Molecular characterization of *Staphylococcus aureus* isolated from milk in Udaipur city (Rajasthan)

Ankita Kumari, Abhishek Gaurav, Nirmal Kumar and Hitesh Kumar

**Abstract**

This study was aimed at the molecular characterization of *Staphylococcus aureus* isolated from milk samples. A total of 100 milk samples comprising of pooled milk (n=25), vendors milk (n=25), pasteurized market milk (n=25) and individual cow milk (n=25) were collected during from Udaipur city, Rajasthan. The *S. aureus* were found in g pooled milk, vendors milk, pasteurized market milk and individual cow milk as 76%, 36%, 0% and 56%, respectively. Out of total 42 *S. aureus* isolates, 10 were multidrug resistant which were further subjected for molecular characterization by PCR. All the 10 isolates were found positive for 16S rRNA and nuc gene. The prevalence of antibiotic resistant genes meca, ermc and aacA-aphD were 40% (4), 70% (7) and 30% (3), respectively.

**Keywords:** Molecular characterization, *Staphylococcus aureus*

**Introduction**

*Staphylococcus aureus* bacteria are Gram positive cocci of 0.5- 1.5 micrometer diameter, forming grape like clusters and facultative anaerobes. The pathogenicity of *S. aureus* is mainly related to a combination of toxin mediated virulence, invasive capacity and antibiotic resistance (Argudin et al., 2010) [1]. Drinking contaminated milk with preformed toxins of *S. aureus* causes rapid onset (IP = 2-8 hours) of vomiting, nausea, abdominal cramps and diarrhoea. *Staphylococcus aureus* is also known for its multidrug resistance and MRSA is one of the most potent drug resistant bacteria that has been causing nosocomial infections and community associated infections and animal diseases (Aklilu et al., 2020) [2]. From a public health point of view, there is a concern about the risk of zoonotic transmission of livestock associated methicillin resistant *S. aureus* (LA-MRSA) strains in animals and man. It has been reported that animal MRSA isolates were significantly more resistant to ciprofloxacin, gentamicin, and clindamycin as compared to human MRSA isolates (Jayaweera et al., 2020) [3].

The PCR is a rapid and reliable tool for the molecular based diagnosis of *S. aureus* infections. Genus specific 16S ribosomal RNA and species specific thermonuclease gene nuc are two important genes to detect *S. aureus*. The frequent and inappropriate use of antibiotics in livestock for therapeutic and growth promoting purpose, results in the emergence of the antibiotic resistance in *S. aureus*. The antibiotic resistance can be easily transferred among healthier commensals and to other animals and humans by close interactions (Sharma et al., 2017) [4]. However, multidrug resistance in *S. aureus* is an emerging and important public health threat as there are fewer, effective antimicrobial agents available for infections caused by these MDR (multidrug resistant) strains.

**Materials and methods**

A total of 100 milk samples comprising of pooled milk (n=25), vendors milk (n=25), pasteurized market milk (n=25) and individual cow milk (n=25) were collected from Udaipur city, Rajasthan. The samples of milk were collected twice in a week from dairy shops, vendors, market and dairy farms from Udaipur city in Rajasthan. The samples were collected in sterile container and transported to the laboratory within 2 hours in chilled condition by using ice packs.

**Molecular characterization**

Isolation of DNA from pure culture was undertaken using by Nucleo-pore gDNAfungal/bacterial mini kit by following the manufacturer’s instructions supplied along...
with the kit. Genomic DNA isolated from S. aureus isolates were used in the PCR. Published primers were used for the detection of 16S rRNA, nuc, ermC, aacA-aphD and mecA genes in S. aureus isolates are described in Table No. 1.

Table 1: The primers used for detection of 16S rRNA, nuc, ermC, aacA-aphD and mecA genes

<table>
<thead>
<tr>
<th>S. No.</th>
<th>Oligo name</th>
<th>Name sequence (5' - 3')</th>
<th>Size of amplified product (bp)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>16S rRNA forward</td>
<td>GTAGGTGCCAAGCGTTATCC</td>
<td>228</td>
<td>Loveseth et al. 2004 [5]</td>
</tr>
<tr>
<td>2</td>
<td>16S rRNA reverse</td>
<td>GCGAATGATGTTGTAACGTTT</td>
<td>279</td>
<td>Barski et al. 1996 [6]</td>
</tr>
<tr>
<td>3</td>
<td>nuc forward</td>
<td>AGCCAGGCTTATGACGAATCC</td>
<td>227</td>
<td>Strommenger et al. 2003 [7]</td>
</tr>
<tr>
<td>4</td>
<td>ermC forward</td>
<td>ATATGTCAATTCCTGCATGT</td>
<td>299</td>
<td>Strommenger et al. 2003 [7]</td>
</tr>
<tr>
<td>5</td>
<td>mecA forward</td>
<td>AAAATCGATGGTAAAGGTTGGC</td>
<td>533</td>
<td>Strommenger et al. 2003 [7]</td>
</tr>
</tbody>
</table>

The PCR procedure to screen the 16S rRNA, nuc, ermC, aacA-aphD and mecA gene in S. aureus isolates was standardized as described by Loveseth et al. (2004) [5], Barski et al. (1996) [6] and Strommenger et al. (2003) [7] with certain modifications. Followed by preliminary trials, the reaction mixture was optimized to contain 12.5 µl 2X PCR master mix, 10 nmol of each forward and reverse primer, 10.5 µl nuclease free water and 1 µl of DNA template. The reaction was performed in the thermal cycler with preheated lid (lid temp. = 105°C). The cycling conditions of 16S rRNA, nuc, ermC, aacA-aphD and mecA gene were comprised of 30 cycles of denaturation, annealing and extension which are described in Table No 2.

Table 2: Steps and conditions of thermal cycling for different primer pairs in PCR

<table>
<thead>
<tr>
<th>Primers (Forward and reverse)</th>
<th>Cycling conditions</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>Initial denaturation</td>
</tr>
<tr>
<td>16S rRNA (F)</td>
<td>940°C for 5 minutes</td>
</tr>
<tr>
<td>16S rRNA (R)</td>
<td>940°C for 5 minutes</td>
</tr>
<tr>
<td>ermC (F)</td>
<td>940°C for 5 minutes</td>
</tr>
<tr>
<td>ermC (R)</td>
<td>940°C for 5 minutes</td>
</tr>
<tr>
<td>aacA-aphD (F)</td>
<td>940°C for 5 minutes</td>
</tr>
<tr>
<td>aacA-aphD (R)</td>
<td>940°C for 5 minutes</td>
</tr>
<tr>
<td>mecA (F)</td>
<td>940°C for 5 minutes</td>
</tr>
<tr>
<td>mecA (R)</td>
<td>940°C for 5 minutes</td>
</tr>
</tbody>
</table>

Results and discussion
Out of the 42 isolates, 10 MDR S. aureus isolates were selected for molecular characterization by targeting the virulence and resistance genes. First of all, detection of 16S rRNA gene was done by standardizing the PCR protocol as per the method described by Loveseth et al. (2004) [5]. Electrophoresis analysis revealed a specific amplification of 228 bp product of the 16S rRNA gene. 16S rRNA gene is species specific gene used in identification of S. aureus (Monday and Bohach 1999) [8]. In our study, the detection of 16S rRNA gene revealed its presence in all the 10 MDR isolates recovered from milk and milk products. Similar findings were also reported by Elsayed et al. (2015) [9], Can et al. (2017) [10], Darwish et al. (2018) [11] and Gencay et al. (2010) [12] who confirmed all presumptive S. aureus isolates by detection of 16S rRNA gene. For molecular identification of S. aureus isolates, molecular targeting of species specific nuc gene of S. aureus coding for the extracellular thermostable nuclease protein of S. aureus was done, which revealed that all the MDR S. aureus isolates were positive for nuc gene. The detection of the nuc gene was carried out as per the method described by Barski et al. (1996) [6] and electrophoresis analysis revealed a specific amplification of 279 bp product of the nuc gene. Kabir et al. (2017) [13] and Javid et al. (2018) [14] also reported 100% prevalence of nuc gene among S. aureus isolates. While, slightly lower prevalence of nuc gene among S. aureus isolates was observed by Kaleorey et al. (2007) [15] and Saraia et al. (2018) [16] as 97.29% and 77.94%, respectively. Thus, the simultaneous detection of both 16S rRNA and nuc gene in S. aureus should be used for the molecular identification of S. aureus. Aminoglycosides resistance in S. aureus may occur as a response to the impermeability catalysed by a bifunctional protein encoded by aacA-aphD gene. PCR assay for the detection of aacA-aphD gene in S. aureus was standardized with primers reported by Strommenger et al. (2003) [7] with slight modifications and electrophoresis analysis revealed a specific amplification of 227 bp product. In our study, 30% prevalence (3/10) of aminoglycosides resistance gene was observed in MDR S. aureus which was in line with the reports of Gulzar et al. (2018) [17] and Zehra et al. (2017) [18] who found prevalence of the aacA-aphD gene as 32.5% and 33.3%, respectively. Higher Prevalence was reported by
Adwan et al. (2014) [19] and Ruban et al. (2017) [20] as 74.5% and 88%, respectively. While, lower prevalence rates were revealed by Hizlisoy et al. (2018) [21] and Zehra et al. (2019) [22] as 9.4% and 7.69%, respectively. Macrolides, lincosamides and streptogamin are antimicrobial groups collectively known as MLS agents. These MLSs are frequently used for the treatment of staphylococcal food poisoning. The MLSs have inhibitory effects on bacterial protein synthesis. The \textit{erm}(A) and \textit{erm}(C) genes are more commonly responsible for the resistance against MLS. PCR assay for the detection of \textit{erm}C gene in \textit{S. aureus} was standardized with primers reported by Strommenger et al. (2003) [7] with slight modifications and electrophoresis analysis revealed a specific amplification of 299 bp product. In total, out of 10 isolates of MDR \textit{S. aureus}, 7 were found to contain \textit{erm}C gene. Wang et al. (2015) [23] reported 92.6% prevalence of \textit{erm}C gene among \textit{S. aureus} isolates. While, Adwan et al. (2014) [19] and Asadollahi et al. (2014) [24] and Fashti et al. (2016) [25] reported prevalence of \textit{erm}C gene in 54.5%, 57% and 20.5% isolates of \textit{S. aureus}.

Presence of \textit{mec}A gene is considered as a reliable method to detect methicillin resistance. Virulent \textit{S. aureus} strains include methicillin resistant \textit{Staphylococcus aureus} (MRSA) strains, which have become resistant to most antimicrobial agents including beta lactams, aminoglycosides, macrolides and fluoroquinolones. Therefore, the spread of MRSA has now considered as an emerging threat to human health. In our investigation, it was observed that out of 10 MDR \textit{S. aureus}, 4 isolates were found to be positive for \textit{mec}A gene giving a prevalence rate of 40%. PCR assay for the detection of \textit{mec}A gene was standardized with primers reported by Strommenger et al. (2003) [7] with slight modifications and electrophoresis analysis revealed a specific amplification of 533bp product of the \textit{mec}A gene. Similar results were reported by Mistry et al. (2016) [26] and Elkenany (2018) [27] who found 48.71% and 54.5% isolates as positive for \textit{mec}A gene, respectively. While, lower prevalence of MRSA were reported by Hoque et al. (2018) [28], Enany et al. (2013) [29], Gindonis et al. (2013) [30] and Normanno et al. (2007) [31] as 20%, 18.18%, 1.8% and 3.75%, respectively. On the other hand, Keyvan et al. (2020) [32] reported higher prevalence of MRSA in which they observed 75.4% of the isolates to be positive for \textit{mec}A gene. Thus, MRSA related management should be applied in dairy farms by detecting the changes in the pattern of the methicillin resistance in bovine staphylococci.
Conclusion
In the current study, out of the 42 isolates, 10 MDR S. aureus isolates were selected for molecular characterization by targeting the virulence and resistance genes. Firstly, detection of 16S rRNA and nuc genes was done by standardizing the PCR protocols. In all the multidrug resistant isolates collected from the different sources of milk samples like pooled milk, vendors milk and individual cow milk, all the MDR isolates (10) were found to be positive for 16S rRNA and nuc genes. Further, the detection of antibiotic resistance genes aacA-aphD, ermC and mecA was carried out. Out of the 10 MDR isolates, only three (30%) isolates were found positive for aacA-aphD gene (all in individual cow milk). Similarly, out of the 10 MDR isolates, 7 (70%) were found positive for ermC which included four isolates from pooled milk, one from vendors milk and two from individual cow milk. While, among the 10 MDR isolates, four isolates (40%) was found to be positive for mecA (three in pooled milk and one in vendors milk). Thus, the high prevalence of multidrug resistant S. aureus isolates is a matter of concern for the public health. So, the antibiotics should be used judiciously in animal husbandry practice to prevent the emergence of antibiotic resistant bacterial strains.

References
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