Original Research Article

Isolation, characterization and antibiotic resistance of Bacillus spps. from bovine mastitis in the region of north Karnataka, India

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ABSTRACT

The present investigation was carried out to isolate the Bacillus spps. from clinical and subclinical Bovine mastitis milk and to determine antimicrobial susceptibility. The samples were collected from North Karnataka, India (09 districts) from March 2012 to August 2012. A total of 392 Milk samples suffering from mastitis were screened and a total of 221 Bacillus spps. were recovered. The molecular characterization confirmed that the isolates belong to Bacillus subtilis, Bacillus methyloptrophicus, Bacillus gaemokensis, Bacillus cereus and Bacillus mycoides. The isolates were subjected to the antibiotic resistance screening. The antibiotic resistance test showed that the isolated Bacillus spps. were resistant Methicillin (100%) followed by Penicillin G (91.40%), Oxacillin (80.54%), Cefixime (54.75%), Cefaclor (51.13%), Ampicillin (50.67%), Ceftriaxone (35.29%), Streptomycin (28.50%), Erythromycin (20.36%), Amikacin (17.64%), Norfloxacin (13.12%), Gentamicin (12.21%), Amoxyclov (10.40%), Cefpodoxime (8.59%), Tetracycline (7.69%), Chloramphenicol (6.33%), Azithromycin (5.42%), Ciprofloxacin (4.07%), Oflloxacin (2.26%) and all Bacillus spps. were susceptible to Vancomycin. The present study demonstrated the presence of alarming level of resistance of frequently and commonly used antimicrobial agents to the isolated bacteria. Therefore, an examination of the antibiotic resistance profiles of the isolates must be done earlier to the use of antibiotics in both to choose appropriate antibiotic for treatment and prevention of Bovine mastitis.

Keywords
Bovine Mastitis, Milk, Bacillus spps., Antibiotics, Resistance

Introduction

Bovine Mastitis is a common disease entity of dairy cows, accompanied by physical, chemical, pathological and bacteriological changes in milk and glandular tissue (Samad, 2008). It is a harmful disease affecting the dairy industry worldwide and is a matter of great concern for leading milk producing country like India because of the losses incurred due to high morbidity, discarded milk, treatment costs and reduced milk production, thus drawing in more attention
towards its treatment and control (Nihar et al., 2013). Apart from the economic losses, mastitis can have serious implications on public health. Mastitis which is mostly caused by the interaction of multiple pathogenic agents (primarily bacteria), can expose human beings to various organisms through infected milk, thus serving as a media for transmission of various zoonotic diseases like T.B, brucellosis, diphtheria, scarlet fever and Q fever (Mahantesh and Kaliwal, 2011).

Mastitis is produced by a variety of pathogenic microorganisms. The majority of cases in bovines are infectious and it has been estimated that up to 200 microbial species are potential causative agents (Quinn et al., 1994, Blowey and Edmondson, 1995). Cows and herds vary in susceptibility and extent, type and duration of infection, although some of mammary pathogens can be isolated from the environment of the cow, manure and bedding, water supplies, soil and inadequately cleaned milking machines (Jain, 1979 Philpot, 1979). In bovine mastitis bacteria isolated with greatest frequency are Staphylococcus aureus, Staphylococcus spp., Bacillus spp., Corynebacterium spp., Escherichia coli, Streptococcus spp., Pseudomonas spp., and Klebsiella spp. (El-Khodery et al., 2008).

Variation in prevalence of mastitis might be due to the different regions, breeds, therapeutic practices, management conditions and presence of microorganisms in environment (Sadashiv and Kaliwal, 2013). Bacteria belonging to the genus Bacillus have been associated with bovine, ovine, and porcine abortions worldwide. (Agerholm et al., 1995; Kirkbride et al., 1993).

The success of bovine mastitis therapy basically depends on the understanding of clinical presentation and antimicrobial susceptibility of the etiological agent, among various other factors (Miltenburg et al., 1996) and the increased antimicrobial resistance of the organisms in animals treated with antibiotics and their zoonotic transmission continues to be a matter of great concern globally (Unakal and Kaliwal, 2010). The important reason for therapy failure in the management of mastitis could arise from various factors involving pathological changes in the udder, etiology, lower efficacy of antimicrobials, and improper veterinary services (Adesola, 2012)

Isolation, identification and characterization of mastitis pathogens are a fundamental aspect of milk quality and udder health control programs. There is a need to discuss public health and food safety issues associated with food borne pathogens found in the dairy environment. Because of worries about antimicrobial residues, antimicrobial resistance, milk quality and animal welfare, there is an increasing demand for development and evaluation of the milk culture method and rapid and accurate identification of bacterial species. Antibiogram 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 (Nadeem et al., 2013). Identification of mastitis pathogens, and their antimicrobial susceptibility is important when selecting appropriate treatment regimen (Sadashiv and Kaliwal, 2014). Therefore, the present investigation was designed to isolate, characterize the Bacillus spp. and their antibiotic resistance, isolated from clinical and subclinical Bovine mastitis milk.
Materials and Methods

Study area

The North Karnataka is located within 15°00' North (N) and 18°30' North (N) latitudes and 74° East (E) and 77°50' (E) East longitude. The border is bounded by Maharashtra and Goa States in the north and northwest and the State of Andhra Pradesh in the east. This region is mainly called as Bayaluseeme region comprising the plains of the Deccan plateau.

Source of milk samples

The samples were collected from North Karnataka, India (09 districts) from March 2012 to August 2012. The lactating cattle of the dairy farms of the North Karnataka Region has been examined from dairy herds in different smallholder farms as well as large scale farms randomly. The study includes Holstein Freshein (H.F), Jersey, Dharwari and Murrah. A Total of 392 milk samples were collected fortnightly. Surf Field Mastitis Test (SFMT) and increased pH of the milk have been done to confirm the clinical and subclinical mastitis.

Surf Field Mastitis Test (Muhammad et al., 2010)

The samples were subjected to Surf Field Mastitis test (SFMT). The principle of the test is that when detergent is added into milk sample, it causes rupture of somatic cell and release DNA and other cell contents. DNA is acid in nature, while detergent contains alkyl-arylsulfonate, which is basic in nature. DNA and detergents unite to form a gel; consistency of gel depends upon the number of somatic cells. More cells more thick gel and vice versa. For this purpose, 3% surf solution (pH = 10.3) was prepared by adding three grams of commonly used detergent powder (Surf Excell, Uniliver, India) in 100 mL of water. Quarter milk samples and surf solution were then mixed in equal quantities in petri-dishes separately for each quarter. The change in consistency of milk indicated mastitis, while no change in consistency of milk indicated healthy samples. The mastitis was graded into further four categories based on the severity of disease from lower to higher intensity as, + = moderate, ++ = severe, +++ = more severe, ++++ = very severe.

Sampling method

Quarter foremilk samples were collected aseptically for bacteriological assay as described by Honkanen-Buzalski. Before sampling, teat ends were disinfected with cotton swabs soaked in 70% ethanol and allowed to dry and the first streams of milk were discarded. Milk samples were collected in sterile 15 ml tubes. The milk samples were transported in a cold container to the laboratory of the P. G. Department of studies in Microbiology and Biotechnology, Karnataka University, Dharwad for further analysis.

Identification and biochemical characterization

A total of 392 Milk samples suffering from mastitis were brought to the laboratory. The isolation of Bacillus spps. was carried out using the standard method (Fall, 2011). Briefly, 100μl of aseptically collected milk samples from each sample was spread over a Nutrient Agar and incubated at 37°C for 24-48 hrs. After incubation, the selected colonies were subjected to Gram nature, morphological character.
Antibacterial Resistance Test

Antibiotic resistance screening was done as per the guidelines of National Committee for Clinical Laboratory Standards (NCCLS). Kirby- Bauer’s disc diffusion technique was adapted for antibiogram. The antibiotic discs and Mueller- Hinton Agar were purchased from Hi-Media Pvt. Ltd, Mumbai. The following antibiotics are used for resistance test – Amikacin, Amoxyclav, Ampicillin, Methicillin, Oxacillin, Penicillin G, Cefaclor, Cefixime, Cefpodoxime, Ceftriaxone, Ciprofloxacin, Norfloxacin, Ofloxacin, Gentamicin, Azithromycin, Erythromycin, Streptomycin, Vancomycin, Tetracycline and Chloramphenicol.

Molecular characterization

The strains identified were further subjected for molecular identification to confirm by analysing 16S rDNA sequence. Three strains were selected for 16S rDNA sequencing each representing from group of similar phenotypic characters.

Isolation of DNA

DNA Extraction was carried out using method modified from Mary Suchita Xalxo, 2 ml of overnight grown Nutrient broth culture was centrifuged at 10,000 rpm at 4°C for 10 minutes. The pellet was re suspended in 10 min 10mM Tris, 100 mM Sodium chloride solution and centrifuged at 10,000 rpm 4°C for 10 minutes. After discarding the supernatant, the pellet was re suspended in 100 µl of T50E20 buffer containing 20µl of lysozyme (50mg/ml) and incubated at 37°C for 20 min, in that solution 1µl of RNase (10 mg/ml) was added and incubated at room temperature for 20 minutes. To this mixture 100µl of SDS (2% in T50E20) was added and incubated at 50°C for 45 min with proper mixing. 2µl of Proteinase K (20mg/ml) was added and incubated at 55°C for 30 min. The sample was extracted in same volume phenol, Chloroform and Iso-amyl alcohol (25:24:1) and DNA was precipitated with one volume of isopropanol and 0.1 volume of 3M of Sodium acetate. The pellet was washed with 70% Ethanol, dried and dissolved in 100 µl of T10E1 buffer and stored at -20°C for further use. Concentration of DNA was determined using UV-1800 spectrophotometer (Schimadzu Corporation). The DNA was stored at -20°C for further use.

Polymerase chain reaction

PCR amplification was performed using Applied Biosystem verti thermal cycler. The primers for PCR amplification were obtained from Sigma-Aldrich.

Universal Primer (Lane, 1991)

27 forward – 5’
AGAGTTTCCTGGCTCAG 3’

1492 reverse – 5’
ACGGCTACCTTGTTACGATT 3’

The PCR was performed in 20µl reaction mixture containing 2µl of 10X assay buffer, 1µl dNTP mix of 2.5 mM, 0.5µl of mgcl2, 1µl each of forward and reverse primer (5pmol), 0.5µl of Taq polymerase, 1µl of template DNA and 13.5µl of HPLC grade water with the following amplification for 16s rDNA initial denaturation at 95°C for 4 min followed by 38 cycles of denaturation, annealing and extension (94°C for 1 min, 59.9°C for 2 min and 72°C for 2 min) and final extension at 72°C for 20 min followed by
hold for infinity at 4°C. The presence of PCR products was determined by 2.5% agarose gel electrophoresis and to analyse the size of amplified PCR product DNA markers of 100bp was used which was provided by the Puregene. The amplified product was sent for sequencing to SciGenom Labs Pvt Ltd, Cochin, Kerala.

**Construction of phylogenetic tree.**

By using the sequence the bacteria were identified and constructed phylogenetic tree by using NCBI(http://blast.ncbi.nlm.nih.gov/Blast.cgi?PROGRAM=blastn&BLAST_PROGRAMS=megaBlast&PAGE_TYPE=BlastSearch&SHOW_DEFAULTS=on&LINK_LOC=blasthome) and MEGA 5 Software.

**Results and Discussion**

A total of 221 Bacillus species were recovered from 392 milk samples based on Gram nature and morphological character. The partial amplification of 16S rDNA confirmed on the agarose gel electrophoresis. (Fig. 1).

By using NCBI and neighbour joining method in MEGA 5 the strains were identified as Bacillus subtilis (Fig. 2), Bacillus methylotrophicus (Fig. 3), Bacillus gaemokensis (Fig. 4), Bacillus cereus (Fig. 5) and Bacillus mycoides (Fig. 6).

The antibiotic susceptibility test to the revealed that the highest number of Bacillus spps. was resistant to Methicillin (100%) followed by Penicillin G (91.40%), Oxacillin (80.54%), Cefixime (54.75%), Cefaclor (51.13%), Ampicillin (50.67%), Ceftriaxone (35.29%), Streptomycin (28.50%), Erythromycin (20.36%), Amikacin (17.64%), Norfloxacin (13.12%), Gentamicin (12.21%), Amoxyclav (10.40%), Cefpodoxime (8.59%), Tetracycline (7.69%), Chloramphenicol (6.33%), Azithromycin (5.42%), Ciprofloxacin (4.07%), Ofloxacin (2.26%) and all Bacillus spps. were susceptible to Vancomycin (Table 1).

Bovine Mastitis is a result of interaction between three elements like bacteria, cow and environment (Mohammed et al., 2012). In the present study B.subtilis, B.methylotrophicus, B.gaemokensis, B.cereus and B.mycoides have been isolated and characterized. Similarly Mohammed et al., (2012) also isolated Bacillus coagulans, B. cereus, B. subtilis, B. licheniformis, B. circulans, B. lentus, B. myoides, B. amyloliquefaciens, B. megaterium from Bovine mastitis. Nieminen et al., (2007) also reported the presence of B. pumilus B. licheniformis and B. cereus. Parkinson et al., (1999) reported the Presence of Bacillus cereus from the mastitis milk. Bacillus cereus is recognised as being ubiquitous in the farm environment and the numbers of Bacillus cereus spores in soil rises throughout the winter (Davies and Wray, 1996). It is a common contaminant of milk at all stages of processing (Crielly et al., 1994). The organism is not generally considered to be a primary mastitis pathogen, but causes mastitis after accidental introduction to the udder (Parkinson et al., 1999). The presence of Bacillus spps. in the 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 soil, water and manure these bacteria infect animals via teat canals (Mohammed et al., 2012). Therefore, the present study suggest that mastitis can be controlled by hygienic
conditions, cleaning manure, keeping the animals away from the stagnant water, washing udder before milking with germicidal solution.

The development of molecular biological methods, such as nucleic acid analysis, protein patterns or fatty acid profiles, has added possibilities for the rapid identification of bacteria (Busse et al., 1996). Species-specific DNA sequences can be used for the identification of bacterial species. The 16s-23s rDNA has proven useful for identification of strains and species (Gurtler & Stanisich, 1996). The 16s rDNA sequencing makes it possible to identify and distinguish closely related bacterial species. In the present study the characterization of the Bacillus sps. have been done by using the 16s rDNA sequence. Similarly, many molecular tools like 16s-23s rRNA spacer regions used by Forsma et al., (1997) PCR–DGGE method used by Ying et al., (2009), 16s rDNA sequencing used by Shea Beasley (2004) for the identification of the bacterial species. The present molecular identification work suggest that, the 16s rDNA sequencing is more accurate for the species identification.

In the mastitis the improper or incomplete treatment of animals also contributes significantly to the development of bacterial resistance against them. The usage of antibiotics correlates with the emergence and maintenance of antibiotic-resistant traits within pathogenic strains (Shitandi et al., 2004). These traits are coded for by particular genes that may be carried on the bacterial chromosome, plasmids (Rychlik et al., 2006), hence these are easily transferred among isolates. In the present study, In vitro antimicrobial susceptibility test of twenty one antimicrobial agents was conducted and studied against isolated 221 Bacillus sps. The most commonly used antibiotics on conventional dairies were Penicillin, Cephalosporin and Tetracycline’s. For mastitis, Penicillin, Ampicillin and Tetracycline’s were commonly used (Mohammed et al., 2012). The antibiotic resistant of the present study revealed that the isolated Bacillus sps. showed resistant to multi drugs. These results were in line with the reports of Mohammed et al., (2012), were the resistance of Ampicillin(84%), Cefotaxime (77%), Ceftizoxime (55%), Amikacin and Ofloxacin (25%) and Tetracycline (17%). The reports were higher to the reports of Firaol et al., (2013) to the Penicillin G (66.67%), lower to Chloramphenicol (88.89%) and Gentamycin (100%). From the study, a large number of isolates were found to be resistant to previous and established antibiotics compared to the newer developed antibiotics. Appearance of resistance against a particular antibiotic in a specific region may be due to its frequent and long-term use (Moon et al., 2007; Kumar et al., 2010).

The resistance to the Penicillin G is also in line with the work of Nadeem et al., (2013). The resistance to Penicillin G must be of concern, since this antibiotic represents the main antibiotic group recommended for mastitis treatment and regular use of antibiotics for the treatment of cows may result in the spread of resistant strains (Hulya et al., 2006; Nadeem et al., 2013). The prevalence of antibiotic resistance usually varies between isolates from the different sampled places and even between isolates from different herds on the same farm(Waage et al., 2002).
Table 1 Antibacterial Resistance pattern for isolated *Bacillus Sps.*

<table>
<thead>
<tr>
<th>Bacterial Isolates</th>
<th>Antibiotics Used</th>
<th>AK</th>
<th>AM</th>
<th>M</th>
<th>OX</th>
<th>P</th>
<th>CF</th>
<th>M</th>
<th>CTR</th>
<th>CI</th>
<th>N</th>
<th>O</th>
<th>G</th>
<th>A</th>
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<th>S</th>
<th>V</th>
<th>TE</th>
<th>C</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Bacillus Spps.</strong></td>
<td>22</td>
<td>39</td>
<td>23</td>
<td>112</td>
<td>00</td>
<td>178</td>
<td>20</td>
<td>11</td>
<td>12</td>
<td>19</td>
<td>78</td>
<td>09</td>
<td>29</td>
<td>5</td>
<td>27</td>
<td>12</td>
<td>45</td>
<td>63</td>
<td>00</td>
</tr>
<tr>
<td><strong>%</strong></td>
<td>17.6</td>
<td>4</td>
<td>10.4</td>
<td>0</td>
<td>67</td>
<td>0</td>
<td>10</td>
<td>80.5</td>
<td>91</td>
<td>51</td>
<td>54</td>
<td>75</td>
<td>8.5</td>
<td>9</td>
<td>35.29</td>
<td>4.0</td>
<td>13</td>
<td>12</td>
<td>2.2</td>
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**Fig.1** Agarose gel electrophoresis to confirm the amplified DNA
Fig. 2 Phylogenetic tree of *Bacillus subtilis*

Fig. 3 Phylogenetic tree of *Bacillus methylothericus*
**Fig. 4** Phylogenetic tree of *Bacillus gaemokensis*

**Fig. 5** Phylogenetic tree of *Bacillus cereus*
Antibiotic resistance is carried on plasmids and transposons which can pass from one species to another (Werckenthin et al., 2001). The possible reason for detection of this species of resistant bacteria at high prevalence supports its contagious nature that gives the chance to circulate and infect the udder once introduced to a herd (Sharif et al., 2009).

It is possible that mastitogenic bacteria can lose the sensitivity to antibiotics over the time or even acquire sometimes this feature (Edward et al., 2008). It is necessary to monitor mastitis pathogens to assess any changes in their antibiotic resistance patterns. Careful use of antibiotics can avoid the increase and dissemination in antimicrobial resistance arising from the use of antimicrobial drugs in animals (Fluit et al., 2006; Schwarz et al., 2001). Also previous investigations of Malinowski et al., (2006) showed that recovery rates from acute mastitis equals “zero” if the pathogens were resistant to antibiotics used to intrammary treatment. Lack of stringent regulations and monitoring in the dispensing and use of antimicrobials in veterinary establishments and mass inoculation of herds of animals by some farmers has risen as a contributory factor to increase antimicrobial resistance (Alemu et al., 2012).

Some natural products like Bacteriocin may be used as an alternative type of antibiotic(Kaur et al., 2012). These natural agent which may inhibit growth of huge number of microbes. They exist in various ecological niches. (Suneel and Kaliwal 2013) have Reported the bacteriocins produced by the Lactococcus garvieae shown antibacterial effect against the B. cereus and B.subtilis.
The present study demonstrated the presence of alarming level of resistance of frequently and commonly used antimicrobial agents to the isolated bacteria from Bovine mastitis. Important reasons for the failure of treatment of mastitis are the indiscriminate use of antibiotics without in vitro sensitivity of causal organisms (Amritha and Kaliwal, 2013). Therefore, an examination of the antibiotic resistance profiles of the isolates may serve as a major tool in evaluating both the hygienic conditions employed during milking and the health hazards that humans may encounter when infected by antibiotic resistant strains (Deresse Daka et al., 2012) and also implementation of a systematic application of an in vitro antibiotic susceptibility test, earlier to the use of antibiotics in both treatment and prevention of Bovine mastitis is very important.

*Bacillus spps.* were isolated and characterized from the collected milk samples. Many isolates of *Bacillus spps.* were showed multi drug resistant. It is difficult to treat as many strains are resistant to antibiotics used in mastitis. The development of antibiotic resistance in the bacteria that affects animal health is of growing concern in veterinary medicine. Therefore, the present study suggests the examination of the antibiotic resistance profiles of the isolates must be done earlier to the use of antibiotics in both to choose appropriate antibiotic for treatment and prevention of Bovine mastitis.

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