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Original Research Article

Isolation and Identification of Major Pathogen from Clinical and Subclinical Mastitis Milk Sample and Their Antibiotic Sensitivity Evaluation

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A B S T R A C T

The present study was conducted to isolate and identify the major pathogens from clinical and subclinical mastitis milk samples and their antibiotic sensitivity evaluation. A total of 360 samples were collected from Bihar Agricultural University dairy farm Sabour, Bhagalpur and adjoining villages and screened for presence of mastitis. Five samples were found positive for presence of clinical mastitis (15%) and twenty nine samples (85%) for subclinical mastitis which shows prevelance of subclinical mastitis in this area. Upon microbiological testing and 16S ribosomal DNA sequencing the major pathogen associated with clinical and subclinical mastitis were identified as coagulase negative *Staphylococcus sps.* (*S. saprophyticus*, *S. haemolyticus*, *S. agnetis*) 54%, followed by coagulase positive *Staphylococcus aureus* (26%) and Streptococcus and *Bacillus* sps. (6%).Antibiotic senstivity testing was done and majority of the pathogens were found sensitive to Enrofloxacin, Ciprofloxacin, Tetracyclin, Streptomycin and Penicillin and they were found resistant to commonly used antibiotics Ampicillin, Cloxacillin, Amoxycillin and Chloramphenicol. Our study indicates the need of field based screening of subclinical mastitis and antibiotic sensitivity testing of the bacterial pathogens before administration of drugs in order to reduce the economic loss caused by mastitis and also to reduce development of antibiotic resistance in bacteria.

Keywords

Mastitis, Sub clinical mastitis, Clinical mastitis, Antibiotic

Introduction

Mastitis is inflammation of mammary gland and is characterized by physical, chemical and usually bacteriological changes in glandular tissue (Santos *et al.*, 2003). Defense cells migrate from the blood tothe mammary gland to combat the infectious agents, which increases milk somatic cell count (Rainard and Riollet, 2006). There are two forms of mastitis prevalent in terms of level of severity; clinical and subclinical. Clinical form of mastitis shows visible symptoms whereas subclinical form does not show any visible symptoms. Mastitis causes a great deal of economic losses, due to reduction of milk yield, decreased milk quality, higher production and medication costs, loss of milking days, reduced milk price, increased labour (Seegers *et al.*,

2003). Mastitis can also affect product shelf life (Barbano *et al.,* 2006) and cheese making properties (Ma *et al.,* 2000). It is one of the most prevalent and costly disease for dairy farmers and industry. In United states annualeconomic loss to dairy industry by mastitis is estimated to be 2 billion dollars (Bogni *et al.,* 2011). Out of the total economic loss 70 per cent loss is due to reduction in milk production and discard of milk from sick animals. Other causes are the elimination of milk containing residues of antibiotics used in treating sick animals, loss of genetic stock by culling cows early and therefore more expensive replacement, veterinary fees, cost of medicines and payment of extra labour hours. In India, annual economic loss to dairy industry by mastitis is estimated to be Rs. 7165.51 crores out of which loss subclinical mastitis account for 57.93 per cent (4151.16 crores) of the total economic loss due to mastitis [PDADMAS 2011].

Etiological agents of mastitis are widespread ranging from various bacteria, viruses (Wellenberg *et al.,* 2002), and fungi (Farnsworth, 1977) the most common cause are gram-positive and gram negative bacteria (Zecconi *et al.,* 2005). The major bacterialagents involved in causing mastitis are identified as Staphylococcus aureus, streptococci, coliforms, Mannheimia species, Arcanobacterium pyogenes, Pasteurellaspecies, coagulase-negative staphylococci and Corynebacteriumbovis (Dingwell *et al.,* 2003). Microbiological testing is necessary for identification of etiological agent of mastitis. Indiscriminate use of antibacterial drugs without testing in vitro drug sensitivity of causal organism leads to failure of treatment and development of resistant bacteria which can transmit to humans via the food chain, in bulk milk with sub-clinical mastitis will cause a public health problem (Owens et. al., 1997 and Solet. al., 2000). This study was done with the purpose to isolate and identify etiological agents of clinical and subclinical mastitis from positive milk samples and evaluation of their antibiotic senstivity in order to suggest suitable antibiotic treatment to the farmers. This will reduce dairy production cost and prevent antibiotic resistance development in bacteria.

Materials and Methods

Collection of milk samples

Milk samples were collected from Bihar Agricultural University dairy farm Sabour, Bhagalpur and adjoining villages. Milk samples were collected from every quarter of cow udder aseptically using sterile vials and stored at 4°C until processed.

Sodium lauryl sulphate test (SLS test)

This test was used for clinical and subclinical mastitis detection. This test is similar to California mastitis Test in principle the difference is that in this 3% sodium lauryl sulphate is used instead of CMT reagent. This solution (test reagent) was prepared by adding 3 gm of sodium lauryl sulphate powder to 100 ml of distilled water. The suspension was heated to 50°C so as to make a clear solution. The pH of the solution was adjusted to 8.0 by using HCL or NaOH as per the need. A pinch bromophenol blue was added to give blue color to the solution. One milliliter of 3% sodium lauryl sulphate solution was mixed in petri dish with equal quantity of milk drawn from every quarter. This was mixed by rotation and examined for clot formation.

Surf Field Mastitis test

This test was also done in order to detect clinical and subclinical mastitis. Two

milliliters of surf (surf excel 3%) solution was mixed in petri dish with equal quantity of milk drawn from every quarter. This was mixed by rotation and examined for clot formation.

Culture of microorganisms

Milk samples found positive in SLS test and Surf field mastitis were further inoculated on nutrient agar, Macconkey's agar and Staphylococcus medium no. 110.The inoculated plates were incubated aerobically at37^{o}C for 24-48 hours. The bacterial isolates were identified further on the basis of their morphological, gram staining characteristics and biochemical reactions for the identification of causative agent.

Gram staining of bacterial culture

Bacterial colony was mixed with drop of water on glass slide and thin smear was prepared and dried. Smear was flooded with crystal violet solution for two minutes.

Then the slide was washed with distilled water and gram's Iodine was applied for one minute. After that 95% alcohol was applied until the color runs off. Finally dilute carbolfuschin was applied for about one minute. Then the slide was washed with distilled water and examined under oil immersion. Identified gram positive and negative bacteria were subjected for biochemical test.

Catalase test

A loopful of test culture was mixed with 2-3 drops of 3 percent hydrogen peroxide on a clean glass slide and examined for the release of nascent oxygen in the form of gas bubbles. A positive reaction was indicated by the effervescence of oxygen within 1-2 minutes.

Coagulase test

A small drop of distilled water was placed on glass slide and one or two colonies of culture was mixed in water to make a smooth suspension. A drop of citrated plasma was added to the suspension and mixed with needle. A positive reaction was indicated by clumping of cocci within 5-10 seconds.

Identification of bacterial culture using 16S rDNA sequencing

DNA was isolated from bacterial culture. Quality was evaluated on 1.2% Agarose Gel. Isolated DNA was amplified with 16S rRNA Specific Primer (8Fand 1492R) Fig.1. The PCR amplicon was enzymatically purified and further subjected to Sanger Sequencing. Bi-directional DNA sequencing reaction of PCR amplicon was carried out with 704F and 907Rprimers using BDT v3.1 Cycle sequencing kit on ABI 3730xl Genetic Analyzer. Consensus sequence of 1533 bp 16S rDNA was generated from forward and reverse sequence datausing aligner software. The 16S rDNA sequence was used to carry out BLAST alignment search tool of NCBI Genbank database. Based on maximum identity score first Fifteen sequences were selected and aligned using multiple alignment software program ClustalW. Distance matrix was generated using RDP database and the Phylogenetic tree was constructed using MEGA5. 16S ribosomal DNA sequences were submitted in NCBI database.

Antibiotic Senstiivity Test

Milk samples found positive in SLS test and Surf field mastitis were subjected to antibiotic sensitivity test using Masti Test ABST Test kit-Himedia (Cat. No. KO91-1KT). Standard procedure given in the kit

literature was followed to study the antibiotic sensitivity and resistance of bacteria present in Mastitis milk sample.

Results and Discussion

Among 360 samples tested 41 milk samples were confirmed to bepositive for bovine mastitis by sodium lauryl sulphate test (SLS test) and surf field mastitis test. Microorganisms could be isolated from 34 (83%) samples, while 7 (17%) samples did not yield any isolate.

Among 34 mastitis cases 5 cases (15%) were of clinical mastitis with visible symptoms of udder inflammation namely redness, heat, swelling, pain, and clots or discoloration of milk and rest 29 cases (85%) were of subclinical mastitis with no visible symptoms (Table 1). These findings are inline with reports of Patel *et al.,* (2012) and PDADMAS 2011 which reported 46.8% and 57.93% of incidence of subclinical mastitis respectively.

Genomic DNA was isolated from 11 bacterial isolates and 16S ribosomal DNA sequencing was done. 16S ribosomal DNA sequences of isolated bacteria were submitted in NCBI database: Staphylococcus saprophyticus strain-4b (Accession no. KY819040), Staphylococcus aureus strain-7T1 (Accession no. MF661886), Staphylococcus haemolyticus strain-7T4 (Accession no.MF661887), Staphylococcus aureus strain 10-T3 (Accession no. KY941096), Staphylococcus agnetis strain-11T1 (Accession no. MF661888), Staphylococcus agnetis strain 11-T4 (Accession no. MF661893), Staphylococcus haemolyticus strain-15a (Accession no. KY819096), Staphylococcus saprophyticus strain-15b (Accession no.KY819138), Staphylococcus aureus strain-S22 (Accession no.KY432815), Bacillus sp. strain S41 (Accession no.KY435720) and Bacillus cereus Strain-1 (Accession no. MG754417).

Among the isolated bacteria coagulase negative *Staphylococcus* sps. (CNS) was the most prevalent 19 (56%) followed by coagulase positive *Staphyococcus aureus* 9(26%), *Streptococcus* spp. 4 (12%) and *Bacillus* sps. 2(6%).

In our study subclinical cases were more (83%), so prevalence of coagulase negative *Staphylococcus* sps. among the bacterial isolates is in line with previous reports of Jakee-El *et al.,* (2013); Pyorala *et al.,* (2009) who reported that CNSis generally high in subclinical mastitic samples, but low insamples from animals with clinical mastitis. Reports on theclinical characteristics of coagulase negative staphylococcalmastitis are scarce as CNS have been ignored in many studieson clinical mastitis, so that CNS infection mostly remained subclinical (Pyorala *et al.,* 2009). In another report Bhalerao *et al.,* (2000) noted that major pathogenicorganisms were *Staphylococcus aureus* (54.55%) following by the *Streptococci* (36.36%), *E. coli* (4.55%) and *Klebsiella* (2.27%).

The identified species of CNS in the current study were *Staphylococcus haemolyticus, Staphylococcus saprophyticus, Staphylococcus agnetis* which is in line with previous reports where it has been reported that the common staphylococci isolated from mastitic herds were *S. chromogenes, S. hyicus, S. simulans, S. epidermidis, S. hominis, S. haemolyticus, S. xylosus, Staphylococcus warneri, Staphylococcus sciuri, Staphylococcus capitis, S. saprophyticus* and *S. lentus* (Sawanta *et al.,* 2009; Thorberg *et al.,* 2009; Piessens *et al.,* 2011).

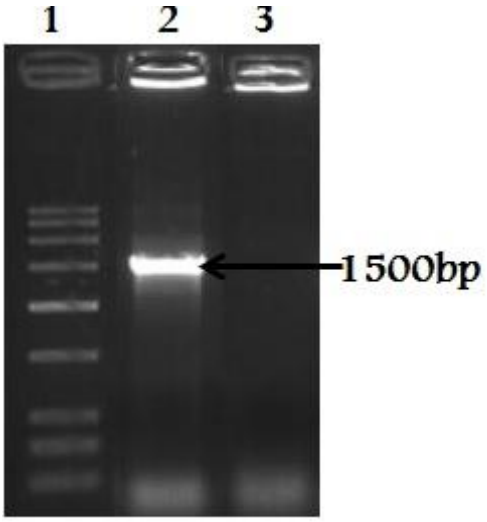
Table.1 Screening of Mastitic milk sample for presence of Mastitis by Sodium Lauryl Sulphate test (SLS test), Surf field mastitis test and bacteriological culture

Sample Collected		No. of samples +ve in Sodium lauryl sulphate Test(SLS Test)	No. of samples +ve in Surf Field Mastitis Test	Bacteriological Culture	Subclinical Cases	Clinical Cases
BAU Dairy Farm	160	16	16	12	10	2
Farmers Field	200	25	25	22	19	3
Total	**360**	**41**	**41**	**34**	**29**	**5**

Table.2 Antibiotic sensitivity testing of Samples

Sample Name	Ampicillin/ Cloxacillin	Amoxycillin/ Cloxacillin	Gentamcin	Enrofloxacin	Ciproflox acin	Tetracycl in	Chloramphe nicol	Streptomyci n/Penicillin
22-T1	Resistant	Resistant	Sensitive	Sensitive	Sensitive	Sensitive	Resistant	Sensitive
41-T1	Resistant	Resistant	Moderate Resistance	Sensitive	Sensitive	Sensitive	Resistant	Sensitive
24-T3	Resistant	Moderate Resistance	Resistant	Sensitive	Sensitive	Sensitive	Resistant	Sensitive
7-T4	Moderate Resistance	Resistant	Moderate Resistance	Sensitive	Sensitive	Sensitive	Resistant	Sensitive

Fig.1 16s Ribosomal DNA PCR



Lane1: DNA Ladder; Lane2: 16S PCR product; Lane 3: Negative control

In our study majority of the bacterial pathogens were found to be sensitive to Enrofloxacin, Ciprofloxacin, Tetracyclin and Streptomycin/Penicillin and resistant to Ampicillin/Cloxacillin, Amoxycillin/Cloxacillin and Chloramphenicol. Our result is concordance with previous report of Patel *et al.*, 2012 who have found Gentamicin, Enrofloxacin and Ceftriaxone as most effective drugs against mastitis. So in present study we conclude that there is higher incidence of

subclinical mastitis in field condition which reflects the bad quality of milk availability. This is due to lack of regular screening for sub-clinical mastitis which is beingnot practiced in the field by the farmers. Coagulase negative Staphylococcus sps. (CNS) are most prevalent (56%) form of pathogen followed by coagulase positive *Staphyococcus aureus* (26%), Streptococcus spp. (12%) and Bacillus sps. (6%). Most of the bacterial pathogensare sensitive to Enrofloxacin, Ciprofloxacin, Tetracyclin Streptomycin and Penicillin so these antibiotics can be used for mastitis treatment. Thus our present study indicates the need of screening of subclinical mastitis at field level as it degrade milk quality, productivity of dairy farming posing great economic loss to the farmers. Antibiotic sensitivity testing before administraton of antibiotics is required as most of the mastitic pathogenshave been found to be resistant to commonly used antibiotics Ampicillin, Cloxacillin, Amoxycillin and Chloramphenicol. Administration of antibiotics after ding antibiotic sensitivity testing will reduce treatment cost and the economic loss caused to the dairy farmers.

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