment regimen in the therapeutic success of bovine *Staphylococcus aureus* mastitis. *J. Dairy Sci.*, **89**: 1877–1895.

Boerlin, P., Kuhnert, P., Hussy,D. and Schaellibaum, M. 2003. Methods for Identification of *Staphylococcus aureus* Isolates in cases of Bovine mastitis. *J. Clin. Microbiol.,* **41**: 767–771.

Bramley, A.J., Patel, A.H., O'Reilly, M., Foster, R. and Foster, T.J. 1989. Roles of alpha-toxin and beta-toxin in virulence of *Staphylococcus aureus* for the mouse mammary gland. *Infect. Immun*., **57:** 2489–2494

Chandrasekaran, D., Venkatesan, P., Tirumurugaan, K.G., Nambi, A.P, Thirunavukkarasu, P.S., Kumanan, K., Vairamuthu, S. and Ramesh, S. 2014. Pattern of antibiotic resistant mastitis in dairy cows. *Vet. World,* **7**: 389-394.

Chatterjee, S.S. and Otto, M. 2013. Improved understanding of factors driving methicillin-resistant *Staphylococcus aureus* epidemic waves. *Clin. Epidemiol.*, **5**:205-217.

Clinical and Laboratory Standards Institute. 2012. Performance standards for Anti-microbial susceptibility testing; 22nd Informational supplement, CLSI document M100-S22. CLSI, Pennsylvannia, pp. 70-82.

Deurenberg, R.H. and Stobberingh, E.E. 2008. The evolution of *Staphylococcus aureus*. *Infect. Genet. Evol.*, **8**: 747–763.

Devriese, L.A., van Damme, L.R. and Fameree, L. 1972. Methicillin (Cloxacillin)-resistant *Staphylococcus aureus* strains isolated from Bovine mastitis cases. *Zentralbl. Vet. Med. A.,* **19**: 548-605.

Devriese, L.A. and Hommez, J. 1975. Epidemiology of methicillin-resistant *Staphylococcus aureus* in dairy herds. *Res. Vet. Sci.*, **19**: 23–27.

Dhakal, I. P. 2006. Normal Somatic Cell Count and Subclinical Mastitis in Murrah Buffaloes. *J. Vet. Med. Series B.*, **53**: 81–86.

European Food Safety Authority. 2012. Technical specifications on the harmonised monitoring and reporting of antimicrobial resistance in methicillin-resistant *Staphylococcus aureus* in food-producing animals and food. *EFSAJ.*, **10**: 2897-2957.

Feßler, A.T., Billerbeck, C., Kadlec, K. and Schwarz, S. 2010. Identification and characterization of methicillin-resistant coagulase-negative staphylococci from bovine mastitis. *J. Antimicrob. Chemother.,* **65**: 1576–1582.

Fernandez, G., Barreal, M.L., Pombo, M.B., Ginzo-Villamayor, M.J., Gonzalez-Manteiga, W., Prieto, A., Lago, N. and González-Palencia, J. 2013. Comparison of the epidemiological behavior of mastitis pathogens by applying time-series analysis in results of milk samples submitted for microbiological examination. *Vet. Res. Commun.,* **37**: 259–267.

Fluit, A.C. 2012. Livestock –associated *Staphylococcus aureus*. *Clin. Microbiol. Infect.*, **18**: 735-744.

Food and Agriculture Organization of the United Nations. 2014. Impact of mastitis in small scale dairy production systems. Animal Production and Health Working Paper No. 13. Rome. pp. 1-44.

Fournier, C., Kuhnert, P., Frey, J., Miserez, R., Kirchhofer, M., Kaufmann, T., Steiner, A. and Graber, H.U. 2008. Bovine *Staphylococcus aureus*: association of virulence genes, genotypes and clinical outcome. *Res. Vet. Sci.*, **85**: 439–448.

Joshi, S., Ray, P., Manchanda, V., Bajaj, J., Chitnis, D.S., Gautam,V., Goswami, P., Gupta, V., Harish, B.N., Kagal, A., Kapil, A., Rao, R., Rodrigues, C., Sardana, R., Sulochana Devi, Kh., Sharma, A. and Balaji, V. 2013. Methicillin resistant *Staphylococcus aureus* (MRSA) in India: Prevalence and susceptibility pattern. *Indian J. Med. Res.*, **137**: 363-369.

Juhász-Kaszanyitzky, E., Jánosi, S., Somogyi, P., Dán, van der Graaf-van Bloois, L., van Duijkeren, E., and Wagenaar, J. 2007. MRSA Transmission between Cows and Humans. *Emerg. Infect. Dis.*, **13**: 630-632.

Krithiga, N., Antony, P. X., Mukhopadhyay, H. K., Pillai, R.M., Vijayalakshmi, P., Thanislass, J. and Subbareddy, K. V. 2011. Species characterization and antibiogram profile of staphylococci isolates in clinical bovine mastitis. *Anim. Sci. Report.*, **5** (1): 3-8.

Kuehn, J.S., Gorden, P.J., Munro, D., Rong, R., Dong, Q., Plummer, P.J., Wang, C. and Phillips, G.J. 2013. Bacterial Community Profiling of milk samples as a means to understand culture-negative Bovine clinical mastitis. *PLoS One.,* **8**(4): 1-10.

Kurjogi, M.M. and Kaliwal, B.B. 2014. Epidemiology of Bovine mastitis in cows of Dharwad district. *Int. Sch. Res. Notices.*, **1**: 1-9.

Kutar, K., Verma, A.K., Sharma, B., Kumar, A. and Yadav, S.K. 2015.Analysis of *mecA* gene and antibiotic resistance in *Staphylococcus aureus* isolates from bovine mastitis. *Indian J. Comp. Microbiol. Immunol. Infect. Dis.,* **36**: 22-27.

Le Maréchal, C., Seyffert, N., Jardin, J., Hernandez, D., Jan, G., Rault, L., Azevedo, V., François, P., Schrenzel, J., van de Guchte, M., Even, S., Berkova, N., Thiéry, R., Fitzgerald, J. R., Vautor, E. and Le Loir, Y. 2011. Molecular basis of virulence in *Staphylococcus aureus mastitis. PLoS One.,* **6**: e27354.

Le Thanh, B. V., Lebeau, C. J., Messier, S., Malouin, F. and Scholl, D. 2008. Epidemiologic association of *Staphylococcus aureus* virulence markers with intra-mammary infection chronicity. In: Lam T.J.G.M. (ed.). Mastits control: From Science to Practice, Wageningen Academic Publishers, Hague, Netherlands, pp. 167-174.

Lee, J.H., Jeong, J.M., Park, Y.H., Choi, S.S., Kim, Y.H., Chae, J.S., Moon, J.S., Park, H., Kim, S. and Eo, S.K. 2004. Evaluation of the methicillin-resistant *Staphylococcus aureus* (MRSA)-Screen latex agglutination test for detection of MRSA of animal origin. *J. Clin. Microbiol.*, **42**: 2780-2782.

Mir, A.Q., Bansal, B.K. and Gupta, D.K. 2014. Subclinical mastitis in machine milked dairy farms in Punjab: prevalence, distribution of bacteria and current antibiogram. *Vet. World.*, **7**: 291-294.

Monecke, S., Kuhnert, P., Hotzel, H., Slickers, P. and Ehricht, R. 2007. Microarray based study on virulence-associated genes and resistance determinants of *Staphylococcus aureus* isolates from cattle. *Vet. Microbiol.*, **125**: 128–140.

National Mastitis Council, 2004. Microbiological Procedures for the Diagnosis of Bovine Udder Infection and Determination of Milk Quality, 4 Edn. National Mastitis Council, Verona, pp. 47.

Park, K. T., Song, J. W., Chung, Y. S., Shin, S., Park, Y. K. and Park, Y.H. 2016. Characterization of Methicillin-resistant *Staphylococcus aureus* isolated from bovine mastitic raw milk in Korea. In: Annual meeting of International Association for Food Protection 2016, St. Louis, USA, July 31- Aug 3, 2016, P1-159.

Patel, J.V., Bhingaradia, B.V., Patel, B.B., Patel, S.B., Patel, P.B. and Vahora, S.P. 2012. Study on prevalence of mastitis and antibiotic sensitivity of bacterial isolates recovered from Cross-bred cows of Anand district of Gujarat. *Indian J. Dairy Sci.*, **65**: 467-471.

Pexara,A., Solomakos, N., and Govaris, A. 2013. Prevalence of Methicillin-resistant *Staphylococcus aureus* (MRSA) in milk and dairy products. *J. Hellenic Vet. Med. Soc.*, **64**: 17-34.

Piccinini, R., Borromeo, V. and Zecconi, A. 2010. Relationship between *Staphylococcus aureus* gene pattern and dairy herd mastitis prevalence. *Vet. Microbiol.*, **145**: 100–105.

Pilla, R., Snel, G.G., Malvisi, M. and Piccinini, R. 2013. Duplex real-time PCR assay for rapid identification of *Staphylococcus aureus* isolates from dairy cow milk. *J. Dairy Sci.,* **80**(2): 223-226.

Prashanth, K., Rao, K.R., Vivek Reddy, P.V., Saranathan, R. and Makki, A.R. 2011. Genotypic characterization of *Staphylococcus aureus* obtained from human and bovine mastitis samples in India. *J. Global Infect. Dis.*, **3**: 115-22.

Quantum GIS Development Team. 2015. QGIS 2.10.1 Pisa Geographic Information System. Open Source Geospatial Foundation Project. http://www.qgis.org/

Radostis, O.M., Gay, C.C., Hinchkliff, K.W. and Constable, P. D. 2006. Veterinary Medicine: A Textbook of the Diseases of Cattle, Sheep, Pigs, Goats and Horses, 10th Edn., Saunders, Oxford, pp. 673 - 749.

Resch, G., François, P., Morisset, D., Stojanov, M., Bonetti, E.J., Schrenzel, J., Sakwinska, O. and Moreillon, P. 2013. Human-to-Bovine jump of *Staphylococcus aureus* CC8 Is associated with the loss of a β-hemolysin converting prophage and the acquisition of a new Staphylococcal Cassette Chromosome. *PLoS One,* **8**: e58187.

Roberson, J.R., Fox, L.K., Hancock, D.D. and Besser, T.E. 1992. Evaluation of methods for differentiation of coagulase-positive staphylococci. *J. Clin. Microbiol.,* **30**: 3217-3219.

Ruegg, P. and Reinemann, D.J. 2002. Milk quality and mastitis tests. *Bovine Pr.,* **36**: 344 -356.

Sakwinska, O., Giddey, M., Moreillon, M., Morisset, D., Waldvogel, A. and Moreillon, P. 2011. *Staphylococcus aureus* host range and human-bovine host shift. *Appl. Environ. Microbiol.*, **77**: 5908–5915.

Sharma, A., Chhabra, R. and Sindhu, N. 2012. Prevalence of Sub clinical mastitis in cows: Its etiology and antibiogram. *Indian J. Anim. Sci.*, **46**: 348 – 353.

Sharma, N., Rho, G.Y., Hong, Y.H., Lee, T.Y., Hur, T.Y. and Jeong, D.K. 2012. Bovine mastitis: an Asian perspective. *Asian J. Anim. Vet. Adv.,* **7**: 454–476.

Singh, R.S., Kumar, R. and Yadav, B.R. 2011. Distribution of pathogenic factors in *Staphylococcus aureus* strains isolates from intra-mammary infections in cattle and buffaloes. *Indian J. Biotechnol.*, **10**: 410-416.

Spoor, L.E., McAdam, P.R., Weinert, L.A., Rambaut, A., Hasman, H., Aarestrup, F.M., Kearns, A.M., Larsen, A.R., Skov, R.L. and Fitzgerald, J.R. 2013. Livestock origin for a human pandemic clone of community-associated methicillin-resistant *Staphylococcus aureus*. *mBio.,* **4** : e00356-13.

Taponen, S. and Pyörälä, S. 2009a. Coagulase-negative staphylococci as cause of bovine mastitis—Not so different from *Staphylococcus aureus*? *Vet. Microbiol.*, **134**: 29–36.

Taponen, S. Salmikivi, L., Simojoki, H., Koskinen, M.T. and Pyörälä, S. 2009b. Real-time polymerase chain reaction-based identification of bacteria in milk samples from bovine clinical mastitis with no growth in conventional culturing. *J. Dairy Sci.*, **92**: 2610–2617.

Tille, P. M. 2014. Bailey and Scott's Diagnostic Microbiology, 13th Edn. Elsevier Mosby Inc., Missouri, USA, pp 193-246.

Tripathi. A. 2015. Prevalence and antimicrobial susceptibility pattern of methicillin resistant staphylococcus aureus in central India. *MedPulse.,* **2**: 45-48.

Weese, J.S. and Duijkeren van, E. 2010. Methicillin-resistance *Staphylococcus aureus* and *Staphylococcus pseudintermedius* in veterinary medicine. *Vet. Microbiol.*, **140**: 418-429.

World Health Organization. 2012. The evolving threat of antimicrobial resistance - Options for action, World Health Organization, Geneva,pp 1-119.

Yamada, T., Tochimaru, N., Nakasuji, S., Hata, E., Kobayashi, H., Eguchi, M., Kaneko, J., Kamio, Y., Kaidoh, T. and Takeuchi, S. 2005. Leukotoxin family genes in *Staphylococcus aureus* isolated from domestic animals and prevalence of *lukM-lukF-PV* genes by bacteriophages in bovine isolates. *Vet. Microbiol.*, **110**: 97–103.