Molecular typing of virulence associated gene (spa) of *S. aureus* isolated from cattle clinical mastitis

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Abstract

The present study was undertaken to determine virulence associated protein-A (spa) gene in 30 *S. aureus* isolated from cattle with clinical mastitis. All the isolates were characterized by polymerase chain reaction to determine the frequency of spa (spa-X) gene wherein 93.33% isolates produced spa (X-region) gene amplicon. Of the 30 isolates, 19 isolates produced single amplicon and 9 isolates produced double amplicons. The size of amplicons obtained were 80, 200, 220, 250, 280, 300 and 320bp with a calculated number of 3, 4, 7, 3, 6, 13 and 1 repeats, respectively. The amplicon of 300bp was detected in 13 isolates followed by 220bp amplicon in seven, 280bp amplicon in six, 200bp amplicon in four, 250bp amplicon in three, 80bp amplicon in 3 and 320bp amplicon in one isolate.

Keywords: *Staphylococcus aureus*, mastitis, cattle, protein-A, spa gene

Introduction

Mastitis is a multifactorial and a costly problem affecting all milk producing animals in world [1]. Mastitis, the most important disease of dairy animals is responsible for heavy economic losses due to reduced milk yield (up to 70%), milk discard after treatment (9%), cost of veterinary services (7%) and premature culling (14%) [2].

Protein A, a surface protein of *S. aureus* binds to the IgG molecules by their Fc portion and inhibits phagocytosis of bacteria and thus contributes to the development of the disease. It is encoded by *spa* gene which is considered as one of the important virulence factors in development and severity of mastitis [3, 4]. The gene encoding protein A (*spa*) is composed of some functionally distinct regions: Fc binding region, X-region and C-terminus, a sequence required for cell wall attachment. The X-region of the *spa* gene includes a variable number of 24-bp repeats [5, 6] and hence can be used as a molecular marker typing in studying the genetic diversity among the strains of *S. aureus* for epidemiological tracing of source of infection and comparing the differences in virulent phenotypes among various strains.

The present investigation was designed to study the polymorphism of *spa* gene (X-region) typing in *S. aureus* isolates from clinical mastitic milk of cows.

Materials and Methods

Isolation of *S. aureus*

Sampling

A total of 60 milk samples from cows with clinical mastitis were collected directly from teats to sampling tube at the time of milking, each about 5-10 ml in amount, from different localities of Bikaner (Rajasthan). These samples were immediately taken on ice to laboratory for further processing.

Isolation and identification

The samples were inoculated in nutrient broth over night and then swabbed on nutrient agar followed by overnight incubation at 37 °C. Bacterial colonies were closely observed for their morphology, color and consistency. Gram’s staining was used as primary identification test and suspected colonies were streaked on mannitol salt agar, incubated aerobically at 37 °C for 24 h and identified as described [7, 8]. Of the 60 samples, 30 isolates of *S. aureus* were obtained which were further confirmed genotypically by 23S rRNA ribotyping using species specific Primer 1: 5’- ACG GAG TTA CAA AGG ACG AC 3’ and Primer 2: 5’- AGC TCA GCC TTA ACG AGT AC 3’ [9].
**Amplification of spa gene**

The amplification of *spa* gene encoding protein-A was done as described [10] with slight modifications using 5′-CAAGCACCACAAGAGGAA-3′ (F) and 5′-CACCAGGTATTACGACAT-3′ (R) primers. PCR was performed in 0.2 ml thin-walled PCR tubes. The reaction mixture (total volume 25 µL) was prepared by mixing: 11.9 µl deionised water, 5.0 µl 10x Buffer, 2.5 µl MgCl2, 0.8 µl dNTP-mix (10mM), 0.8 µl of each primer (10 pM/µl), 0.2 µl TaqDNA polymerase (5U/µl) and 3 µl template DNA (25ng/µl). Amplification was carried out in a Veriti thermal cycler (Applied biosystem) as follows: initial 34 cycle of amplification (denaturation at 94 °C for 60 s, primer annealing at 55 °C for 60 s and primer extension at 70 °C for 60 s), and final extension at 72 °C for 5 min. The PCR products were resolved in 1.2% agarose gels prepared in 1x TBE buffer containing 0.5 µg/ml of ethidium bromide along with 100bp ladder as molecular marker. The amplification products were electrophoresed for 60 min at 100 V. The gel was then visualized under gel documentation system (ENDURO GDS).

**Fig 1:** Agarose gel electrophoresis of amplicons of 23S rRNA ribotyping of *S. aureus* isolates obtained from cattle with clinical mastitis; M – Molecular marker (1250bp); CM 1- CM7: Isolates from cattle with clinical mastitis.

**Fig 2:** Agarose gel electrophoresis of amplicons of *spa* gene (X-region) of *S. aureus* isolates obtained from cattle with clinical mastitis; M – Molecular marker (100bp); CM 1- CM7: Isolates from cattle with clinical mastitis.

**Fig 3:** Agarose gel electrophoresis of amplicons of *spa* gene (X-region) of *S. aureus* isolates obtained from cattle with clinical mastitis; M: Molecular marker (100bp); CM 12- CM22: Isolates from cattle with clinical mastitis.
Results

The ribotyping produced an amplicon of 1250 bp in all the 30 isolates confirming them to be Staphylococcus aureus (Fig.1).

In the present investigation amplification of X-region of spa gene produced amplicons in 28 isolates of S. aureus with specific primers whereas two isolates (CM3, CM24) did not amplify this region and were considered spa (x-region) gene deficient (Fig.2 and 3). The size of amplicons obtained were 80, 200, 220, 250, 280, 300 or/and 320bp with calculated number of repeats of 3, 4, 7, 3, 6, 13 and 1 respectively. Most of isolates produced single amplicons whereas nine isolates (CM4, CM7, CM8, CM11, CM15, CM20, CM22, CM25, CM28) produced two amplicons (Table 1). The amplicon of 300bp was detected in 13 isolates (seven single and six in double combination) followed by 220bp in seven, 280bp amplicon in six, 200bp amplicon in four, 250bp amplicon in three, 80bp amplicon in 3 and 320bp in one isolate.

Table 1: spa gene (X-region) polymorphism in S. aureus isolates from cattle with clinical mastitis

<table>
<thead>
<tr>
<th>S. No.</th>
<th>Isolates numbers</th>
<th>Total isolates</th>
<th>spa (X-region) gene amplicon size (bp)</th>
<th>No. of tandem repeats</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>CM1,CM5,CM17,CM18,CM26,CM29,CM30</td>
<td>7</td>
<td>300</td>
<td>11</td>
</tr>
<tr>
<td>2.</td>
<td>CM2,CM9,CM10</td>
<td>3</td>
<td>280</td>
<td>10</td>
</tr>
<tr>
<td>3.</td>
<td>CM4</td>
<td>1</td>
<td>250,320</td>
<td>8,11</td>
</tr>
<tr>
<td>4.</td>
<td>CM6,CM13,CM14</td>
<td>3</td>
<td>80</td>
<td>1</td>
</tr>
<tr>
<td>5.</td>
<td>CM7,CM8,CM22,CM28</td>
<td>4</td>
<td>220,300</td>
<td>7,11</td>
</tr>
<tr>
<td>6.</td>
<td>CM11,CM15</td>
<td>2</td>
<td>280,300</td>
<td>10,11</td>
</tr>
<tr>
<td>7.</td>
<td>CM12,CM27</td>
<td>2</td>
<td>220</td>
<td>7</td>
</tr>
<tr>
<td>8.</td>
<td>CM16</td>
<td>1</td>
<td>250</td>
<td>8</td>
</tr>
<tr>
<td>9.</td>
<td>CM19,CM21,CM23</td>
<td>3</td>
<td>200</td>
<td>6</td>
</tr>
<tr>
<td>10.</td>
<td>CM20</td>
<td>1</td>
<td>220,250</td>
<td>7,8</td>
</tr>
<tr>
<td>11.</td>
<td>CM25</td>
<td>1</td>
<td>200,280</td>
<td>6,10</td>
</tr>
<tr>
<td>12.</td>
<td>CM3, CM24</td>
<td>2</td>
<td>Not obtained</td>
<td>-</td>
</tr>
</tbody>
</table>

Discussion

Protein A, encoded by the spa gene, is one of the virulence factors involved in the Staphylococcal pathogenesis. It has been suggested that the number of 24 bp tandem repeat Amplification of this region thus produces amplicons of variable sizes depending on the number of repeats. This feature of spa gene provides a base for differentiation among various strains of Staphylococcus aureus isolates. The amplicon sizes obtained in the investigation is almost similar to those reported by Salasie et al. (2004) [11] who from 35 S. aureus from milk sample of cattle obtained amplicons of 100, 150, 200, 230, 240, 250, 270, 290 and 340 bp sizes. Similarly Salem-Bekhit et al. (2010) [12] obtained amplicon of 290, 280, 200, 100bp sizes in 68 S. aureus isolates collected from milk samples of cattle. Yadav et al. (2015) carried out spa typing of S. aureus of 16 isolates from cattle and observed amplicon sizes of 120, 150, 200, 250, 280, 300, and 330 bp. We did not obtained spa amplicons in two (6.66%) isolates. Such spa negative S. aureus isolates have earlier been reported by some workers. In an investigation out of 94 isolates of S. aureus collected from raw milk from four dairy farms of Brazil Santos et al. (2014) recorded only 44 (46.8%) isolates positive for spa gene. The absence of spa-X region gene has also been reported Momtaz et al. (2010) [13] from bovine clinical and subclinical mastitis, Salem-Bekhit et al. (2010) [14] in bovine mastitis isolates, Shakeri et al. (2010) [16] in healthy carriers and human patients and Bhati et al. (2016) [17] in bovine subclinical mastitis. Nine isolates produced two bands of spa which are in conformity to the earlier observation by Bhati et al. (2016) [17] who recorded two spa bands in one isolate of S. aureus isolated from subclinical cases of bovine mastitis. The two spa bands have also been reported Rathore et al. (2012) [18] form camel skin wounds.

Conclusion

The present study revealed polymorphism in spa X-region gene amplicons of S. aureus obtained from clinical mastitis cases. A wide degree of polymorphism was observed in the isolates. In the present study of the 30 isolates 22 were considered to be pathogenic since they possessed seven or more repeats.

References


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