MOLECULAR ANALYSIS OF VIRULENT GENES (COA AND SPA) OF
Staphylococcus aureus INVOLVED IN NATURAL CASES OF BOVINE
MASTITIS

Ahrar Khan1*, Riaz Hussain2, Muhammad Tariq Javed1 and Fazal Mahmood1

1Department of Pathology, University of Agriculture, Faisalabad-38040, Pakistan;
2University College of Veterinary and Animal Science, The Islamia University of Bahawalpur, Bahawalpur, Pakistan.
*Corresponding author’s e.mail: ahrar1122@uaf.edu.pk

The present study was undertaken to determine the distribution and genotypic characteristics of Staphylococcus aureus isolates recovered from naturally occurring mastitis in cattle and buffaloes. For this purpose a total of 1445 lactating cattle (653) and buffaloes (792) present at two experimental livestock farms Okara (Bahadarnagar) and Sahiwal (Qadiarabad), in and around district Faisalabad and slaughtered at an abattoir due to low milk yield and were screened for mastitis. California Mastitis Test (CMT) was used to detect sub clinical mastitis. The positive quarter milk samples were collected for culturing of S. aureus isolates. Staphylococcus aureus isolates were identified on the basis of growth features, biochemical characteristics, coagulase test and as well as amplification of coagulase (coa) and spa (spa-X) genes specific to its virulence. S. aureus isolates (n=265) were characterized by Polymerase chain reaction to determine the frequency of coagulase (coa) and spa (spa-X) genes. From these isolates the amplification of the coagulase (coa) gene yielded three different PCR products approximately 204bp to 490bp while spa (spa-X) gene produced five different products ranging in size from 190bp to 320bp. PCR revealed that from all the coagulase positive S. aureus isolates 261(98.5%) had spa (spa-X) gene. The results of the present study indicated that S. aureus isolates recovered from bovine mastitis were genetically different within and among the various herds which may provide essential and valuable strategies to control staphylococcal infections in future.

Keywords: Bovine mastitis, Staphylococcus aureus, PCR, Virulent genes

INTRODUCTION

Mastitis is a disease of high importance with relatively higher incidence among the clinical and sub-clinical health disorders in dairy animals (Karahan et al., 2011; Islam et al., 2012; Kenar et al., 2012). Infection of mammary glands results in long term production loss in dairy industry (Jamil et al., 2011; Hussain et al., 2012a). Prevalence of mastitic pathogens varies from one dairy herd to other but bacterial pathogens are the most prominent and act as first line of infection throughout the world (Unnerstad et al., 2009). Mastitis is caused by a wide range of bacteria mainly Staphylococcus aureus followed by Streptococcus agalactiae and E. coli (Hussain et al., 2012b,c). S. aureus is the major pathogen identified worldwide under different management conditions (Karahan and Cetinkaya, 2007; Taponen and Pyorala, 2009). Intra-mammary infections due to S. aureus cause huge economic losses (Guler et al., 2005; Nazifi et al., 2011). In dairy animals S. aureus causes pustular inflammation of skin and different other organs and is an important pathogen causing mastitis of serious nature (Nagase et al., 2002). Mastitis caused by S. aureus is highly contagious disease and is characterized by congestion, hardness of udder and formation of milk clots in infected animals. Intra-mammary infections with S. aureus are usually associated with increased somatic cell count (Akhtar et al., 2012). S. aureus remains major problem in variety of tropical and semi-tropical management conditions. S. aureus poses different virulent proteins and spreads easily with in dairy herds (Marjan et al., 2009; Karahan et al., 2011; Maksymiec and Mikolajczyk, 2012). S. aureus produces coagulase and spa proteins which are virulence factors and induce mastitis (Karahan et al., 2011). S. aureus produces an important cell wall surface protein A (spa) which impairs the process of opsonisation and phagocytosis by binding with immunoglobulin G (Gao and Stewart, 2004). The important pathological feature of S. aureus infection is formation of abscesses in the deep mammary tissue. These lesions become foci for bacterial shedding in chronically infected animals, refractory to antibiotic therapy and finally results an increase in vascular permeability (Ibrahim et al., 2011). Various phenotyping and genotyping techniques have been employed for sub-typing of S. aureus isolates of bovine and human origin such as gene typing (Wang et al., 2009), plasmid analysis and amplification of specific gene regions (Kalroey et al., 2007; Saei et al., 2009). In spite of intensive control strategies the eradication of this pathogen is difficult. Therefore, the control of S. aureus mastitis is vital importance and remains necessary (Waller et al., 2009). No reports are available...
about the molecular analysis of virulent genes of *S. aureus* recovered from bovine mastitis in Pakistan. Therefore, the present study was conducted to determine the distribution and genotypic characteristics of *S. aureus* isolates recovered from naturally occurring cases of mastitis in cattle and buffaloes.

**MATERIALS AND METHODS**

**Isolation and identification of pathogens:** This study was carried out on 1445 animals including lactating cattle (653) and buffaloes (792) present at two experimental livestock farms Okara (Bahadarnagar) and Sahiwal (Qadiarabad), different private herds present at Colony-I (Chekara, Aminpur Road), Colony-II (Chak 225RB, Satyana Raod), Faisalabad and slaughtered at Faisalabad abattoir. Milk of these animals was examined for the presence of mastitis using California Mastitis Test (CMT) following the standard protocol (Schalm et al., 1971). Positive samples were subjected to bacterial isolation and purification following standard laboratory procedures. For bacterial isolation, a loopful of 0.1ml of milk sample was separately cultured on Nutrient broth (Oxoid), Nutrient agar (Oxoid), Staph-110 agar medium (Oxoid) and 5% sheep blood agar. The pathogens were identified on the basis of cultural and morphological features of their primary growth (National Mastitis Council Inc., 1990). Suspected colonies of *Staphylococci* were kept at -20°C in Nutrient broth with 20% glycerol. All the Staphylococcal isolates were biotyped using commercially available kits (API, BioMerieux, France). All the isolates were also subjected to tube coagulase test.

**DNA extraction:** *S. aureus* DNA was isolated from different bacterial cultures grown on blood agar base supplemented with 5% sheep blood. Different well defined colonies (4-5) from pure culture on the basis of biotyping were removed and mixed in distilled water. This mixer was then boiled for 20-25 min and stored at -20°C for further use in PCR. The bacterial DNA was also extracted using commercially available DNA extraction kit (Vivantis, USA).

**PCR amplification of coagulase and spa genes:** PCR analysis of *S. aureus* for coagulase and spa (spa-X) genes was carried out following method previously described (Goh et al., 1992) with some modifications. The coagulase gene (coa) was amplified with a pair of primers (Invitrogen, USA): Coag-2 (5'-CGA GCC CCA GAT TCA ACA AG-3') and Coag-3 (5'-AAA GAA AAC CAC TCA CAT CA-5'). For amplification of coagulase gene (coa) each sample after initial denaturation at 94°C for 5 min was subjected to 35 PCR cycles consisting of 30s at 94°C, annealing at 55°C for 2 min and elongation at 72°C for 60s. The specific X region of the spa gene present in *S. aureus* was amplified using the primers 5-GCT AAA AAG CTA AAC GAT GC and 5'-CGA GCC CCA AAT ACA GTT GTA CC (Enzynomics, Korea) in 25 µl final volume containing 17 µl of 1X master mix, 4 µl of template DNA and 2µl each forward and reverse primers. Amplification was carried out as follow: initial denaturation at 94°C for 5 min followed by 20 cycles of denaturation for at 94°C for 30s, annealing for 30s at 55°C and extension for 1.30 min at 70°C. The final extension was performed at 72°C for 5 min. The PCR reaction was performed by using PCR master mix (Enzymomics, Korea) in 25 µl final volume containing 17 µl of 1X master mix, 4 µl of template DNA and 2µl each forward and reverse primers. Amplification was carried out in Q-Cycler (Model, England). The electrophoresis and separation of amplified PCR products were carried out at 90 volt for 60 min using 1% agarose gel. The amplified PCR products were stained with etidium bromide 0.5µg/ml (Sambrook et al., 2002), visualized and photographed under UV illuminator gel documentation system (Syngene, NIFSAT).

**RESULTS**

**Amplification of coagulase (coa) gene:** The results of PCR for amplification of coagulase (coa) gene are presented in Table 1. The PCR products obtained were categorized into

<table>
<thead>
<tr>
<th>Genes/PCR product (bp)</th>
<th>No (%)</th>
<th>Abattoir</th>
<th>Okara</th>
<th>Sahiwal</th>
<th>Colony-I</th>
<th>Colony-II</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>coa (n=265)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>204</td>
<td>39(14.7)</td>
<td>9</td>
<td>4</td>
<td>3</td>
<td>11</td>
<td>12</td>
</tr>
<tr>
<td>355</td>
<td>147(41.4)</td>
<td>33</td>
<td>17</td>
<td>16</td>
<td>42</td>
<td>39</td>
</tr>
<tr>
<td>490</td>
<td>79(29.8)</td>
<td>15</td>
<td>11</td>
<td>13</td>
<td>21</td>
<td>19</td>
</tr>
<tr>
<td><strong>spa (n=261)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>190</td>
<td>53(20.3)</td>
<td>14</td>
<td>5</td>
<td>7</td>
<td>13</td>
<td>14</td>
</tr>
<tr>
<td>240</td>
<td>39(14.9)</td>
<td>13</td>
<td>6</td>
<td>7</td>
<td>6</td>
<td>7</td>
</tr>
<tr>
<td>270</td>
<td>47(18.0)</td>
<td>17</td>
<td>6</td>
<td>5</td>
<td>10</td>
<td>9</td>
</tr>
<tr>
<td>290</td>
<td>67(25.6)</td>
<td>15</td>
<td>9</td>
<td>11</td>
<td>13</td>
<td>19</td>
</tr>
<tr>
<td>320</td>
<td>55(21.1)</td>
<td>13</td>
<td>7</td>
<td>6</td>
<td>13</td>
<td>16</td>
</tr>
</tbody>
</table>
Virulent genes of *S. aureus* involved in bovine mastitis

three classes on the basis of their molecular size (Table 1). The PCR amplification and electrophoresis pattern showed that all the coagulase positive and negative staphylococcal isolates approximately produced 204bp to 490bp PCR products. The results also showed that the majority of isolates yielded 355bp band size (Fig. 1). Coagulase negative 39 (14.7%) *S. aureus* isolates on the basis of coagulase test were also characterized by PCR and found to be positive 32 (82.1%) for coagulase gene. During coagulase gene analysis it was observed that PCR product with 355 bp size was the predominant and frequently present in all *S. aureus* isolates obtained from different areas of district Faisalabad.

**Amplification of spa (spa-X):** With the exception of four from coagulase positive and negative *S. aureus* isolates, all isolates from different areas were confirmed positive for spa (spa-X) gene by PCR (Table 1). All the isolates produced five different PCR amplicons of size ranging from 190bp-320bp (Fig. 2). Among spa amplicons, PCR products with 290bp, 320bp and 190bp were frequently obtained. Some *S. aureus* genotypes were less frequently and some were more frequently observed in different herds on the basis of geographic location of different herds.

**DISCUSSION**

The control of bovine mastitis is vital not only in Pakistan but also in the world as mastitis is the major cause among various diseases involved in reduction of milk production (Hussain *et al.*, 2012a). Therefore, it is essential to investigate the pathogens using molecular techniques as vibrant components to control intra-mammary infections. In dairy industry the mastitis can be reduced by identification of exact pathogenesis and virulent factors present in infectious microorganisms. The molecular typing of infectious agents is known to be essential part of infection control strategies and is crucial to track and spread of contagious infections from one region to others or among different herds.

Molecular analysis using 3’ end region of the coagulase gene produced three different kinds of PCR amplicons in the present study. The variation in size of PCR amplicons of coagulase gene could be due to polymorphism among different isolates obtained from different herds. Previously various studies also confirmed different PCR products using molecular analysis of 3’ end region of the coagulase gene (Goh *et al.*, 1992; Kalorey *et al.*, 2007; Reinoso *et al.*, 2008), however, reason for this polymorphism is still unknown (Saei *et al.*, 2009). It could be due to deletion or addition mutations by which different nucleotides are inserted or deleted particularly in 3’ end region of the coagulase gene. Further this variation in gene products could also be due to the antigenic features of coagulase enzyme which has vital role in antigenic variations and prevent coagulase positive *S. aureus* isolates against anticoagulase effects. Amplification

![Figure 1.](image1.png) **Figure 1.** PCR amplification and agarose gel electrophoresis of coagulase (coa) gene present in *S. aureus* isolates stained with ethidium bromide. M: 100 bp DNA marker (Fermentas). Lane 1 and 3 negative samples. Lane 2: 355bp. Lane 4: 204bp.

![Figure 2.](image2.png) **Figure 2.** PCR amplification and agarose gel electrophoresis of spa (spa-X) gene present in *S. aureus* isolates stained with ethidium bromide. M: 100 bp DNA marker (Fermentas). Lane 1: 240bp, Lane 2: 270bp, Lane 3: 290bp, Lane 4: 190bp and Lane 5: 320bp.
of spa (spa-X) gene produced five different PCR amplicons ranging between 190bp-320bp. Among different cell surface proteins produce by \textit{S. aureus}, spa (sap-X) is important and regarded as appropriate target to determine the difference among \textit{S. aureus} isolates in a very short period (Lange et al., 1999; Reinoso et al., 2008; Karahan et al., 2011). The results of our study indicate some genotypes of \textit{S. aureus} occur infrequently in particular dairy herds. The occurrence of such types of \textit{S. aureus} genotypes might be less adapted to udder and easily eliminated from mammary gland and also differed on geographic distribution (Joo et al., 2001). The presence of particular \textit{S. aureus} genotypes in different herds could be due to increased resistance against the host’s immune response particularly neutrophilic activities (Moon et al., 2007). The results of the current study indicated that some \textit{S. aureus} genotypes were frequently observed in those herds that prevailed close to each other in particular area. The possible reason about the presence of these isolates could be linked to the poor management practices adopted by the farmers, existence of common pathogens in the same area, feeding and grazing of healthy and infected animals together and trading of animals between the herds. It can be concluded from the results of the present study that different \textit{S. aureus} obtained from mastitic milk showed genetic diversity and homogeneity in \textit{coa} and \textit{spa} genes on the basis of location of herds.

Acknowledgement: The authors highly acknowledge the grant provided by Higher Education Commission of Pakistan under Project No. 20-979/R&D/07 entitled “Histomorphometry and molecular pathobiology of naturally occurring mastitis in buffaloes and cows”.

REFERENCES


Virulent genes of *S. aureus* involved in bovine mastitis


