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Coexpression of Methicillin-resistant *S. aureus* and ESBL Producing *E. coli* in Mastitic Milk of Buffaloes

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ABSTRACT

Background: Mastitis is mostly caused by mixed infections, of which *S. aureus* and *E. coli* are amongst the most important bacteria. Detection of resistant strains of these bacteria is a major public health concern due to the possibility of infection *via* contaminated milk and milk products intended for human consumption. This study describes simultaneous occurance of methicillin-resistant *S. aureus* and extended spectrum β-lactamase producing *E. coli* in mastitic milk samples of buffaloes in Jabalpur, India.

Methods: A total of 408 buffaloes milk samples were screened for mastitis by California mastitis test (CMT) test. A total of 102 mastitic milk samples were collected for isolation of *Staphylococcus aureus* (*S. aureus*) and *Eschirichia coli* (*E. coli).* Polymerase chain reaction was performed for molecular characterization of methicillin resistant *S. aureus* for mecA gene and ESBL producing *E. coli* for *bla* TEM gene, respectively.

Result: On the basis of cultural and biochemical characteristics, 31/102 samples yielded *S. aureus* and 10/102 samples yielded *E. coli*. Out of 31 *S. aureus* isolates, mecA gene was positive in 05 (16.1%) and out of 10 ESBL producing *E. coli* isolates *bla* TEM was positive in 02 (20%) isolates. Coexpression of *bla* TEM and mecA genes was recorded only in 01 (10%) isolate.

Key words: Buffaloes, Coexpression, ESBL, Mastitic milk, Polymerase chain reaction.

INTRODUCTION

Mastitis is inflammation of parenchyma of mammary glands or udder and is characterized by physical, chemical and usually bacteriological changes in milk and pathological changes in glandular tissues of udder (Radostits *et al.*, 2000). Mastitis is considered to be the most common cause of injudicious antibiotic use in dairy animals such as wrong drug dose, or duration contribute to the increase in antimicrobial resistance without improving the outcome of treatment (Williams, 2000).

Mastitis is generally classified into clinical and subclinical mastitis. Clinical mastitis is characterized by local (*e.g.* swelling of the udder, heat and pain) or systemic (*e.g.* fever, anorexia, depression) while, subclinical mastitis is characterized by symptoms with milk abnormalities (*e.g.* milk clots, flakes, watery secretions, blood) (Gruet *et al.*, 2001). Bacteria causing mastitis are of two types based upon their primary reservoir and mode of transmission. Environmental bacteria include coliform species like *E. coli, Klebsiella* sp. and species of Streptococci. These arise from the environment in which the buffalo lives, when teats are exposed to mud and manure and dirty bedding materials. Contagious Bacteria like *Staphylococcus aureus*, *Streptococcus agalactiae* are transmitted among cows by contact with infected milk (Cremonesi *et al.*, 2006).

In India the prevalence of bovine mastitis due to *Staphylococcus aureus* (*S.aureus*) is around 30-40% (Patel *et al.,* 2012 and Sharma *et al.,* 2012). Mastitis caused by *Staphylococcus aureus* bacteria is extremely difficult to control by treatment alone because of property of methicillin-resistant *S. aureus* (MRSA) to impart resistance to all β-lactam anti-microbial agents. *S. aureus* organisms colonize abnormal teat ends or teat lesions. It is capable of causing peracute, acute, subacute, chronic, gangrenous and subclinical types of mastitis. The acute form of the disease usually occurs shortly after parturition and tends to produce gangrene of the affected quarters with high mortality (Rahman *et al.*, 2010 and Sharma and Maiti, 2010).

Escherichia coli (E. coli) is also reported to be one of the commonest pathogens in mastitis and the infected quarter shows swelling, pain with discharge of watery or bloody milk. Necrosis of the mammary epithelium occurs during severe, naturally occurring clinical *E. coli* mastitis, as well as during severe experimental *E. coli* mastitis (Bradley and Green, 2001). The multiplicity of the cause and emergence of resistance due to indiscriminate and prolonged use of antibiotics in absence of antibiogram is a major hurdle in the control of mastitis (Jeykumar *et al.*, 2013). Early identification of the prevalence and distribution

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of causative pathogens is one of the important prerequisites to effectively prevent diseases and to guide treatment. So far, the prevalence of Methicillin resistant *S. aureus* and Extended spectrum beta lactamase *E. coli* in mastitic milk samples of buffaloes in Jabalpur region of Madhya Pradesh is not known.

Therefore, the present study was conducted to determine the prevalence of Methicillin resistant *S. aureus* and Extended spectrum beta lactamase *E.coli* in mastitic milk samples of buffaloes by means of phenotypic and genotypic characterization.

MATERIALS AND METHODS

A total of 408 buffaloes milk samples were screened from private dairy farms and adopted villages in and around Jabalpur city for the presence of subclinical mastitis by 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. Isolation of Methicillin-resistant *S.aureus* and ESBL producing *E. coli*was carried out according to the protocol published by EFSA, 2012 and Markey *et al.* (2013) respectively.

Pre-enrichment of *S. aureus* was performed 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 HiChromeMeReSa 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. Pre-enrichment of *E. coli* was performed in buffered peptone water and incubated at 37°C for 18 hrs; followed by enrichment in Lauryl Tryptose broth and incubated at 37°C for 48 hrs. The MacConkey+ Cefotaxime agar plates were used as selective agar and were incubated at 37°C for 24 hrs.

The presumptive isolates of *S. aureus* and *E. coli* were phenotypically characterized by performing biochemical tests. The genotypic characterization of the Methicillin-resistant *S. aureus* (MRSA) was done using PCR for the detection of mecA gene (EFSA, 2012) and ESBL producing *E. coli* for*bla* TEM 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 mMTris, 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 amplification of the mecA gene of *S. aureus* with a size of 162 bp and *bla* TEM gene of *E. coli* with a size of 867 bp confirms resistance.

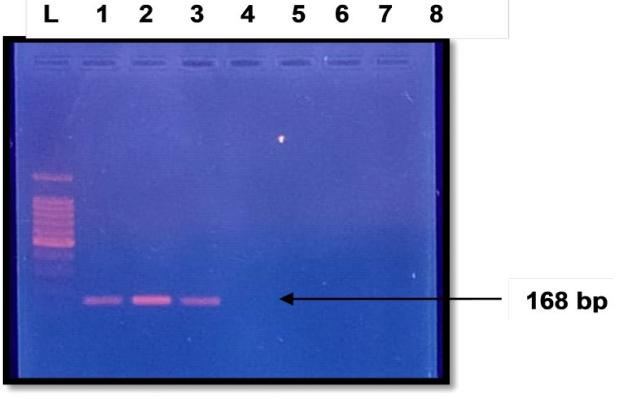
RESULTS AND DISCUSSION

Prevalence of subclinical mastitis in buffaloes

A total of 408 buffaloes milk samples from private dairy farms and adopted villages in and around Jabalpur citywere screened for mastitis by California Mastitis test (CMT). A total of 102 quarter or composite samples of milk with CMT score ≥1 were collected. Out of the 102 milk samples collected from dairy buffaloes, a total of 92 samples were of subclinical mastitis. A prevalence of 22.54% of subclinical mastitis respectively was found in 102 mastitic buffalo milk samples. In India, the estimated prevalence of SCM was 46.35% obtained from the meta-analysis studies described during the period 1995-2014 as reported (Bangar *et al*., 2015). The overall prevalence of SCM in buffaloes on animal basis was 23.85% and CM 8.67%, in organized farms as reported by Sharma *et al.* (2018).

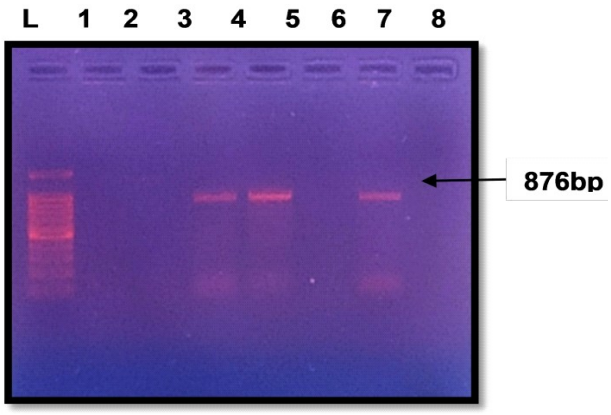
Genotypic characterization of Methicillin-resistant *S. aureus* (MRSA) and ESBL producing *E. coli* by *bla*TEM gene

After screening of 102 milk samples for methicillin resistance in *S. aureus*, 05 isolates were found MRSA positive after phenotypic and genotypic characterization for both *S. aureus* and methicillin resistance. The prevalence of 4.9% MRSA in buffalo milk was recorded. In the present study, 05 (4.9%) of *S. aureus* samples were found to be carrying a *mec A* gene, thereby confirming them as MRSA as depicted in Plate 1. Song *et al.* (2016) also confirmed MRSA by detection of the mecA gene from 25.4% *S. aureus* samples. 13.9% confirmed as MRSA, in a similar study, conducted by Chandrasekaran *et al.* (2014) in Tamil Nadu India, MRSA was detected in 3.0% (12/409) of the mastitis milk samples. Similarly, Shrestha *et al.* (2021) found, two mastitis milk samples 6.9% (2/29) of *S. aureus* were found to be carrying a *mecA* gene. Likewise, Li *et al.* (2015) conducted a study in Northwestern China in 2014, a high



L : 100 bp DNA ladder (Promega).
Lane 1 and 2: Amplified product of (mecA) gene.
Lane 3: Positive control.

Plate 1: Agarose gel electrophoresis showing amplified product (168bp) ofmecA gene.



L : 100 bp DNA ladder (Promega).
Lane 3 and 4: Amplified product of (*bla* TEM) gene.
Lane 6: Positive control.

Plate 2: Agarose gel electrophoresis showing amplified product (867 bp) of *bla* TEM gene.

prevalence (56.5%, 121/214) of *S. aureus* was determined, but only one isolate (0.46%) was found to be MRSA. Shrivastava *et al.* (2017) studied prevalence and characterization of Methicillin-Resistant *S. aureus* (MRSA) mastitis in dairy cattle, strains were positive for mecA gene was 16.47% which is considered as a gold standard for the confirmation of methicillin resistance.

The present study was designed to detect the presence of ESBL producing *E. coli* in mastitic buffalo milk by characterization of beta lactamase gene. 10 (10%) isolates were found to be phenotypically positive for *E. coli*. These isolates were checked for resistance, for *bla*TEM (867bp) by PCR assay as depicted in Plate 2. Out of the 10 *E. coli* isolates, 02 (20%) were found positive for *bla*TEM gene. In the present study, 02 ESBL positive isolates (20%) were isolated from organized dairy farms carrying a *bla* TEM gene Similarly, Olowe *et al.* (2015) performed PCR in *E. coli* isolates obtained from animal fecal samples in Nigeria and detected *bla* TEM and *bla* CTX gene in 48 (42.10%) and 51 (44.70%) isolates, respectively. Liu *et al.* (2018) from China reported 09.60% *E. coli* isolates from pigs as ESBL producer harbored at least one type of beta lactamase, with *bla* CTX"M, *bla* TEM, being detected in 90.90% and 68.18%, respectively. Abboud *et al.* (2021) investigated the etiology of the main mastitis causing pathogen and identified their antimicrobial resistance (AMR) ESBL gene, blaTEM in (83.3%) *E. coli* isolates.

Coexpression of methicillin-resistant *S. aureus* (MRSA) and ESBL producing *E. coli*

The present study was designed to detect the Coexpression of Methicillin-resistant *S. aureus* (MRSA) and ESBL producing *E. coli* in mastitic buffalo milk by characterization of beta lactamase gene of *E. coli* and methicillin resistant gene of *S.aureus.*

Out of 05 MRSA isolates and 02 ESBL isolates, Coexpression of *bla* TEM and mecA genes was recorded in only 01 (10%) isolate.

Similarly, Bandyopadhyay *et al.* (2015) described intramammary infection of methicillin resistant *S. epidermidis* (MRSE), methicillin resistant *S. aureus* (MRSA) and ESBL producing *E. coli* in two cows with subclinical mastitis and one with clinical mastitis in two different districts of West Bengal, India. In total, three such cases of bovine mastitis MRSE, one MRSA and three ESBL producing *E. coli* were isolated. Detection of simultaneous occurrence of MRSE, MRSA and ESBL producing *E. coli* in bovine mastitis in the present study indicates a major concern for dairy industry and public health as well.

However, very less reporting on simultaneous infection of MRSA and ESBL-producing *E. coli* in the same mastitic animal available to compare with the present findings.

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Conflict of interest

All authors declare that they have no conflict of interest.

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