

ISOLATION AND IDENTIFICATION OF MICROORGANISMS FROM BOVINE MASTITIS INFECTED MILK SAMPLES AND THEIR ANTIBIOGRAM

Mahenthiran R.* and Michael A.

Department of Microbiology, PSG College of Arts and Science¹, Coimbatore-641014.

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***Correspondence for
Author**

Mahenthiran R.

Department of
Microbiology, PSG
College of Arts and
Science¹, Coimbatore-
641014.

ABSTRACT

Bovine Mastitis is an inflammatory reaction of the udder tissue which is caused by the bacterial infection. Bovine mastitis is one of the devastating disease causing heavy economic loss to the dairy industry in worldwide. The present study investigated the current status of clinical mastitis among dairy cattles in Coimbatore. The mastitis infected milk samples (n=64) were assessed at based on positive reaction to California mastitis test (CMT)(Strong positive 35.93%), surf field mastitis test (SFMT)(Strong positive 40.62%), NaOH test (Strong positive 37.5%),PH and bacteriological evaluation of milk samples. The major pathogens isolated from the milk samples were *S.aureus*, *E.coli*, *Klebsiellasp*, *coagulase negative staphylococci*

(CNS), *Streptococcus species*, *Bacillus sp*, *Pseudomonas species* and *Proteus sp*. Antibioqram studies were also conducted for the isolates by using twelve antibiotics like amoxycillin, methicillin, amikacin, kanamycin, endrofloxacin, penicillin-G, Vancomycin, chloramphenicol, streptomycin, gentamicin, tetracycline and oxytetracycline. which were used in the regular veterinary practice for the treatment of mastitis. The bacterial examination revealed that the predominant cause is *Staphylococcus aureus* (26.50%). The most effective antibiotic was chloramphenicol (63.68%). Antibioqram studies of mastitis pathogens are important to suggest suitable antibiotic treatment to provide quality milk to the consumers and to prevent antibiotic resistance, potential health risk for humans.

KEY WORDS: Bovine mastitis, CMT, SFMT, NaOH, Bacterial pathogens, Antibioqram.

INTRODUCTION

Mastitis in dairy animals occurs when the udder becomes inflamed and bacteria invade the teat canal and mammary glands. These bacteria multiply and produce toxins that cause injury to the milk secreting tissue, besides, physical trauma and chemical irritants. These cause increase in the number of leukocytes, or somatic cells in the milk, reducing its quantity and adversely affecting the quality of milk and milk byproducts¹¹. The economic losses due to mastitis comprises of reduced milk yield (up to 70%), milk discard after treatment, cost of veterinary services (7%) and premature culling (14%)⁵. The disease is usually classified as subclinical, acute, subacute and chronic based on etio-pathological findings and observations¹⁴. Under the classification disease large numbers of infectious agents are responsible in causing the disease in dairy animals. Bacterial agents like *Staphylococcus spp.*, *Streptococcus spp.*, *Escherichia coli*, *Corynebacterium spp.*, *Klebsiella spp.*, *Pseudomonas spp.*, Mycoplasmal agents, fungal agents, viral agents are responsible for the disease. About 95% of intramammary infections are caused by *Staphylococcus spp.* and *Streptococcus spp.*. The remaining 5% are caused by other organisms²⁴. The potential impact of transmission of resistant bacteria to humans via the food chain, as in bulk milk with sub-clinical mastitis is a public health problem¹⁹. Indirect determination of the number of somatic cells in milk. Among the indirect tests, CMT, WST, SFMT are commonly used for indirect somatic cell count as indicator of subclinical mastitis both in cows and does³. Maintaining hygiene with antimicrobial therapy plays a role in mastitis control by reducing the levels of herd infection. Among different tests, California Mastitis Test (CMT), White Slide Test (WST) and Surf Field Mastitis Test (SFMT) are considered as simple, easily applicable, rapid indirect screening tests for determining SCM⁴. Reagents of these tests contain detergents which change the structure and conductivity of cell membrane and nucleus of somatic cells, stimulate proteolytic enzymes, and increase milk viscosity⁷. The purpose of study is to do the different indirect tests and isolation bovine mastitis causing pathogens and their antibiogram profile from mastitis infected milk samples¹⁶.

MATERIALS AND METHODS

1. Collection Mastitis milk samples

64 Bovine Mastitis infected cow was observed physical observation and milk samples were collected in and around Coimbatore. Milk samples were collected aseptically and transfer to the laboratory for further process.

2. Physical examination of milk sample

Immediately after collection, milk samples were subjected to physical examination with naked eyes to detect any abnormalities in colour, odour, consistency and presence of clot, blood, lumps and any other visible abnormalities²⁵.

3. Indirect screening tests

a) pH determination

In this study pH was used as one of the important parameter as the indicator of mastitis where the normal milk pH is 6.6 – 6.7 where as milk with higher pH indicates the positive test for mastitis¹⁷.

b) California mastitis test

The procedure of CMT was followed in this study as per manufacturer's instruction (M&S Industries, Pune, India). In brief, about 2 ml milk was drawn from bottle into the cup and an estimated equal volume of CMT reagent was squirted from a polyethylene wash bottle^{12,14}. 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^{20,23}. The peak of reaction was obtained within 10 seconds^{10,1}. The score was recorded while continuing to rotate the paddle. The test result was interpreted based on the thickness of the gel formed by CMT reagent and milk mixture, and scored as, weak positive (+), distinctive positive (++), and strong positive (+++). Quarters with CMT score of (+) or above were judged as positive^{2,6,7}.

b) Surf field mastitis test

The samples were subjected to surf test. For this purpose, 3% Surf solution was prepared by addition of three grams of commonly used detergent powder (Surf Excel, Unilever, India.) in 100 ml of water. Milk samples and surf solution were then mixed in equal quantities in glass slides^{10,12}. This test was performed and scored following the method described by Muhammad *et al*^{14,1}. Mixing was accomplished by gentle circular motion of the paddle in a horizontal plane². 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 6, 7.

c) NaoH test

The test is based on the increase in number of leukocytes in mastitis milk. The WST was performed as per procedure described by Aminul Islam *et al.*,^{3,9}. 2ml of milk and 1ml of

WST reagent (4% NaOH) was placed on a glass slide using with a toothpick for 20-25 seconds. The gel formation indicative of positive reaction. On the other hand, milky and opaque and entirely free of precipitant was indicative of negative reaction⁷.

4. Isolation and identification of bacterial agents

After the conformation using with different screening tests each milk samples were carried out by culturing the milk samples. One loopful of milk samples were streaked on to 5% sheep blood agar, McConkey agar, Mannitol salt agar, Eosin Methylene Blue agar and nutrient agar plates¹⁵. The inoculated plates were incubated at 37°C under aerobic condition for 24-48 hours. Suspected colonies were subcultured and examined their colony characteristics, morphological, microscopic observation and biochemical tests²¹.

5. Antibacterial susceptibility testing

Microbiologically Isolated bacteria were subjected to antimicrobial susceptibility test by disc diffusion method to identify the most effective drugs for mastitis treatment in the study area. Antibiotic susceptibility screening was done as per the guidelines of National Committee for Clinical Laboratory Standards (NCCLS)^{1, 2}. Kirby- Bauer's disc diffusion technique was adapted for antibiogram. The antibiotic discs and Mueller- Hinton Agar were purchased from Hi-Media, Mumbai. The plates were prepared as per the manufacturer's instructions and checked for sterility by incubating the plates overnight at 37°C. All the isolated bacteria were tested in-vitro for their sensitivity to 14 different antibiotics, that are commonly used in veterinary practice ^{8, 17}. Commercially available antibiotics used in this study, amoxicillin (10µg), amikacin (30µg), kanamycin (30µg), Methicillin (5µg), enrofloxacin (5µg), penicillin (10units), streptomycin (10µg), gentamycin (10µg), cloxacillin (10µg), chloramphenicol (30µg), tetracyclin (30µg), oxytetracyclin (30µg)^{19, 22}.

6. Statistical analysis

The scores from the Indirect tests (CMT, SFMT and NaOH) and laboratory tests were recorded and compiled using the computer software programme (Microsoft Excel, 1998) and standard deviation method.

RESULT

1. Analysis of milk

A total of 64 bovine mastitis infected milk samples were screened PH and SCC level by surf field mastitis test (SFMT) and California mastitis test¹⁸. The results were showed in figure 1.

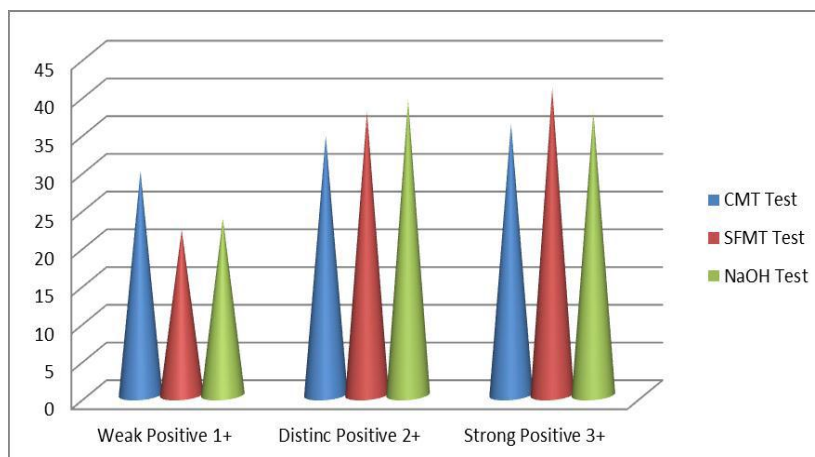


Figure:1 Comparison of Screening tests to determine the severity of mastitis infected milk samples

2. Bacteriological analysis

8 different types of microorganisms were isolated from 64 bovine mastitis infected milk samples and the percentage of bacteriological analysis^{15, 21} showed in figure 2.

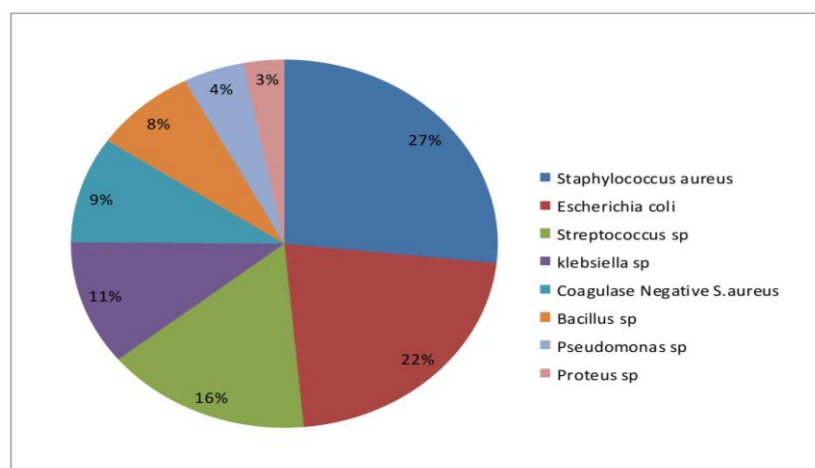


Figure 2: Percentage of different bacterial isolates from the bovine mastitis milk samples

3. Antibacterial susceptibility test

All the isolated bacteria were subjected to Antimicrobial susceptibility using 12 different antibiotics (AM, M, AK, K, EN,P,S,G,V,C,T, and O) commonly used in Veterinary Practice²². The antibiotic susceptibility test revealed that highest number of *staphylococcus aureus* were susceptible to Cloxacillin 70.58% and the least number of *S. aureus* were susceptible to penceillin G 5.88%. The antibiotic susceptibility tests showed that highest number of *E.coli* were susceptible to Amoxycillin 85.71% and least susceptible to

Endrofloxacin and Tetracyclin 35.71%.The antibiotic susceptibility test suggested that highest number of *Streptococcus species* weresusceptible to chloramphenicol 80% and least susceptible to Kanamycin 30%.

In antibiotic susceptibility test *Klebsiella species* showed highest susceptibility to amoxicillin 85.71%, and least susceptible to amikacin 28.57%.The antibiotic susceptibility test for coagulase negativestaphylococcus were highest number of susceptibility to Cloxacillin and Chloramphenicol 66.66% and least number of susceptibility to Streptomycin16.66% respectively.The antibiotic susceptibility test indicated that highest number of other *bacillus species* weresusceptible to Amoxycillin, Amikacin and Tetracyclin 80% and least to kanamycin 40%.The antibiotic susceptibility test suggested that highest number of *Pseudomonasspecies*wereSusceptibleAmikacin, Endrofloxacin and Chloramphenicol 66.66% (Figure 3)and least to Kanamycin, 33.33%.The antibiotic susceptibility test suggested that highest number of *Proteus species* wereSusceptible Endrofloxacin and Gentamycin 100% and least to Kanamycin and Chloramphenicol 50%. All isolates were resistant to Methicillin.

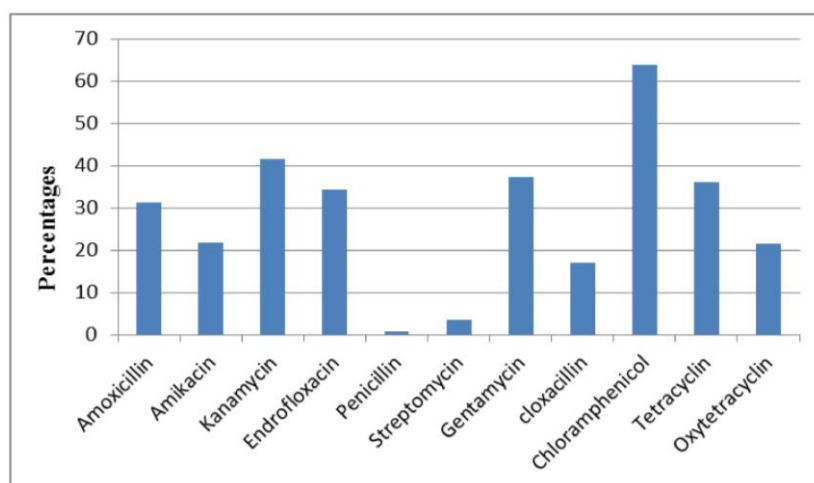


Figure 3: Percentage recovery of bacterial isolates from mastitis infected milk samples

DISCUSSION

Successful breeding from the health as well as economic point of view is highly dependent on the healthy udder of dairy animals. The affected cows and buffaloes produce less milk in lactation and in the entire life. Inflammation of mammary gland, increased cells count can be detected easily using surf test, NaOH test and pH meter and CMT test. In the present study 64 bovine mastitis infected milk samples were examined for severity and infection causing agent. Stage of the mastitis infection was tested using indirect screening tests like PH

determination pH of normal milk is 6.6 or 6.7. In this study tested the infected milk samples were showed above 7.0 because infection is present the pH increase to alkalinity which approximates to that of blood (7.2 ± 0.2). Next important three tests (CMT, SFMT and NaOH) were showed the somatic cell count increasing level based on the gel formation. These three tests used solution was anionic in nature and the infected milk samples having the acidic in nature. When the mixture of these CMT, NaOH and SFMT reagent with infected milk samples. A chemical reaction causes gel formation and the gel formation was divide into four types weak positive (+) distinctive positive (++) and strong positive (+++) and these CMT, NaOH, SFMT and pH test are a good and economic methods used for the diagnosis of mastitis and farmers should use these techniques to test the animal before purchasing and avoid buying the animals which shows positive for these test⁷. Strong positive results for these tests were 37.5% (NaOH test), 40.62% (SFMT test) and 35.93% (CMT test). Mastitis is a result of interaction between three elements like bacteria, cow and environment. In the present study staphylococcus species may be due to the incomplete milking and especially when it is associated with the painful lesions or any wounds on the outer surface of the udder. Staphylococcus is an opportunistic pathogenic bacteria which survive on the skin of the udder and can infect the udder via teat canal or any wound¹. Further, the prevalence of enterobacteria species in the present study may be due to the poor hygienic condition in the herds and this infection is becoming more and more frequent which tends to follow the infection of staphylococcus species. The prevalence of streptococcal species may be due to poor dairy practicing methods, it is the contagious organism which infects other healthy animals in the herds. The prevalence of bacillus species in the present study may be due to the environmental factors like soil water and manure, these are the main source of bacteria and when animals are exposed to water, soil and manure these bacteria infect animals via teat canals. Similar findings showed that higher incidence of *E.coli* may be due to poor hygienic conditions as *E.coli* originate from the cows environment and infect the udder via the teat canal. Moreover, staphylococci, pseudomonas and mixed growth were the second after *E.coli*. their presence was also an indication of sub-standard hygiene of farm management². Therefore, the above findings indicate that mastitis can be controlled by hygienic conditions in the herds like keeping the animals away from the stagnant water, cleaning manure, use of germicidal solution for washing udder before milking and culling of infected animals⁸. In the study 8 different types of pathogens were found there are *Staphylococcus aureus*, *Escherichia coli*, *Coagulase negative staphylococcus*, *Klebsiella species*, *Streptococcal species*, *Bacillus species*, *Pseudomonas species* and *Proteus species*. In present study, *Staphylococcus*

aureus(26.5%) was isolated as top ranking pathogen from cases positive for mastitis. In previous studies, it was also reported as major pathogen (Mahantesh. *Met al.*, 2011:Nadeem Akram*et al.*, 2013) Further in the present investigation antibiogram studies were also conducted for the isolates by using fourteen antibiotics which were used frequently in this area for the treatment of mastitis and Chloramphenicol (63.68%) was found to be more effective antibiotic among all the tested antibiotics against all the bacteria isolated in the present study followed by Amoxicillin, Amikacin, kanamycin, Methicillin, enrofloxacin, penicillin, streptomycin, gentamycin, vancomycin, chloramphenicol, tetracycline, oxytetracyclin.

CONCLUSION

In conclusion hygiene should be maintained at every aspect of dairy farms and screening tests like CMT, WST and SFMT are easily applicable and cost effective tests for regular screening of clinical mastitis cost,easy availability and adoptability. Proper isolation and identification of the causative organism play significant role in prevention and control of the diseases. Therefore, establishing an antibiogram of pathogens is very important from the clinical and economic points of view. Therefore, the findings of the present study showed that Chloramphenicol is the most effective antibiotic which can be used for control of bovine mastitis infected cows in the area of research. Present findings suggest need to routinely investigate and record the epidemiology of bovine mastitis and antibiogram sensitivity of bacterial isolates.

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