Chapter - 5

Isolation and identification of pathogenic bacteria in milk of mastitis affected cow and buffalo
ISOLATION AND IDENTIFICATION OF PATHOGENIC BACTERIA IN MILK OF MASTITIS AFFECTED COW AND BUFFALO

INTRODUCTION

Cow and buffalo mastitis, distinct as inflammation of the mammary gland, can have an infectious or non-infectious etiology. It is characterized by physical, chemical, bacteriological and cytological changes in milk. Pathological changes in glandular tissues of the udder and effects on the quality and quantity of milk. This disease is mainly caused by microorganisms usually bacteria, including gram-negative and gram positive bacteria, mycoplasma, yeast and algae. Mastitic organisms are widespread on different body sites of cow, milker’s hands, milking cans and in the milk samples. Teat apices have been reported the most common site from where these organisms have been isolated. Inflammatory reaction caused by various types of bacteria that expand entry into the teat canal and mammary gland and it is the most significant economic use up on the world wide dairy industry. Many infective agents have been implicated as cause of mastitis in dairy cattle. It is customary to divide them for enhanced relativity to control them. It is classified as contagious mastitis and environmental mastitis. Contagious mastitis is caused by *Streptococcus agalactiae*, *Staphylococcus aureus*, *Mycoplasma bovis* and *Corynebacterium bovis* have been found living on skin of teat and inside the udder. The usual source of contagious pathogens is the infected glands of other cow in the herd. The hands of milker’s can act as a source of *S. aureus*. Environmental mastitis caused by environmental *Streptococcus* spp. including *Streptococcus uberis* and *Streptococcus dysgalactiae*, which are most prevalent; less prevalent is *Streptococcus equins* (formerly referred to as *Streptococcus bovis*), Environmental coliforms include the Gram-negative bacteria *Escherichia coli*, *Klebsiella* spp., *Enterobacter* spp., and *Arcanobacterium*, have been found in soil and feed (Bradley, 2002; Schroeder, 2009; Mekonnen and Tesfaye, 2010; Bhatt et al., 2011; Behiry et al., 2012; Ikiz et al., 2013; Hawari et al., 2014; Mbindyo et al., 2014 and Alekish, 2015).
140 species of microorganisms were identified as etiological agents of Mastitis out of these major mastitis incidences were due to *Staphylococci* (*Staphylococcus aureus* and *Staphylococcus epidermidis*), *Streptococci* (*Streptococcus agalactiae*, *Streptococcus dysgalactiae* and *Streptococcus uberis*), *Escherichia coli*, *Pseudomonas* spp., *Corynebacterium*, *Mycoplasma* and *Mycobacterium tuberculosis*. Opportunistic pathogens result in mild form of mastitis and it is include coagulase negative *Staphylococci* (CNS). CNS has been traditionally considered minor mastitis pathogens, especially in comparison with the major pathogens such as *Staphylococcus aureus*, *Streptococci* and Coliforms, *Staphylococcus* occur commensally and isolated from milk but usually illicit minor immune response in cattle and infections caused are slight. They include *Staphylococcus chromogenes*, *Staphylococcs epidermidis*, *Staphylococcus saprophyticus* and *Staphylococcus simulans* (Watts, 1988; Quinn et al., 1999, Vliegher et al., 2003, Nascimento et al., 2005, Taponen et al., 2006; Tenhagen et al., 2006; Piepers et al., 2007; Sharma et al., 2007; Malinowski and Klossowska, 2010; Smulski et al., 2011; Zenebe et al., 2014; Balkrishna et al., 2016 and Marama et al., 2016).

Isolation of mastitis pathogens is a fundamental aspect of milk quality and udder health control programs, There is a current need to discuss public health and food safety issue associated with food borne pathogens found in or related to dairy products. In Rajasthan previously no research work have been carried out on the isolation of pathogenic bacteria associated with the milk of mastitis affected cow and buffalo. So present research works carried out on “Isolation and identification of pathogenic bacteria in milk of mastitis affected cow and buffalo” for the better health of cow and buffalo.
RESULTS

Present research work showed isolation and identification of pathogenic bacteria in milk of mastitis affected cow and buffalo. Total 1688 milk samples of cows and buffalos mastitis were collected from different dairy farms of the Udaipur during the period of April 2013 to April 2017.

Pathogenic bacteria were isolated and identified on the basis of Standard microbial procedure. *Staphylococcus aureus* bacteria shows yellow colour colonies on the Mannitol salt agar and ferment the Mannitol. In the catalase test *S. aureus* show positive catalase test and form bubble formation (Plate 1: Fig 1, 2 and 3).

*Staphylococcus aureus* bacteria show hemolysis on the blood agar. Due to hemolysis of blood a clear zone is form around the *S. aureus* colonies (Plate 2: Fig 1 and 2).

*Staphylococcus aureus* show irregular cluster of the cocci under the microscope. *Streptococcus* show chain like structure under the microscope and bacillus species look like rod shape. *Escherichia coli* bacterial colonies show green metallic sheet on the eosin methylene blue agar (Plate 3: Fig 1, 2; Plate 4: Fig 1, 2, 3 and Plate 3: Fig 1 and 2).

Coagulase test is used to differentiate *Staphylococcus aureus* (positive) from coagulase negative *Staphylococcus* (CNS). Coagulase is an enzyme produced by *S. aureus* that converts fibrinogen in plasma to fibrin. Positive coagulase test of *S. aureus* show clot formation (Plate 6: Fig 1, 2 and 3).

Isolated bacterial colonies were used to preserve as the slant preparation for the further use. Pathogenic bacteria were isolated from the cows and buffaloes mastitis milk samples were preserved in the nutrient agar slant for the further use of the bacteria (Plate 7: Fig 1 and 2).
### Table 1: Prevalence of pathogenic isolates in the mastitis milk sample of cow and buffalo

<table>
<thead>
<tr>
<th>Isolates</th>
<th>Number of isolates</th>
<th>Prevalence (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Gram Positive</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Staphylococcus aureus</em></td>
<td>414</td>
<td>24.53</td>
</tr>
<tr>
<td>Coagulase negative <em>Staphylococcus</em></td>
<td>252</td>
<td>14.93</td>
</tr>
<tr>
<td><em>Streptococcus</em> spp.</td>
<td>179</td>
<td>10.60</td>
</tr>
<tr>
<td><strong>Gram negative</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Coliform</td>
<td>264</td>
<td>15.64</td>
</tr>
<tr>
<td>Non coliform</td>
<td>214</td>
<td>12.68</td>
</tr>
<tr>
<td>Mix infection</td>
<td>209</td>
<td>12.38</td>
</tr>
<tr>
<td>No Growth</td>
<td>156</td>
<td>9.24</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td>1688</td>
<td>100</td>
</tr>
</tbody>
</table>

The number and prevalence of the pathogenic isolates recovered from the milk of mastitis affected cows and buffaloes were presented in the Table 1. Out of 1688 milk samples of cow and buffaloes were collected from the different dairy in Udaipur, 209 samples considered contaminated and showed mix microbial infection. There are 156 milk samples showed no growth under the In vitro condition tested for bacterial isolation.

A total 1317 a single bacterial isolates were recovered from the 1688 mastitis milk samples of cow and buffalo isolates. Out of 100%, 78.38 % bacterial isolates recovered as a single bacterial from the milk sample, 12.38% showed mix microbial infection and 9.24 % possess no growth on the culture media. Bacteria
isolated from the mastitis affected cow and buffalo milk sample were indentifying as *Staphylococcus aureus*, coagulase negative *Staphylococcus*, *Streptococcus* spp., coliform and non coliform bacteria. Coliform bacteria include *Escherichia coli*, *Klebsiella* spp. and *Enterobacter* spp. Non coliform bacteria comprise *Pseudomonas* spp., *Pasteurella* spp. and *Proteus* spp.

The present research work on isolation and identification of bacteria isolates revealed that *Staphylococcus aureus* pathogenic bacteria found most prevalent isolates in the cow and buffalo mastitis milk samples. Out of 1688 mastitis milk samples of cows and buffaloes, 24.53% (414) *S. aureus* were frequently isolated from the mastitis cow and buffalo milk samples. After the *S. aureus*, Coliform bacteria include *Escherichia coli*, *Klebsiella* spp. and *Enterobacter* spp. second most prevalent isolates, found 15.64 % frequent (264 number of isolates recovered) in the bovine mastitis milk samples.

Out of 1688 milk samples, a total 252 number of coagulase negative *Staphylococcus* (CNS) bacteria isolated and found 14.93% frequent isolates recovered from the cow and buffalo mastitis milk samples. It is found most often after the *Staphylococcus aureus* and coliform bacteria. A total of 214 samples positive for the non coliform bacteria and 12.68% frequently isolated from the mastitis affected cow and buffalo milk samples.

Present research work indicates higher incidence of *Staphylococcus* spp. and found total 666 number of isolates out of the 1688 mastitis affected milk samples. The frequency of occurrence the incidence found 39.46% in mastitis milk samples of cow and buffalo in Udaipur.
DISCUSSION

Cow and buffalo mastitis is the most prevalent disease and a major monetary drain on the dairy industry. It is result from the inflammation of the mammary gland and mainly associated with bacterial infection, being influence by multiple factors related to management, housing and milking (Bramley et al., 1996; Bradley et al., 2002; Pol and Ruegg, 2007; Behiry et al., 2012; Singh et al., 2014 and Gomes & Henriques, 2016).

The main etiological pathogens responsible for mastitis include contagious pathogens, environmental and opportunistic bacteria. Contagious mastitis pathogens have been reported to found on the udder or teat surface of infected cows and buffaloes and are primary source of infection between uninfected and infected udder quarters, usually during milking. *Staphylococcus aureus* is a major pathogen of the mammary gland isolated from the milk of mastitis affected cow and buffalo. In the present research work indicated that *S. aureus* found most prominent bacteria causing cow and buffalo mastitis. The research finding indicates of high prevalence of *S. aureus* and concerning about spreading mastitis in cow and buffalo. The present research work finding of high prevalence of *S. aureus* found agreement with previous studies and still recognized as most prevalent pathogen causing intramammary infections in dairy cow and buffalo worldwide (Watts, 1988; Nickerson, 1993; Lafi et al., 1994; Oliveira et al., 1998; Barkema et al., 2006; Reiekerink et al., 2006; Roesch et al., 2006; Sharma et al., 2007; Getahun et al., 2008; Reiekerink et al., 2008; Sumathi et al., 2008; Lakew et al., 2009; Malinowski and Klossowska, 2010; Rahman et al., 2010; Smith et al., 2010; Nayak et al., 2011; Ebrahimi et al., 2013; Zenebe et al., 2014; Marama et al., 2016; Singh et al., 2016; Tessema, 2016; Ceniti et al., 2017; Swarnakar et al., 2017 and Vlkova et al., 2017).

Mastitis in cattle has been associated with various infectious agents. The leading microbes implicated in mastitis include *Staphylococcus* spp. and coliforms bacteria among all other pathogens. The staphylococcal enterotoxins are
recognized agents of the staphylococcal food poising syndrome, food poising in
dairy products and may be involve in other types of infections with squeal of
shock in humans and animals. The present research finding exhibited agreement
with previous research study that *Staphylococcus* spp. and coliform bacteria found
most frequent in milk of mastitis cow and buffalo (Akineden *et al.*, 2001;
Argudin, 2010; Chatterjee and Otta, 2013; Yadav *et al.*, 2015 and Pekana *et al*.,
2017).

Several researchers have been reported on the isolation of other member of
*Staphylococcus* spp. such as coagulase negative *Staphylococcus*. The role of
cogulase negative *Staphylococcus* in mastitis has greatly increased during last
year’s due to this group of bacteria reported to cause subclinical mastitis.
Coagulase negative *Staphylococcus* has been considered to be minor mastitis
causing pathogens, especially in comparition with major mastitis pathogens such
as *Staphylococcus aureus* and coliforms. The main reason for this that mastitis
caused by coagulase negative *Staphylococcus* is very mild and usually remain
subclinical (Smith *et al.*, 2001; Taponen *et al.*, 2006; Ebrahimi *et al.*, 2007;
Sharma *et al.*, 2007 and Alekish, 2015). In the current study about isolation and
identification of mastitis pathogens coagulase negative *Staphylococcus* found
third most prevalent bacteria and 14.93% frequently isolated from the mastitis
affected cow and buffalo milk samples. Coagulase negative *Staphylococcus*
prevalence was found most prevalent after the *Staphylococcus aureus* and
coliform bacteria.

Previously research reported that *Streptococcus* spp. identified with the
frequency of 1.8 to 14% associated with the mastitis (Martins *et al.*, 2010 and
Oliveira *et al.*, 2011). In the present research work exhibited that *Streptococcus*
spp. found 10.6% frequent in the milk of mastitis affected cow and buffalo milk
which found similarity with the above researchers.
Explanation of Plate - 1

Fig. 1: Yellow colour (YC) colonies of *Staphylococcus aureus* (Sa) are showing in the mannitol salt agar petridis. Colour change of the medium background of the *S. aureus* colonies became yellow due to *S. aureus* ferment manitol in the medium.

Fig. 2: *Staphylococcus aureus* (Sa) is showing yellow colour (YC) colonies in the Mannitol salt agar petridish.

Fig. 3: Catalase test of *Staphylococcus aureus* is showing bubble (B) formation and indicates as positive result.
Explanation of Plate - 2

Fig. 1: Yellow colour colonies of *Staphylococcus aureus* on blood agar (BA) medium are indicating hemolysis (H) of blood around the colonies.

Fig. 2: *Staphylococcus aureus* is showing hemolysis (H) of blood around the colonies on the blood agar (BA) medium.

Fig. 3: *Staphylococcus aureus* colonies are showing high hemolysis (H) on the blood agar (BA).
Explanation of Plate - 3

Fig. 1: Photograph of mix bacterial infection showing *Streptococcus* (St) and *Bacillus* (B) bacteria.

Fig. 2: Photograph of *Streptococcus* (St) bacterial colonies.
Explanation of the Plate - 4

Fig. 1 and 2: *Staphylococcus aureus* (Sa) colonies are showing *S. aureus* colonies are present in the irregular cluster form of the bacteria.

Fig. 3: High magnified view of *Staphylococcus aureus* (Sa) colonies.
Explanation of Plate - 5

Fig. 1: *Escherichia coli* (Ec) bacteria colonies are showing green metallic sheet (GMS) on the eosin methylene blue (EMB) agar.

Fig. 2: *Escherichia coli* (Ec) bacterial colonies are showing on the eosin methylene blue (EMB) agar.
Explanation of Plate - 6

Fig. 1: Clot formation (CF) is showing in the tube and indicating positive coagulase test of the test organism.

Fig. 2: Clot formation (CF) is showing in the positive tube and control tube does not show clot formation in the coagulase test of the test organism.

Fig. 3: Clot formations (CF) are showing in the test tube and showing positive coagulase test.
Explaination of Plate - 7

Fig. 1 and 2: Photograph of prepared slant (S) of the test organism.