



## Subclinical Bovine Mastitis in Rural, Peri-Urban and Suburban Regions of Jaipur District of Rajasthan, India

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### ABSTRACT

A cross section study was carried out from June 2013 to May 2014 on a total of 110 lactating cows of rural, peri-urban and suburban regions of Jaipur District of state of Rajasthan, for sub clinical mastitis by using California Mastitis Test (CMT), White side test (WST), Surf Field Mastitis Test (SFMT) and Somatic cell count (SCC). Prevalence of subclinical bovine mastitis in animal level was recorded as 67.27, 64.55, 63.64 and 74.55 % by CMT, WST, SFMT and SCC, respectively whereas 39.55, 38.86, 37.95 and 45.23 % by CMT, WST, SFMT and SCC, respectively in the level of quarters. *Staphylococcus* species (46.3%) occupied the prime position among the bacterial isolates followed by *Streptococcus* species (9.76%), *Escherichia coli* (6.1%), mixed growth (32.96%) and sterile growth (4.88%). Antibiotic susceptibility test revealed highest sensitivity towards Enrofloxacin. However, antibiotics showing higher rate of resistance patterns were Streptomycin, Penicillin G, Ampicillin, Cloxacillin, Amoxicillin, Kanamycin and Lincomycin. This reflects the poor quality of milk available to the consumers, lack of adequate hygienic practices, pre-emptive prophylactic regimen and indiscriminate use of antimicrobials.

**Keywords:** Subclinical mastitis, bovine, CMT, SFMT, WST, SCC, bacterial isolates, antibiotic sensitivity test

Mastitis is an inflammation of the mammary gland (Suojala *et al.*, 2011) The incidences of mastitis highly affects the economics of dairy industry, due to sudden drop in milk yield, increase in treatment costs, recovery time and finally culling of the affected cows (Bar *et al.*, 2008; Hertl *et al.*, 2011). Mastitis compromises animal welfare as well as its treatment, associated with human health hazard (Fogsgaard *et al.*, 2011; Rasmussen *et al.*, 2011). Bovine mastitis is categorized as one of the most problematic diseases affecting the dairy industry throughout the world (Bachaya *et al.*, 2011). It was the most prevalent and cost sparing diseases of dairy animals worldwide, with an annual economic loss of over 1.7 billion dollars in the USA (Sahoo *et al.*, 2012) and 526 million dollars in India (Varshney and Naresh, 2004). It is characterized by heat, redness, swelling, hardness and pain with abnormalities in milk like increased somatic cells, especially leukocytes,

in the milk and by pathological changes in the mammary tissue (Ranjan *et al.*, 2010). Various forms of clinical and subclinical mastitis occur in bovines. In clinical mastitis all the five cardinal signs of udder inflammation (redness, heat, swelling, pain and loss of milk production) are recorded, while in the sub-clinical form no obvious manifestations of inflammation are found. Subclinical mastitis is 3-40 times more common than clinical mastitis that causes greater overall loss in most dairy herds (Bachaya *et al.*, 2011). In 2004, Varshney and Naresh reported 70% economic losses due to subclinical mastitis. The situation has been compounded by the continued indiscriminate use of antibiotics without culture and sensitivity testing of milk. This may be attributed to callous approach of the dairy farmers, who instead of consulting qualified veterinarian, prefer to take over the counter supply of medicine by the drug retailers. Veterinarians who do not capitalize

on the available diagnostic tests are no less responsible for increase in the incidence of mastitis (Ranjan *et al.*, 2010). However, the detection of mastitis is difficult. Clinical mastitis is confirmed by observation of clinical signs by the farmer (direct detection) (Hokmabad *et al.*, 2011). Subclinical mastitis can be recognized by indirect detection: the somatic cell count in milk (Hokmabad *et al.*, 2011) or by animal-side milk tests (Bachaya *et al.*, 2011), but most of the farmers in Jaipur (rural), Rajasthan are not acquainted with these practices. Therefore it is indispensable to recognize and enumerate the causative organisms to review the sufficiency of the therapeutic armory, evade auxiliary complications and acclimatize management practices for the efficient control of mastitis.

The purpose of this investigation was to elucidate the prevalence of subclinical mastitis in apparently healthy dairy cows in rural, peri-urban and suburban regions of Jaipur district of state of Rajasthan, to find out the major causative agents causing subclinical mastitis and to study their drug sensitivity. The study was conducted on 110 cows in the Jaipur rural region of state of Rajasthan, India.

## MATERIALS AND METHODS

### Location

The study was conducted on randomly selected apparently healthy cows who visited Teaching Veterinary Clinical Complex, M.J.F. College of Veterinary and Animal

Science, Chomu, Jaipur during a period of 11 months from June 2013 to May 2014. Cows which were tested under this study mostly belong to rural, peri-urban and suburban regions of Jaipur district of state of Rajasthan. The study area was found at and around 26.9260° N longitude, 75.8235° E latitude with an altitude range of 431 m above sea level. Jaipur has a semiarid subtropical climate, receiving over 650 millimetres (26 in) of rainfall annually only during the monsoon. The average daily temperature is around 30°C during summer and 15-18°C during winter.

### Animals

In the present study, a total number of 110 (440 quarters) apparently healthy cows without any clinical signs of mastitis were screened for SCM in and around Jaipur District during the period of 11 months from June 2013 to May 2014. Animals were managed under extensive and semi-intensive production system. The traditional extensive production system consists of indigenous breeds graze for feed with minor supplementations. On the other hand, in the semi-intensive production system, the animals are mainly belongs to crossbred cattle. They are reared indoors with occasional grazing in the field. They are supplemented with concentrates in addition to the natural pasture, crop by products, straw. This type of dairy husbandry system is booming and becoming an important source of milk supplies to households, nearby Dairy plants and a means of income generation in rural and peri-urban

**Table 1 :** Animal wise and quarter wise prevalence of subclinical mastitis detected by four screening tests.

Tests used	Types	Sample tested	Positive cases	Prevalence (in %)	95% Confidence Intervals	Chi-square value	P-value
California mastitis test (Score > 1+)	Animal wise	110	74	67.27	55.97-76.75	13.127	0.000*
	Quarter wise	440	174	39.55	34.25-44.89	19.236	0.000*
White side test (Score > 1+)	Animal wise	110	71	64.55	53.16-74.31	9.309	0.000*
	Quarter wise	440	171	38.86	33.59-44.20	21.827	0.000*
Surf field mastitis test (Score > 1+)	Animal wise	110	70	63.64	52.23-73.49	8.182	0.000*
	Quarter wise	440	167	37.95	32.71-43.28	25.536	0.000*
Somatic cell count (SCC > 5 × 10 <sup>5</sup> )	Animal wise	110	82	74.55	63.65-83.07	26.509	0.000*
	Quarter wise	440	199	45.23	39.784-50.615	4.009	0.000*

**Table 2.:** Bacteria isolated from 82 numbers of cases of SCM in cows.

Bacteria	Number of isolates	Percentage (%)
<i>Staphylococcus</i> Spp.	38	46.3
<i>Streptococcus</i> Spp.	8	9.76
<i>E. coli</i>	5	6.1
<i>Staphylococcus</i> spp.+ <i>Streptococcus</i> spp.	10	12.2
<i>Staphylococcus</i> spp.+ <i>E. coli</i>	9	11
<i>Streptococcus</i> spp. + <i>E. coli</i>	5	6.1
<i>Staphylococcus</i> spp.+ <i>Streptococcus</i> spp. + <i>E. coli</i>	3	3.66
Negative growth	4	4.88

areas of Jaipur District, Rajasthan, India. Manure removal is generally made on a daily basis. Although milking is done by hand, pre- and post-milking hygienic protocols, such as washing of udder and subsequent drying are not followed. All the tested animals were apparently healthy during preceding lactations. Full hand method of milking was performed twice a day (6 and 18 h). Among the 110 animals 64, 25, 13 and 8 were crossbred Holstein Friesian, Haryana, Rathi and Non-descript breeds, respectively.

#### White side test (WST)

One drop of 4 per cent sodium hydroxide and five drops of milk from each quarter were placed on the glass slide and mixed with a glass rod (Doxey, 1985). Results were read after 20 sec, according to the change in viscosity of milk as negative, 1+, 2+ and 3+. Samples scoring 1+, 2+ or, 3+ considered as positive case for subclinical mastitis.

#### Somatic cell count (SCC)

It was done as described by Schalm *et al.* (1971). Milk was mixed thoroughly before testing. Ten microliter of milk from each quarter was spread over 1 cm<sup>2</sup> marked square area on a glass slide. The milk film was left undisturbed at room temperature until it dried, and then the smear was fixed in Xylol for 5 min and stained with Loffer's methylene blue reagent. Cell counting was made under oil immersion as per the procedure described by Dhakal (2006). Animal sample, showing somatic cell count more

than  $5 \times 10^5$  of somatic cells, is considered as positive case for subclinical mastitis as per criteria cited by International Dairy Federation (IDF) and Hegde *et al.* (2013).

**Table 3.** Antibiotic sensitivity pattern in term of high to moderate and mild to resistant antibiotic sensitivity for selection of antibiotics for therapeutic use.

List of the antibiotics with its MIC ( $\mu$ g)	Bacterial Isolates			
	H, Mo		Mi, R	
	No.	%	No.	%
Enrofloxacin (Ex, 10)	76	92.7	6	7.3
Ciprofloxacin (Cf, 10)	73	89	9	11
Amikacin (Ak,30)	70	85.4	12	14.6
Ceftriaxone (Ci, 30)	66	80.5	16	19.5
Chloramphenicol (C, 30)	60	73.2	22	26.8
Cephotoxime (Ce, 30)	56	68.3	26	31.7
Gentamicin (G, 10)	47	57.3	35	42.7
Pefloxacin (Pf, 5)	40	48.8	42	51.2
Cephalexin (Cp, 30)	37	45.1	45	54.9
Neomycin (N, 30)	31	37.8	51	62.2
Lincomycin (L, 10)	25	30.5	57	69.5
Kanamycin(K, 30)	23	28	59	72
Amoxycillin (Am, 10)	22	26.8	60	73.2
Cloxacillin(Cx, 10)	21	25.6	62	74.4
Ampicillin(A, 10)	18	22	64	78
Penicillin G (PG, 10)	13	15.9	69	84.1
Streptomycin(S, 10)	10	12.2	72	87.8

#### Surf Field Mastitis Test (SFMT)

This test was performed and scored following the method described by Muhammad *et al.* (2010) in brief, about 2 ml milk from each quarter was drawn from bottle into test cup and an estimated 2 ml of 3% solution of household detergent (Surf Excel®, Uniliver, India Ltd.). Mixing was accomplished by gentle circular motion of the paddle in a horizontal plane for few seconds. The reaction developed almost immediately with milk containing a high concentration of somatic cells. The peak of reaction was obtained within 30 seconds and immediately scored as 1+, 2+ and 3+.



### California mastitis test (CMT)

The California mastitis test was carried out as screening test for selections of samples for culture following the method described by Schalm *et al.* (1971) and Quinn *et al.* (1994). A squirt of milk, about 2 ml from each quarter was placed in each of four shallow cups in the CMT paddle. An equal amount of the commercial reagent was added to each cup. A gentle circular motion was applied to the mixtures, in a horizontal plane for 15 sec. The reaction was interpreted according to Schalm *et al.* (1971), Quinn *et al.* (1994) and David *et al.* (2005). The results of visible reactions were classified into 5 scores: (0) = negative, ( $\pm$ ) = trace, (+1) = weak positive, (+2) = distinct positive, and (+3) = strong positive. In this study, CMT score of 1+ and above was considered positive for mastitis and of trace and negative ( $\pm$  and 0) together was considered negative for subclinical mastitis.

### Milk Sampling

Quarters that scored negative and trace were assumed healthy and the quarters with different positive scores through any screening tests were assumed infected. A cow with at least one affected quarter at the time of examination was considered positive for subclinical mastitis. Similar criteria were used to characterize a cow positive for SCM (Sharma *et al.*, 2007). All the animals found positive in SCC were selected for further analysis under bacteriological prevalence and antibiotic sensitivity test. Only one of the infected quarters from each cow was selected for milk sampling except when the cow had four severely infected quarters so one more milk sample was collected. Then the teat end of the selected quarter was swabbed with cotton soaked in 70% ethyl alcohol and 10 milliliters of milk was approximately collected into sterile containers. The first 3-4 streams of milk were discarded. The collecting vial was as near horizontal as possible and by turning the teat to a near horizontal position, 15 ml of milk was collected into the vial. Samples were transported to the laboratory in a special box with ice at 4°C for bacteriological investigation (Quinn *et al.*, 1994).

### Bacteriological culture and identification of microorganisms

The samples were subjected to bacteriological study in the laboratory by inoculating approximately 0.01 ml of

milk sample on to blood agar, nutrient agar, MacConkey's agar, Sabourads dextrose agar and Eosine Methylene Blue agar plates and the plates were incubated "under aerobic conditions" at 37° C for 24 to 48 hours. The staining and cellular morphological features of organisms were ascertained by microscopic examination of Gram stained smears. The bacteria isolated were identified on the basis of their cultural, morphological and biochemical characteristics as per the method of (Cruickshank *et al.*, 1975).

### Antibiotic susceptibility test

All the isolates were subjected to in vitro drug sensitivity test as per method described by Bauer *et al.* (1966). The antimicrobials ( $\mu$ g) available commercially in market like cloxacillin (10), amoxycillin(10), streptomycin (10), penicillin G (10), and neomycin (30) were tested for their in vitro effectiveness against various bacterial isolates. In addition, old and new generation antimicrobials ( $\mu$ g) like chloramphenicol (30), lincomycin (10), gentamicin (10), ciprofloxacin (10), cephalixin (30), enrofloxacin (10), amikacin (30), cefotaxime (30), ceftriaxone (30), pefloxacin (5), kanamycin (30) and ampicillin (10) were also tested. The antibiotic discs (Hi-Media<sup>®</sup>, Mumbai, India) were impregnated on the surface of an agar plate previously inoculated with a standard amount of the organism under scrutiny. The plates were incubated at a temperature of 37°C for duration of 18 - 24 hours. Subsequently, the plates were examined for the zone of inhibition developed around the discs, followed by the diameter of the zone of inhibition was measured in millimeter and compared with the values listed in standard chart provided by the manufacturer, on the basis of which the isolates were categorized as resistant (R), mildly sensitive (Mi), moderately sensitive (Mo) or highly sensitive (H) to the antimicrobial contained in that particular disc (Ranjan *et al.*, 2010).

### Statistical analysis

All collected data were entered in Microsoft Office<sup>®</sup> 2007 excel sheet and analyzed by SYSTAT<sup>®</sup> version 12 computer package program. In the present study chi-square test and confidence intervals were calculated.

## RESULTS AND DISCUSSION

Prevalence of subclinical bovine mastitis in animal level was recorded as 67.27, 64.55, 63.64 and 74.55 % by CMT, WST, SFMT and SCC, respectively whereas 39.55, 38.86, 37.95 and 45.23 % by CMT, WST, SFMT and SCC, respectively in the level of quarters, as summarized in Table 1. The figures on both animal wise and quarter wise prevalence of SCM based on individual tests closely proximated with the observations of Sharma *et al.* (2004 and 2007). However, both lower (Sharma and Sindhu, 2007; Sharma and Maiti, 2010; Supriya *et al.*, 2010; Bachaya *et al.*, 2011; Hegde *et al.*, 2013) and higher (Muhammad *et al.*, 2010) prevalence rates of SCM have been reported in the literature. This large variability of prevalence of SCM found around Jaipur could be due to prevalence of risk factors e.g., a large proportion of cow confined to zero grazing production system, differences in management practices and poor hygienic standards of the dairy environment cum milking conditions. Other factors that could influence the prevalence of SCM could be due to immune responses, genetic variation in disease resistance amongst the breeds, some heritable characteristics such as milk production capacity, teat structure, udder conformation, use of different methods of diagnosing of subclinical mastitis and the definition of infection, which is variable according to Mdegela *et al.* (2005). According to IDF criteria, 15.38 % quarters of cows were suffering from sub clinical mastitis on account of having somatic cell count (SCC) more than 5,00,000 per ml of milk and culturally positive. The prevalence rate of SCM on IDF criteria was lower than cultural examination or SCC alone. These findings are in agreement with the observations of Tuteja (1993) and Sharma and Kapur (2000), Supriya *et al.* (2010) and Hegde *et al.* (2013).

Among all the four indirect tests, SCC showed highest efficacy of 74.55 % with respect to diagnosis of SCM. The efficacy of SCC is followed by CMT, WST and SFMT which in accordance to Sharma *et al.* (2008). Hence all the animals found positive in SCC were selected for further analysis under bacteriological prevalence and antibiotic sensitivity test. The data summarized in Table 2 indicates the relative occurrence of various bacteria isolated from cows. The pathogens isolated from 82 milk samples, found positive under SCC, were *Staphylococcus* spp. 38 (46.3%), followed by *Streptococcus* spp. 8 (9.76%), *Escherichia coli* 5 (6.1%), mixed growth 27 (32.96%) and

no growth were found in 4 (4.88%) milk samples. Among the mixed growth, prevalence of *Staph.* spp. and *Strep.* spp. was the most predominant combination with 12.2% of prevalence. This was followed by *Staph.* spp. + *E. coli* combination, *Strep.* spp. + *E. coli* combination and *Staph.* spp. + *Strep.* spp. + *E. coli* combination with 11%, 6.1% and 3.66% prevalence, respectively.

On cultural examination the *Staph.* spp. was found to be the chief etiological agent causing SCM. This finding is in agreement with the earlier reports of Sharma and Sindhu (2007); Sumathi *et al.* (2008); Sharma and Maiti (2010); Harini and sumathi (2011) and Ranjan *et al.* (2011). The highest incidence of *Staph.* spp. are closely associated with hygiene. It becomes pathogenic whenever the hygienic conditions of the animal or environment become meager. Moreover, the existence of high concentration of *Staph.* spp. in milk also indicates the relatively poor quality of milk, related with unhygienic milking practices as this pathogen is mainly spread during milking via milkers' hands [Bradley 2002]. This also might be due to harboring of the organism in the skin, udder and milk of the infected gland which acts as reservoir (Olmsted and Norcross, 1992). Davidson (1961) have shown that the ability of *Staph.* spp. to bind to epithelial cells of the ductile and alveoli in mammary gland is also an important virulence factor.

*Strep.* spp. was the second largest mastitogen group of isolates recovered from our experiment. This was in accordance with reports of Sahoo *et al.*, (2009) and Sharma and Maiti (2010). *Strep.* spp. which is an obligate parasite of the epithelium and tissue of mammary gland, multiplies in the milk and on the mammary epithelial surfaces, generally causing a subacute or chronic inflammatory reaction with periodic acute flare-ups. The affected tissue eventually is destroyed resulting in reduced milk production (Sharma *et al.*, 2012).

The *E. coli* isolates in the present study accounted for 6.1% share and third most prevalent organism among different isolates of mastitis milk, which is very low with respect to previous literature (Ranjan *et al.*, 2011). Despite *E. coli* is the environmental pathogen, but low or sporadic incidence of *E. coli* has also been reported by various workers (Shukla *et al.*, 1998) as observed in this study. Opsonization of bacteria by IgM with subsequent phagocytosis and killing by neutrophil are some of the factors, which prevent

establishment of *E. coli* mastitis (Gyles and thoen, 1993). These inherent properties of udder defense against *E. coli* infection might be responsible for reduced incidence of *E. coli* mastitis in the present study. Prevalence of *E. coli* is an indication of poor hygienic practices in dairy environment, as these organisms originate from the cow's environment and infect the udder through the teat canal. Contamination of end of the teat is a major predisposing factor in the development of environmental mastitis (Bradley, 2002; Sumathi *et al.*, 2008; Sharma *et al.*, 2012).

Different combinations of the mastitogenic organisms were detected in mixed infection. Most predominant combination of the isolates was *Staph. spp.* and *Strep. spp.* followed by *Staph. spp.* and *E. coli* similar to findings of Srinivasan *et al.* (2013). Therefore, it is of principal magnitude that a particular dairy herd/organized farm should be screened for the disease routinely, espouse an efficient treatment protocol and sound prophylactic measures, that would prevent serious economic losses in terms of decreased milk production, cost of treatment and culling of animals.

The failure of some pathogens to grow in vitro may be due to the fact that certain microorganisms require specific culture media (Ranjan *et al.* 2010). It could also be explained by the possible premedication of the animals with antibiotics (Hawari and Al-Dabbas, 2008) because the withdrawal time may not have been respected.

The data summarized in Table-3 indicates the zone of inhibition and antibiotic sensitivity of the isolates. Out of 82 isolates obtained from cases of SCM from cows tested for their antibiogram, revealed percent of isolates were most sensitive to Enrofloxacin (92.7%), followed by Ciprofloxacin (89%), Amikacin (85.4%), Ceftriaxone (80.5%), Chloramphenicol (73.2%), Cephotoxime (68.3%), Gentamicin (47%), Pefloxacin (40%), Cephalexin (37%) and Neomycin (31%). On the contrary, antibiotics showing higher rate of resistance patterns were Streptomycin, Penicillin G, Ampicillin, Cloxacillin, Amoxicillin, Kanamycin and Lincomycin showing 87.8%, 84.1%, 78%, 74.4%, 73.2%, 72% and 69.5% resistance, respectively.

The emergence of drug resistant organisms causing mastitis due to indiscriminate use of antibiotics is well known. Moreover, due to lack of prophylactic agents,

chemotherapy continues to play a major role in the therapeutic management of the disease. For success of the treatment, sensitivity testing plays a pivotal role. Recently newer antibiotics have been introduced for the treatment of both sub clinical and clinical mastitis. Thus, it has become imperative to control this dreaded disease with most effective antibiotic therapy. Hence the present study was also designed to prove into in-vitro sensitivity of isolated bacterial strains from cases of SCM against a range of traditional as well as newly introduced antibiotics potentially useful for the treatment and control programme (Sharma *et al.*, 2007).

Highest sensitivity of bacteria towards Enrofloxacin is in agreement with Sahoo *et al.*, (2009); Ranjan *et al.*, (2010) and Sharma *et al.* (2012). Isolates in the present study showed moderate sensitivity or resistance to Streptomycin and Penicillin – G. Indiscriminate and frequent use of these antibiotics in animals could be the reason for their ineffectiveness against bacterial isolates. Similar observations of resistance towards Penicillin were also observed by Ranjan *et al.* (2010) and Harini and sumathi (2011).

The distressing level of resistance of organisms to a particular drug might be due to the indiscriminate use of the respective drugs. In view of the diversified gamut of pathogens resulting in mastitis, its control needs the selection of apt antimicrobial by establishing an antibiogram. Hence, the control of mastitis should be directed by administration of distinct regime of antibiotics and holistic advance to the disease management.

Hence the present study concluded that prevalence of subclinical mastitis in rural, peri-urban and suburban provinces of Jaipur was found to be highly contagious. The rationale behind this high prevalence is multifaceted, but there are a few points need scrupulous elucidation such as lack of regular screening for early detection and prophylactic treatment, inadequate housing with improper sanitation of environment, udder and milker's hand, zero grazing, late stage of lactation, high parity; all seem to augment the risk of getting SCM. Although we did not perform any statistics on it, the results of this study provide new information and will hopefully contribute to a possibly lower prevalence of SCM in the future.

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