JOURNAL OF PURE AND APPLIED MICROBIOLOGY, October 2008. Vol. **2**(2), p. 607-613

Early Diagnosis and Milk Quality Assessment of Subclinical Mastitis by Somatic Cell Counts and its Correlation with Pathogenic Bacteria in Crossbred Jersey Cows

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(Received: 12 April 2008; accepted: 04 June 2008)

Sub-clinical mastitis, a highly prevalent chronic disease of crossbred cows in Orissa, reduces milk production to the extent of 10 to 20 % and also significantly decreases the quality of milk by influencing the fat, protein and carbohydrate concentration of milk. Both the microorganisms and toxins produced by microorganisms in subclinical mastitis milk affects the human health. The economic loss due to subclinical mastitis has been estimated up to 40%. The diseases is considered to be highly dangerous as it has no outward clinical manifestation and difficult to diagnose at early stage with the presently known diagnostic methods. Hence it has been attempted to diagnose the subclinical mastitis and assess the milk quality by somatic cell counts (SCC) and correlate the SCC with microbial load of mammary gland for prognosis. The milk samples from 400 quarters of 100 crossbred Jersey cows (2nd lactation) of small herd dairy units in and around Bhubaneswar preliminarily screened for subclinical mastitis by Modified Californian Mastitis Test. The preliminary screening of milk samples revealed that about 41 % of the quarters were positive for subcliniacal mastitis. The total microbial load (× 105 CFU/ml) of the subclinical mastitis milk (21.94±0.38) was significantly (P<0.01) higher than the unaffected cows (1.85±0.02). Bacteria like *Staphylococcus aureus, Streptococcus agalatiae, Bacillus spp., Alcaligen* spp. and *Pseudomonas* spp. were found to be predominant.

Key words: Subclinical mastitis, Diagnosis, Microbial load, Milk composition, Somatic cell counts.

Sub-clinical mastitis poses a serious threat to dairy animals, especially purebred and crossbred cows with high incidence rate and is very difficult to control. Generally producers put an emphasis on clinical mastitis and underestimate the significance of sub clinical mastitis, not realizing that for every clinical case in the herd there are 15 to 40 subclinical cases. Subclinical mastitis accounts for high economic losses in dairy farms due to reduction in milk yield and milk quality (Tyler *et al*., 1989). Global monetary loss due to mastitis alone is put at 140 billion US dollars per annum. In our country the economic losses due to sub clinical mastitis is estimated to be about Rs. 4300/- crores and in Orissa the loss due to both Behera *et al.*: Early Diagnosis and Milk Quality Assessment of Subclinical Mastitis by Somatic Cell Counts clinical and subclinical mastitis is around Rs. 356,557,469 of which large chunk is attributable to subclinical mastitis (Sahoo and Parida, 2002). It is a highly prevalent chronic

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disease of crossbred cows in Orissa, reduces milk production to the extent of 10 to 20 % and also significantly decreases the quality of milk by influencing the fat, protein and carbohydrate concentration of milk. Sometimes the microorganisms and toxins produced by microorganisms and anti-microbial drug residues in sub-clinical mastitis milk seriously affect the human health. The economic loss due to sub-clinical mastitis has been estimated up to 40%. The diseases is considered to be highly dangerous as it has no outward clinical manifestation and difficult to diagnose at early stage with the presently known diagnostic methods. Hence it has been attempted to diagnose the sub-clinical mastitis and assess the milk quality by somatic cell counts (SCC) and correlate the SCC with microbial load of mammary gland for prognosis.

MATERIAL AND METHODS

Screening of milk samples for subclinical mastitis

One hundred crossbred Jersey cows within 3 to 4 months of their 2nd lactation were randomly selected from small herd dairy units in and around Bhubaneswar city, Orissa. The milk samples collected aseptically from 400 quarters of the cows were screened for subclinical mastitis at fortnightly intervals during experimental period by the Modified California Mastitis Test (MCMT) as described by Singh *et al*. (2001). The MCMT reagent comprised of sodium hydroxide 1.5g, teepol 0.5ml, and bromothymol blue 0.10g and distilled water up to 100 ml.

Collection of milk samples

Ten cows both from normal and sub clinical mastitis group were randomly selected and 100 ml of milk samples were collected in sterilized containers by adding hydrogen peroxide as preservative. The milk sampling was conducted at 15 days intervals for two months. Immediately after collection of milk samples transported to the laboratory for further processing and analysis.

Microbiological analysis

The milk samples collected from the crossbred Jersey cows of Bhubaneswar of Orissa state were processed in the laboratory of P.G Department of Microbiology and Department of Animal Nutrition, Orissa University of Agriculture and Technology to determine the total aerobic bacterial load, isolation of different strains of bacteria, making axenic culture of the strains, studying their morphological and physiological parameters, assigning strain no, identification through a series of biochemical characteristics and also other features required for their characterization following standard microbiological techniques of Collins and Lyne (1970) and Hansen and Sorheim (1991).

Enumeration of aerobic bacteria

Enumeration of total aerobic bacteria load in the samples was carried out by 10-fold serial dilution technique followed by spread plating. Diluents were prepared with sterilized phosphate buffer having pH 7.2 to avoid osmotic shock to bacteria. From each dilution tube 100ìl of samples were used for spread plating on presterilised Nutrient Agar (NA) plates. Three replicates from each dilution were used for spread plating to minimize the error. All the plates were incubated at 37^0C for 24-48 hours. Plates having viable colony counts (30-300 colonies per plate) were selected for the enumeration of the milk samples. The total microbial load per ml of sample was estimated by multiplying number of bacterial colonies with dilution factor and expressed as total count/ml of milk.

Pure culture and maintenance of the bacterial isolates

The selected bacterial colonies showing different morphological features were picked up from the NA plates and were restreaked several times on presterilised NA plates to obtain pure culture of the isolates. The pure cultures of the isolates were preserved by restreaking on NA slants at 4°C for future use. Viability of strains was maintained by subculturing the isolates at every 4 weeks interval on fresh NA slants.

Identification of the bacterial isolates

The isolated bacterial strains from the normal and subclinical mastitis milk samples of crossbred Jersey cows of Bhubaneswar were identified and confirmed by colony characteristics on different media, morphological characteristics by staining the culture smears by Gram's staining method, different biochemical tests, various sugar utilization tests, pH tolerance capacity and enzymatic characterization and standard microbiological techniques as described by Collins

and Lyne (1970). The frequency distribution of microorganisms isolated from sub clinical mastitis milk was calculated.

Test for enzymatic activity of bacterial isolates

All the isolates were screened on pseudo-selective media for important exocellular enzyme activities like amylase, lipase, pectinase, protease and DNAse following standard microbiological methods of Collins and Lyne (1970).

Antibiotic sensitivity test of the bacterial isolates of subclinical mastitis milk

An antibiotic sensitivity test was conducted on four pathogenic strains of bacteria viz. *Pseudomonas spp.*, *Proteus penneri*, *Staphycoccus aureu* and *Klebsiella spp.*, isolated from subclinical mastitis milk of crossbred jersey cows by following the disc diffusion method of Bauer *et al*. (1966) for which Muller Hinton Agar was used. The test organisms were spreaded over the surface of pre-sterilized Muller Hinton Agar plates with the help of a sterilized cotton swab. Precaution was taken while sweeping the plates with broth culture of the test organisms to make a uniform lawn culture of the isolate. Selective antibiotic discs were put uniformly throughout the MHA plates. The antibiotic discs were selected depending on their use and mode of action. The pates were incubated at 37°C for 24 hours. The zone of inhibition was measured with the help of a scale and was categorized as sensitive, moderately sensitive and resistant.

Measurement of Somatic Cell Count of milk samples

The milk samples collected on 0, 15th, 30th 45th and 60th day of experimental period were utilized for counting somatic cells by the microscopic method developed by Singh and Ludhri (2001).

Determination of composition of milk samples

Two hundred fifty milliliter of milk samples were collected by using hydrogen peroxides as preservatives from 10 subclinical mastitis and 10 uninfected (normal) cows at 0, 15th, 30th, 45th and 60th day of experimental period. The samples were analyzed for fat by Gerber's butyrometer method (IS: 1224, 1977), crude protein and lactose by Roy and Sen (1994), and pH by using a Sytronic pH meter. The SNF and total solids were calculated by using Richmond's modified formula.

Statistical analysis

The data were analyzed statistically following the methods of Snedecor and Cochran (1998).

RESULTS AND DISCUSSION

Screening of milk samples for the prevalence of subclinical mastitis

The screening results of milk samples for subclinical mastitis collected from small herd dairy owners around Bhubaneswar have been presented in Table 1. Out of 400 quarters on an average of 162.60±2.56 quarters were positive for subclinical mastitis in Modified Californian Mastitis Test during an experimental period of 60 days. The average number of quarters infected with subclinical mastitis was 40.65±0.64 %. The present findings are in concordance with the findings of a number of researchers (Singh and Singh, 1994; Kadar *et al*., 2002) who reported the incidence of subclinical mastitis to the extent of 37 to 46.61%. The high incidence of subclinical mastitis in various small herd dairy units that has been recorded in the present investigation might be due to poor managemental practices or might be due to emergence of antibiotic resistant strains.

Microbial load of milk samples

The average microbial load of milk samples collected at fortnightly intervals (Table 2) was significantly ($P<0.01$) higher in subclinical mastitis milk (21.94±0.38 x 10^5 CFU/ml) compared to normal milk (1.85±0.02× 10^5 CFU/ml). As evident from the percent frequency of various genera of bacterial isolates (Table 3), *Staphylococcus* spp. was the most frequent isolates, accounting for 90% of the isolations followed by *Streptococci* spp. (80%), *Bacillus* spp. (80%), *E. coli* (70%), *Klebsiella* (70%), *Alcaligens* (70%), *Pseudomonas* (60%). Lowest was the *Acenatobacter* spp. (10%). Similar results of high incidence of *Staphylococcal* (Ajariyakhajorn and Smngamnim, 2006) and *Streptococcal* (Leigh, 2003) have been reported. *Staphylococcus* was found to be the most common cause of subclinical mastitis in the present study which might be due to presence of large numbers in various body sites, such as teat surfaces and orifices due to unhygienic

Table 1. Screening of milk samples of crossbred Jersey cows for sub clinical mastitis

Period (d)	No. of cows screened	No. of quarters screened	Normal quarters	Sub clinical mastitis quarters	Percentage of positive quarters
0	100	400	242	158	39.5
15th	100	400	242	158	39.5
30th	100	400	240	160	40.0
45th	100	400	234	166	41.5
60th	100	400	229	171	42.75
Avg. ±S.E.	100.00±0.00	400.00±0.00	237.40±2.56	162.60±2.56	40.65±0.64

conditions and invading properties of *Staphylococcus* within the mammary gland, macrophage and polymorphonuclear cells and the capacity of pathogens to produce a polysaccharide capsule to form the host factors. Further *Staphylococcus* is highly pathogenic organism because it can survive inside the phagocytic cells after being phagocytosed. It is not digested inside the cells because of the loss of hydrolytic enzymes and many other reasons. As antibiotics cannot penetrate into the somatic cells it affects the cell healing by antibiotic therapy. Similar to the present investigation, Silva and Silva (2005) reported high prevalence rate of *Staphylococcus* in milk. It might be due to *Streptococci*, which are unable to survive for longer period in the environment outside the body.

Table 2. Average microbial loads of milk samples collected from normal and sub clinical mastitis cows

Period (d)	Microbial load (x10^{5}CFU/ml)	
	Normal milk	Sub clinical mastitis milk
0	1.80^a±0.11	21.70^b±1.07
15th	1.81^a±0.12	20.59^b±1.39
30th	1.88^a±0.09	22.33^b±1.16
45th	1.88^a±0.09	22.34^b±1.05
60th	1.87^a±0.08	22.76^b±1.1
Overall Avg. ±S.E.	1.85^a±0.02	21.94^b±0.38

Values bearing different superscripts in rows varied significantly ($P<0.01$)

Table 3. Frequency distribution of different organisms in milk samples of subclinical mastitis cows

Types of organisms isolated	No. of positive samples	Percentage of distribution
Staphylococcus aureus	9	90
Streptococcus agalactiae	8	80
Escherichia coli	7	70
Proteus penneri	3	30
Enterobacter cloaceae	3	30
Klebsiella spp.	7	70
Pseudomonas spp.	6	60
Alcaligenes spp.	7	70
Acenetobacter spp.	1	10
Acenetobacter iwoffi	1	10
Moraxella urethalis	2	20
Pasteruella spp.	2	20
Bacillus polymyxa	8	80
Bacillus cereus	7	70

E. coli is not generally pathogenic for udder and causes only mild infection (Singh and Singh, 1994). The low incidence of Gram-negative rods was mainly due to their destruction by mammary gland.

Antibiogram of microorganisms isolated from subclinical mastitis milk

Sensitive test of microorganisms isolated from subclinical mastitis milk (Table 4) revealed that *Proteus penneri* and *Klebsiella* spp. were sensitive to all the antibiotics whereas Staphylococcus spp. was resistant to doxycyclin hydrochloride and vancomycin and Pseudomonas spp. was resistant to only Vancomycin. Gatifloxacin was highly effective antibiotic against the microorganisms responsible for subclinical mastitis.

Enzyme activity of the isolates

The detailed enzymatic activities of 10 bacterial strains isolated from subclinical mastitis milk of Bhubaneswar, Orissa were given in Figure 1. Analysis of the data on the enzymatic activity of all the 10 strains showed that 50%, 60%, 70%, 40% and 70% of the isolates were found to be potential producers of DNAse, caseinase, amylase, lipase and pectinase, respectively. It was also observed that most of these bacteria were capable of producing more than one type of enzymes. The enzymes produced by these bacteria might have contributed towards the deterioration of milk quality in subclinical mastitis by digesting milk nutrients

Table 4. Antibiogram of organisms isolated from subclinical mastitis milk

Species	Sensitive	Moderatively sensitive	Resistant
Pseudomonas spp.	Am, Ce, Pi, Gf	Dc, Ct, G	V
Proteus penneri	Ce, Ct	Am, Dc, Pi, V, G, Gf	
Staphylococcus aureus	Gf	Am, Ce, Pi, Ct, G	Dc, V
Klebsiella spp.	Pi, Gf	Am, Ce, Dc, V, Ct, G	

Antibiotics used:
Am: Amikacin, **Ce**: Cephotaxim, **Dc**: Doxycycline Hydrochloride, **Pi**: Piperacillin/Tazobactum,
V: Vancomycin, **Ct**: Ceftriaxone, **G**: Genetamycin, **Gf**: Gatifloxacin

Somatic cell counts (SCC) of the milk samples

Somatic cell count of the milk is a measure of udder health and milk quality. Though the average SCC vary depending on the season and breeds of animal still it remains in the physiological limits irrespective of parity and stage of lactation. The average SCC ($\times 10^5$cells/ml) in buffaloes, crossbred cows and goats were about 1.0, 1.5 and 0.8, respectively (Singh and Ludri, 2001). The cells counts beyond the physiological limits (> 2.5 to 3.0 lakhs/ml) indicate abnormal cell secretion and some pathological state of mammary gland. According to some researchers, if quarter milk has 1.0 x 10^5 cells/ml, the udder is considered to be healthy. If the counts exceed 2.5×10^5 cells/ml, it can be presumed that there are some disturbances in normal secretion process. A cell count of 3.0 x 10^5 cells/ml or above may indicate that the mammary gland has got infection and cell count above 4.0×10^5 cells/ml indicate the udder is diseased (Hillerton, 1999). In the present investigation the somatic cell counts of the quarters milk samples, which were negative in MCMT varied between 1.4 to 1.45 lakhs/ml and somatic cell counts of the quarter milk samples showing positive for subclinical mastitis milk varied between 2.5 to 2.77 lakh/ml. The average somatic cell counts of subclinical mastitis milk were significantly ($P<0.01$) higher than normal milk (Table 5). The present finding corroborates with the findings of Schukken *et al*. (2003) and contradicts Czerw *et al*. (2004) who observed no correlation between pathogenic bacteria isolated from the mammary gland and the somatic cell counts in milk samples infected with subclinical mastitis.

Milk quality

The fortnightly average percentage of milk fat, protein, lactose, SNF and total solids

Table 5. Average somatic cell counts (x lakhs/ml) of normal and subclinical mastitis milk of crossbred Jersey cows

Period (d)	Somatic cell counts (SCC)	
	Normal milk	Sub clinical mastitis milk
0	$1.40^{a}\pm0.05$	$2.52^{b}\pm0.10$
15^{th}	$1.45^{a}\pm0.05$	$2.56^{b}\pm0.09$
30^{th}	$1.43^{a}\pm0.04$	$2.62^{b}\pm0.09$
45^{th}	$1.44^{a}\pm0.06$	$2.71^{b}\pm0.09$
60^{th}	$1.43^{a}\pm0.05$	$2.77^{b}\pm0.08$
Overall Avg. ±S.E.	$1.43^{a}\pm0.01$	$2.64^{b}\pm0.04$

Values bearing different superscripts in rows varied significantly (P<0.01)

significantly (P<0.01) reduced and pH of milk significantly increased in subclinical mastitis cows compared to normal cows (Table 6). Milk composition progressively decreased as the days of exposure of mammary gland to the infective organisms increased. The decreased fat and protein contents of subclinical mastitis milk might be due to reduced synthetic and secretary capacity of mammary gland by bacterial toxins. Further increased production of lipolytic enzymes by leucocytes, which damaged the fat globules, might have decreased the fat percentage of milk. In mastitis the amounts of á-casein, â-casein and á-lactalbumin may decrease, whereas, amounts of immunoglobulins, serum albumin and k-casein increase resulting in decrease product quality and stability. The decrease in lactose content of milk during subclinical mastitis might be due to leakage of lactose into the extra cellular fluid and blood after the destruction of normal lactose barrier (Karimuribo *et al*., 2005). The reduction in SNF and TSS content of subclinical mastitis milk might be due to reduced milk fat, protein and lactose secretion due to infection of mammary gland. The increased pH of subclinical mastitis milk might be due to leakage of blood bicarbonate into milk following damage to mammary epithelium. The increasing pH may decrease the activity of enzymes responsible for clotting the milk and may have severe implications in production of quality milk products.

Correlation of somatic cell counts with microbial load, milk composition and pH

The somatic cell counts of milk increased significantly with the increase in microbial load of milk samples with a correlation coefficient of 0.99. Similarly the pH of the milk was positively correlated with somatic cell

Table 6. Composition of normal and subclinical mastitis milk of crossbred Jersey cows

Parameters	Normal milk	Sub clinical mastitis milk
Total solids	$14.04^{a}\pm0.03$	$10.63^{b}\pm0.07$
SNF	$9.96^{a}\pm0.03$	$8.21^{b}\pm0.18$
Fat	$4.37^{a}\pm0.04$	$3.96^{b}\pm0.06$
Protein	$3.71^{a}\pm0.01$	$3.31^{b}\pm0.05$
Lactose	$4.77^{a}\pm0.03$	$4.11^{b}\pm0.12$
pH	$6.55^{a}\pm0.01$	$6.84^{b}\pm0.03$

Values bearing different superscripts in rows varied significantly (P<0.01)

Table 7. Correlation coefficient of microbial load, milk composition and milk pH with somatic cell counts

Parameters	Correlation coefficient
Total microbial load	0.99
Total solids %	-0.99
SNF %	-0.98
Fat %	-0.91
Protein %	-0.96
Lactose %	-0.92
pH %	0.97

counts. The milk composition with respect to total solids, SNF, fat, protein and lactose contents were negatively correlated with the somatic cell counts (Table 7).

CONCLUSION

It is concluded that the somatic cell counts can be used as a tool for early diagnosis of subclinical mastitis and assessment of milk quality.

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