Characterization of Staphylococcus aureus Isolates of Bovine Mastitis Origin and Antibiotic Sensitivity Pattern from Northern Plains of India

Pati BK* and Reena Mukherjee
Division of Medicine, ICAR-Indian Veterinary Research Institute, Izatnagar, UP-243122, India

Received Date: August 22, 2016, Accepted Date: October 06, 2016, Published Date: October 17, 2016.

*Corresponding author: Pati BK, Division of Medicine, ICAR-Indian Veterinary Research Institute, Izatnagar, UP-243122, India, E-mail: dr.banajpati@gmail.com

Abstract
Among the various mastitis pathogens, Staphylococcus aureus (S. aureus) is identified as chief etiological agent responsible for subclinical and chronic mastitis and most challenging for the veterinary clinician to treat such cases. In the present study, 435 cows were screened for mastitis by California Mastitis Test (CMT) and Somatic Cell Count (SMC) from the northern tropical region of India. On the basis of cultural and biochemical properties, 61 isolates were presumptively identified as S. aureus. Further genotypically identified as S. aureus by Polymerase Chain Reaction (PCR) and 52 isolates were confirmed by species specific 16S rRNA and for thermonuclease gene (nuc). Those 52 isolates were subjected to antibiotic sensivity test, for which 18 antibiotic discs were used, belonging to 14 different classes of antibiotics. The Sensitivity Intermediate Resistance (SIR) pattern revealed multidrug resistant S. aureus, as the isolates were resistant to more than three different classes of antibiotics. The isolated revealed maximum resistance > 50% towards penicillin, aminopenicillins, betalactum, macrolide, sulpha drugs and tetracycline class of antibiotics. The sensitivity pattern revealed maximum susceptibility towards fluoroquinolones, oxazolidone, glycopeptide and extended spectrum betalactum inhibitors class of antibiotics. SIR pattern revealed 100% susceptibility for vancomycin. The phenotypic and genotypic findings of the present study might help to understand the distribution of prevalent S. aureus infection in the dairy farms and antibiotic sensitivity pattern will help to choose most appropriate antibiotic, hence help to adopt appropriate strategies for the management and control of bovine mastitis.

Keywords: Antibiotic Resistance; Bovine Mastitis; Staphylococcus aureus; Thermonuclease Gene

Introduction
Staphylococcus aureus (S. aureus) is a commensal skin microbota, causes mild to acute disease condition in man and animals. In man S. aureus may cause diseases like skin infections, septicemia, infective endocarditis and septic arthritis [1]. In animal, it causes bovine mastitis in dairy cattle and buffaloes [2]. It is a serious problem for the dairy industry, because of huge economic losses incurred due to poor quality and quantity of milk [3]. To the Indian dairy industry, the loss due to bovine mastitis is around 90 × 10^7 [4]. It is a major contagious pathogen, spreads rapidly in dairy herd during milking and generally leads to subclinical and chronic mastitis [5]. Bovine mastitis is generally associated with the extensive farming system. For implementation of therapeutic and preventive measures, appropriate knowledge of the bacteriological population, antibiotic sensitivity pattern and epidemiological aspect holds key for the management of bovine mastitis in a dairy farm [6]. The bacterial culturing of the raw milk is the standard procedure for diagnosis of the bacterial flora of a particular dairy farm causing intramammary infection, but the method is time consuming. Hence, molecular identification of S. aureus by PCR based on their 16S rRNAs is preferred because 16S rRNA genes are highly conserved throughout bacterial evolution. Both 16S rRNAs and nuc gene can be used for species identification of S. aureus [7–9]. The emergence of resistance to antibiotics in gram-positive pathogens has become a major global issue [10]. Bovine mastitis caused by S. aureus is a serious issue because the concern is the increasing antimicrobial resistance and it is more threatening when considering only few antimicrobial agents are available for the treatment. Therefore, the objective of this study is to characterize S. aureus phenotypically and genotypically with species specific 16S rRNA and also by thermonuclease gene (nuc). The SIR patterns of the milk collected from the characterized samples were also recorded.

Material and Methods

Collection of milk samples
Four hundred and thirty-five lactating crossbred cattle were screened for intramammary infection (IMI) from northern part of India. The initial diagnosis of clinical and subclinical mastitis was based on clinical signs and CMT as described earlier [11]. SCC was performed as per the guidelines of Schalm et al. [12]. The positive animals by CMT point scores were selected for collection of milk samples. The milk samples from affected quarters from each cow were collected after proper disinfection of hand and teat surface with 70% ethyl alcohol. After teat preparation, about 5-10 ml of milk samples were collected in sterile vials, kept in ice box and carried to the laboratory where the samples were kept at 4°C in refrigerator for further studies on SCC, and bacterial isolation.

Isolation and phenotypic characterization of S. aureus

Bacterial isolation: The isolation procedures were carried out under strict sterile environment. The identification of causative organism in collected milk samples was carried as per the method described by Griffin et al. [13]. The causative organism of the milk samples was identified initially on the basis of colony morphology, zone of hemolysis on 5% blood agar after 24 hrs post incubation at 37°C [14].

Culture in selective media: The suspected colonies from 24 to 48 hrs old culture grown in 5% bovine blood agar were further grown on Mannitol Salt Agar (MSA). The colony characteristics were observed after 24 - 48 hours of incubation at 37°C. Further, the colonies picked from MSA were streaked on Baird Parker media. The suspected samples were selected for biochemical testing.

Biochemical test of S. aureus

The biochemical characterization of Staphylococcus aureus was performed by HiStaph™ Identification Kit (HiMedia Lab Pvt. Ltd., India), the test contains voses proskauer’s test, alkaline phosphate, ONPG, urease, arginine utilization and ability to ferment carbohydrate including mannitol, sucrose, maltose, arabinose, raffinose, trehalose, and maltose [15].

Catalase test, Coagulate test and Oxidase test
Catalase test producing bacteria were identified as per method
of Thomas [16]. Catalase production was detected on nutrient agar slants after 24 – 72 hrs of incubation by adding 3% hydrogen peroxide over culture slants. Coagulase test was performed as per procedure described by Gillespie [17]. Oxidase test was performed by filter paper spot method as described by Kovacs [18].

**Genotypic characterization of *S. aureus***

**Identification of *S. aureus* by PCR:** The isolates were identified as *S. aureus* by amplification of 16S ribosomal RNA (rRNA). Briefly, for DNA preparation isolates were incubated overnight in 10 ml brain heart infusion broth (BD, USA), centrifuged (5000 g, 15 min) and resuspended in 5ml TE (10 mM Tris, 1 mM EDTA, pH 8). Total cellular DNA was extracted from 1ml TE with QiAamp DNA Mini Kit (Quiagen, Netherlands) for Gram+ve bacteria according to manufacturer’s protocol. From each sample, 5 µl of total cellular DNA was evaluated by PCR with primers used previously described by Lovseth A et al [19] (Table 1). The Cycling conditions for amplification: 1 × 5 min at 95°C, 1 min at 95°C, 0.5 min at 55°C, 1.5 min at 72°C, and a final extension at 72°C for 7 minutes.

**Thromonuclease (nuc) gene:** Species specific published primer sequence of Brakstad et al. [20] (Table 1) was employed for the amplification of nuc gene for molecular identification of *S. aureus*. The Cycling conditions for amplification: 1 × 5 min at 95°C, 1 min at 95°C, 0.5 min at 55°C, 1.5 min at 72°C, and a final extension at 72°C for 7 minutes.

**Antibiotic sensitivity test:** Antibiotic sensitivity pattern of Staphylococcus was carried out by disc diffusion method of Kirby et al.,[21]. Briefly, each culture was inoculated into sterilized BHI (Brain-Heart Infusion medium) broth incubated at 37°C for 4–6 hours. The turbidity of the inoculum was compared with 0.5 McFarland standards and the optical density of the suspension was measured by spectrophotometry to be 0.08-0.13 OD turbid suspension at 620 nm. Pure broth culture of each isolate was spread on to the Mueller hinton agar plates and kept for drying. Antibiotic discs (BD, BBL, USA and HiMedia, India) used for the study is depicted in table 2, antibiotic disc were aseptically placed on the dried surface of agar. After incubation, the zone of inhibition was measured in order to ascertain sensitivity of the isolates against the antibiotics. Mean was calculated to depict the results.

**Results**

Out of 435 cattle screened by CMT, 186 cattle were found positive for IML. The SCC ranged from 2.81 ± 1.29 × 10^5 to 20.22 ± 1.43 × 10^5 cells per ml of milk samples isolated from normal healthy cows and mastitic cows, respectively.

**Bacterial isolation**

Out of 186 milk samples, 173 samples revealed growth on 5% sheep blood agar plates and of which 112 samples with zone of hemolysis. Grams’ staining of 112 isolates, 96 numbers of samples were found as gram positive cocci. Sixty nine samples out of 96 gram positive cocci engendered lactose fermenting colonies on MSA, which were positive for catalase and negative for oxidase tests. Baird Parker agar plates revealed characteristic black colonies in 63 samples on 24 hours post incubation. The isolated 63 samples were further screened by coagulase test, which revealed viscous clot of rabbit plasma in varying proportions in 61 samples.

<table>
<thead>
<tr>
<th>Primer Code</th>
<th>Gene</th>
<th>Primer Sequence (5’-3’)</th>
<th>Amplicon Size (bp)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>16S F</td>
<td>16S rRNA</td>
<td>GTAGGTGCAAGGCTTACC</td>
<td>228</td>
<td>Lovesth et al., 2004</td>
</tr>
<tr>
<td>16S R</td>
<td>nuc</td>
<td>GGCACATCAGGCGAC</td>
<td>280</td>
<td>Brakstad et al., 1992</td>
</tr>
<tr>
<td>Nuc F</td>
<td>nuc</td>
<td>GGATCGATGGTATGCGT</td>
<td>228</td>
<td>Lovesth et al., 2004</td>
</tr>
<tr>
<td>NucR</td>
<td>nuc</td>
<td>ACCAAGCCTTGAAGAATTACGAC</td>
<td>280</td>
<td>Brakstad et al., 1992</td>
</tr>
</tbody>
</table>

**Table 1:** Primer sequences used for 16S ribosomal RNA gene segment and Thromonuclease gene (nuc gene) of *Staphylococcus aureus*.

<table>
<thead>
<tr>
<th>S No</th>
<th>Antibiotic used</th>
<th>Antibiotic disc</th>
<th>Antibiotic Class</th>
<th>SIR pattern</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Penicillin G</td>
<td>PEN 10 U/disc</td>
<td>Penicillin</td>
<td>%R %I %S</td>
</tr>
<tr>
<td>2</td>
<td>Ampicillin</td>
<td>AMP 10 µg/disc</td>
<td>Amino penicillin</td>
<td>96 0 4</td>
</tr>
<tr>
<td>3</td>
<td>Ampicillin+ clavulanic acid</td>
<td>AMC 20/10 µg/disc</td>
<td>Amino penicillin</td>
<td>37 12 52</td>
</tr>
<tr>
<td>4</td>
<td>Ampicillin/Cloxacillin</td>
<td>APX 30 µg/disc</td>
<td>Aminopenicillin/β lactamase</td>
<td>49 21 30</td>
</tr>
<tr>
<td>5</td>
<td>Ceftriaxone</td>
<td>CRO 30 µg/disc</td>
<td>Cephalosporin</td>
<td>46 44 10</td>
</tr>
<tr>
<td>6</td>
<td>Ceftriaxone / Tazobactum</td>
<td>CIT 30/10 µg/disc</td>
<td>Cephalosporin/β lactam inhibitor</td>
<td>24 11 65</td>
</tr>
<tr>
<td>7</td>
<td>Ciprofloxaxin</td>
<td>CIP 5 µg/disc</td>
<td>Fluoroquinolone</td>
<td>37 40 23</td>
</tr>
<tr>
<td>8</td>
<td>Enrofloxacin</td>
<td>ENR 5 µg/disc</td>
<td>Fluoroquinolone</td>
<td>22 6 72</td>
</tr>
<tr>
<td>9</td>
<td>Ofloxacain</td>
<td>OFX 5 µg/disc</td>
<td>Fluoroquinolone</td>
<td>13 16 71</td>
</tr>
<tr>
<td>10</td>
<td>Cotrimoxazole</td>
<td>COT 25 µg/disc</td>
<td>Sulpha</td>
<td>56 25 19</td>
</tr>
<tr>
<td>11</td>
<td>Gentamicin</td>
<td>GEN10 µg/disc</td>
<td>Aminoglycoside</td>
<td>57 30 13</td>
</tr>
<tr>
<td>12</td>
<td>Erythromycin</td>
<td>ERY 15 µg/disc</td>
<td>Macrolide</td>
<td>66 8 22</td>
</tr>
<tr>
<td>13</td>
<td>Methicillin</td>
<td>MET 5 µg/disc</td>
<td>β - Lactum</td>
<td>65 16 19</td>
</tr>
<tr>
<td>14</td>
<td>Terramycin</td>
<td>TET 30 µg/disc</td>
<td>Tetracycline</td>
<td>54 6 40</td>
</tr>
<tr>
<td>15</td>
<td>Linezolid</td>
<td>LZD 30 µg/disc</td>
<td>Oxazolidinone</td>
<td>22 15 73</td>
</tr>
<tr>
<td>16</td>
<td>Vancomycin</td>
<td>VAN 30 µg/disc</td>
<td>Glycopeptides</td>
<td>0 0 100</td>
</tr>
<tr>
<td>17</td>
<td>Rifampicin</td>
<td>RIF 5 µg/disc</td>
<td>Rifamycin</td>
<td>10 22 68</td>
</tr>
<tr>
<td>18</td>
<td>Chloramphenicol</td>
<td>CHL 30 µg/disc</td>
<td>Chloromycetin</td>
<td>9 11 76</td>
</tr>
</tbody>
</table>

**Table 2:** Antibiotic sensitivity and resistance pattern of *Staphylococcus aureus* isolated from bovine mastitic milk samples.
Biochemical confirmation of *S. aureus*

Sixty one isolates showed characteristic color change on HiStaph™ Identification Kit, the isolates were positive for VP, Alkaline Phosphate, Urease, Arginine utilization and ability to ferment carbohydrate including mannitol, sucrose, maltose, trehalose, and negative for onpg, arabinose and raffinose. They were (n = 61) considered as *S. aureus* based on cultural characteristic and biochemical tests.

Genotypic identification of *S. aureus*

Isolation of bacterial genomic DNA: Isolation of bacterial genomic DNA from Staphylococcal isolates (n = 61) yielded varying concentration of DNA ranged from 45 ng/µl to 1606 ng/µl with purity ranging from 1.50-1.97 as measured by nanodrop spectrophotometer. Amplification was observed in all 52 isolates using MTCC 2940 Staphylococcal strain (SPS) as positive control.

Amplification for 16sRNA was observed in 52 isolates (Figure 1). The amplified products were of nearly 228 bp when resolved in gel electrophoresis. Amplification for thermonuclease (nuc) gene was observed in 52 isolates (Figure 2). The amplified products were of nearly 279 bp when resolved in gel electrophoresis.

Antibiotic resistance/susceptibility pattern of Staphylococcal isolates

The SIR pattern of the used antibiotics of the 52 Staphylococcal isolates revealed maximum resistance for penicillin-G (96%), ampicillin (93%), methicillin (65%), cotrimoxazole (56%) and gentamicin (57%); whereas, the isolates were to chloramphenical (76%), kinezolid (73%), enrofloxacin (72%), oloxicin (71%), reflamin (68%), ceftriaxone / tazobactum (65%), and amoxicillin + clavulanic acid (52%). All the isolates were susceptible to Vancomycin (100%). Intermediate pattern was observed in ceftrixzone (44%), ciprofloxacin (40%) and gentamicin (30%) (Table 2).

Discussion

In the present study, 186 animals were found positive for IMI, out of 435 animals screened. Genotypically 52 isolates qualified for *S. aureus* infection. Bovine mastitis is one of the most significant causes of economic loss to the dairy industry [22]. There is also an increasing public health concern over bovine mastitis because the affected milk is a potential source of pathogens, drug resistant pathogens and antibiotic residue in the human food chain [23]. Mastitis is caused by several pathogens but *S. aureus* is one of the etiologic agent causing intramammary infection in dairy animals in most part of the world [24]. Many researchers reported staphylococci causing bovine mastitis from northern Indian states [25–29]. *S. aureus* can be recognized phenotypically by a number of ways like Staphylocoagulage test, clumping factor test [30] and direct bacteriological examination of milk samples. Moreover, these tests may give positive results for some other species also, hence molecular identification is recognized to be superior to earlier mentioned methods [31]. The identification of *S. aureus* based on their 16s rRNAs is preferred because 16s rRNA genes are highly conserved throughout bacterial evolution. They consist of regions which are common to all eubacteria and other regions which are extremely species specific and for species identification of *S. aureus* has been used by many workers [7–9]. In present study 83.8% (52 out of 61 isolates) of the isolates were identified as *S. aureus* on the basis of amplification of 16s rRNA gene. Similarly, 83.8% (52 out of 61 isolates) of the isolates were identified as *S. aureus* basing on the amplification of nuc gene. Brakstad, et al. [20] also opined that nuc gene for identification of *S. aureus*.

Mastitis is the most common cause for antibiotic use in dairy herds [32,33]. Antibiotics have been used for more than 50 years for treatment of mastitis but safe, effective and economical treatment is still lacking and attributes to resistance among the isolates. Indiscriminate use of antibiotics for the treatment of dairy animals without knowing the antibiotic sensitivity pattern is the causal factor for alarming increase of antibiotic resistance. In the present study the staphylococcal isolates depicted resistance towards most of the commonly used antibiotics which are being used for the treatment of intramammary infection in dairy cattle. We have used 18 antibiotic discs which were belonging to 14 different class of antibiotic. Most of the isolates belonging to...
multiple drug resistant category, as they were showing resistance for more than three classes of antibiotics. Tenover [34], stated that isolates exhibiting in vitro resistance to three or more than three classes of antimicrobials were classified as multidrug-resistant. In our study, maximum resistance was observed against penicillin and ampicillin and the resistance was >90%, followed by erythromycin, methicillin, cotrimoxazole, tetracycline and ceftriaxone. Behiry et al. [35], reported 85.72% of Penicillin G resistance among \textit{S. aureus} isolates from mastitis milk samples in Germany. Biswas et al. [36], recorded mupirocin resistant staphylococci from yak milk. Many workers have reported multidrug resistant staphylococci from northern region of India [37,28].

\textit{S. aureus} develop antimicrobial resistance to most of the commonly used antibiotics, Lindsay [38], the bacteria acquire the resistance through the horizontal gene transfer. Whereas, Pantosti and colleagues [39] were in view, the mechanism through spontaneous mutations and positive selection. In the present study, contrary to the above result, remarkably the isolates were showing susceptibility for chloramphenicol, it may be due to this antibiotic is not being in use for considerably long period of time. Similarly the isolates were susceptible to enrofloxacin and ceftriaxone/azobactum, although linezolid and refampin are also very sensitive but are not used for the treatment of bovine mastitis. All the isolates were vancomycin susceptible. Surprisingly we have observed resistance in few isolates for oxazolidone group, because this antibiotic is never used for the treatment of animal diseases, which is a matter of great concern. The source of acquisition of resistance against these novel antibiotics in these dairy cattle may be from environmental source or dairy workers. All these factors in conjunction raise a disquieting situation in India where regular intensive screening of the cultural agents of mastitis and antibiogram profiling is a prerequiste for developing effective control strategies.

Conclusion

In the present study, 435 cows were screened for mastitis from the Northern tropical region of India. Fifty two isolates were confirmed as \textit{S. aureus}. Isolates were resistant to penicillin, aminopenicillins, beta-lactum, macrolide, sulphla and tetracycline class of antibiotics, and susceptible to fluoroquinolones, oxazolidone, glycopeptides and extended spectrum betalactum inhibitors. Such kind of study might help to understand the distribution of \textit{S. aureus} infection in the dairy farms and selection of most appropriate antibiotic to adopt appropriate strategies for the management and control of bovine mastitis.

Acknowledgments

The authors are thankful to the farmers and dairy owners of northern region for helping and cooperating for the sample collection. Authors are thankful to director, Indian Veterinary Research Institute & Indian Council of Agricultural Research, New Delhi, for proving all the facilities for conducting the research work.

Conflict of Interest

No conflict of interest of any nature.

References


*Corresponding author: Pati BK, Division of Medicine, ICAR-Indian Veterinary Research Institute, Izatnagar, UP-243122, India, E-mail: dr.banajpati@gmail.com

Received Date: August 22, 2016, Accepted Date: October 06, 2016, Published Date: October 17, 2016.

Copyright: © 2016 Pati BK, et al. This is an open access article distributed under the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.