Isolation and molecular characterization of Staphylococcus aureus from pigs in Thrissur district, Kerala

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Abstract
Staphylococcus aureus is a common commensal bacterial pathogen colonizing humans and variety of animal species. Nasal colonization of S. aureus has been detected from pigs in many parts of the world and is having great potential to act as a human pathogen. The present study was conducted to identify the nasal carriers of S. aureus among pigs and their confirmation by biochemical and molecular characterization. Nasal swabs (n=119) were collected from four pig farms located at different places of Thrissur District and Staphylococci were isolated by conventional culture methods. Preliminary identification of Staphylococci was carried out by performing a range of biochemical tests. Molecular characterization of S. aureus was done by genus specific Polymerase Chain Reaction (PCR) targeting the 16S rRNA and species specific PCR targeting the 23S rRNA. The prevalence of S. aureus in the study was 42.86 percent.

Keywords: Isolation-molecular characterization-S. Aureus-prevalence-pigs

Introduction
Staphylococcus aureus is a Gram positive commensal bacterium of humans causing a wide variety of diseases ranging from simple skin and soft tissue infections to severe life-threatening septicemic conditions [1]. It is a pathogen of greatest concern because of its inherent capacity to cause resistance, cause severe infections by production of different types of toxins and its ability to adapt to different environmental conditions [2]. Staphylococcus aureus is considered as the leading cause of nosocomial infections [3]. The primary mode of transmission of S. aureus is by direct skin-to-skin contact and also by indirect contact with contaminated objects or surfaces [4]. An important character of S. aureus is its ability to colonize in healthy individuals without any symptoms [5].

In animals S. aureus is one of the three most pathogenic Staphylococcus species along with S. hyicus and Staphylococcus Intermedius Group (SIG) which include S. pseudintermedius, S. intermedius, and S. delphini [6]. Pig farming is considered as an important risk factor for nasal colonization of humans with S. aureus [7]. Methicillin Resistant Staphylococcus aureus (MRSA) infections are increasingly reported from pigs in many parts of the world, which is considered as a major threat to public health globally. Livestock associated MRSA especially pig associated MRSA is now common in countries with high density pig farming and pigs acted as a major reservoir for transmission of MRSA to humans [8, 9]. Identification of nasal carriers of S. aureus among pigs will be helpful for avoiding the colonization and infection of humans by this deadly pathogen.

This study was conducted with the objectives of determining the prevalence of S. aureus among healthy pigs, to investigate the risk factors for nasal colonization of S. aureus and molecular characterization of the isolates using Polymerase Chain Reaction.

Materials and Method
Sample Collection
Nasal swabs (n=119) were collected from healthy pigs of different age and sex groups from four pig farms located at different places of Thrissur District. All sampling were done using sterile cotton-tipped swabs that were placed in Mannitol Salt Broth (MSB), (Himedia Laboratories). A questionnaire regarding the possible risk factors for nasal colonization of S. aureus was also filled during the farm visit. Swabs were immediately brought to laborato under sterile precautions.
Culture and Isolation of bacteria
The nasal swabs collected in MSB were incubated overnight at 37 °C for enrichment. Following overnight incubation the swabs were streaked onto the Staphylococcus selective Mannitol Salt Agar (MSA) (Himedia) plates. The plates were then incubated aerobically at 37 °C for 24 hrs. Plates were examined after 24 hrs for growth. Plates with yellow colored colonies were selected for further identification. The isolates were identified based on morphology, cultural characteristics and biochemical tests as per Barrow and Feltham (1993) [10] and Quinn et al. (2013) [11].

Biochemical characterization
Gram’s staining was done for preliminary identification of the isolates and further identification of the Gram positive isolates as Staphylococcus aureus was carried out by performing a range of biochemical tests which included catalase test (positive), indole test (negative), methyl red test (positive), Voges-Proskauer test (positive), citrate utilization test (positive), urease test (positive), sugar fermentation test and nitrate reduction test (positive). Sugar fermentation test was performed using seven sugars which included fructose (positive), galactose (positive), sucrose (positive), arabinose (negative), Raffinose (negative), lactose (positive) and trehalose (positive).

Molecular Characterization
Molecular identification of the isolates was done by performing genus and species specific PCR. Genus specific PCR was performed by using the primer pairs given in Table.1, targeting the 16S rRNA of Staphylococcus with a predicted amplicon size of 756 bp [12]. Species specific PCR was carried out using the primer pairs given in Table.1, targeting the 23S rRNA with a predicted amplicon size of 1318 [13]. The PCR amplification was performed using a total volume of 25μl reaction mixture containing 3μl of DNA template, 1μl each of forward and reverse primers, 12.5μl of 2X master mix and 7.5μl of nuclease free water. The cycling conditions for both the PCR are mentioned in table 2 and 3.

Table 1: Oligonucleotide primer pairs used for detection of Staphylococcus isolates

<table>
<thead>
<tr>
<th>Organism</th>
<th>Primer</th>
<th>Primer sequence (5'-3')</th>
<th>Size of amplicon (bp)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Staphylococcus</td>
<td>F</td>
<td>AACCTCTGTATTAGGGAAGAACA</td>
<td>756</td>
<td>Ciftci et al., 2009 [12]</td>
</tr>
<tr>
<td>spp.</td>
<td>R</td>
<td>CCACCTTCCTCCGGTTGTCACC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>S. aureus</td>
<td>F</td>
<td>GGACGACATTAGACGATCA</td>
<td>1318</td>
<td>El-Razik et al., 2010 [13]</td>
</tr>
<tr>
<td></td>
<td>R</td>
<td>CGGCGACCTATTTTCTATCT</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 2: PCR protocol used for the identification of Staphylococcus spp.

<table>
<thead>
<tr>
<th>Steps</th>
<th>Temperature (°C)</th>
<th>Time</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial denaturation</td>
<td>94</td>
<td>5 min</td>
</tr>
<tr>
<td>Denaturation</td>
<td>94</td>
<td>45 sec</td>
</tr>
<tr>
<td>Annealing</td>
<td>58</td>
<td>45 sec</td>
</tr>
<tr>
<td>Extension</td>
<td>72</td>
<td>90 sec</td>
</tr>
<tr>
<td>Final extension</td>
<td>72</td>
<td>10 min</td>
</tr>
<tr>
<td>Hold</td>
<td>4</td>
<td>Until use</td>
</tr>
</tbody>
</table>

Table 3: PCR protocol for the identification of Staphylococcus aureus

<table>
<thead>
<tr>
<th>Steps</th>
<th>Temperature (°C)</th>
<th>Time</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial denaturation</td>
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<td>5 min</td>
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<tr>
<td>Denaturation</td>
<td>94</td>
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<tr>
<td>Annealing</td>
<td>50</td>
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<tr>
<td>Extension</td>
<td>72</td>
<td>2 min</td>
</tr>
<tr>
<td>Final extension</td>
<td>72</td>
<td>10 min</td>
</tr>
<tr>
<td>Hold</td>
<td>4</td>
<td>Until use</td>
</tr>
</tbody>
</table>

Results and Discussion
A total of 119 nasal swabs were collected from pigs of different age groups and sex. Management activities and hygienic practices followed in the farms were analyzed which revealed that in all the farms except one, swill feeding was followed and the floors were cleaned with water alone. The stocking density of animals in the pens was medium to high.

The nasal swabs were collected as the preferred sample for isolation of Staphylococcus because anterior nares were considered as the most common site of Staphylococcal colonization [14]. The nasal swabs were enriched in MSB, selective enrichment media for isolation of Staphylococcus. The use of selective enrichment inhibits the growth of unwanted organisms. After incubation the colour of the broth was changed from red to yellow indicating the growth of bacteria fermenting mannitol. De Neeling et al. (2007) [15] also used Mannitol salt broth for enrichment of the nasal swabs whereas Dierikx et al. (2016) [16] and Furono et al. (2018) [17] used Muller Hinton broth with 6.5 percent NaCl for pre-enrichment and phenol red mannitol salt broth with 5 mg/L cefitoxime and 75 mg/L aztreonam.

Yellow coloured colonies were observed on MSA after overnight incubation at 37 °C. Gram’s staining of the isolates revealed that 98 isolates were Gram positive cocci. Staphylococcus organisms were identified as Gram positive grape-like cocci. Gram positive cocci were further characterized by various biochemical tests. S. aureus isolates were positive for catalase test, methyl red test, Voges-Proskauer test, citrate test, urease test, and nitrate reduction test and negative for indole test. Sugar fermentation test result was positive for all sugars except arabinose and Raffinose.

Fig 1: Enrichment in Mannitol Salt Broth

Fig 2: Greenish blue coloured colonies of MRSA on Hicrome MeReSa CHROM agar
Molecular characterization

Molecular characterization of *S. aureus* isolates were performed by genus and species specific PCR. Out of the 98 Gram positive cocci, eighty two isolates (68.9 percent) were positive by genus specific PCR targeting the 16S rRNA as described by Ciftci et al. (2009) and yielded the PCR product with an amplicon size of 756 bp (Fig. 3). Species specific PCR targeting the 23S rRNA of *S. aureus* was performed as described by El-Razik et al. (2010) with the isolates positive by genus specific PCR. The PCR products were yielded out of eighty two *Staphylococcus* isolates, 51 were *S. aureus*. Thus the prevalence of *Staphylococcus aureus* in the present study was 42.86 percent.

![Fig 3: Agarose gel electrophoresis of the PCR amplified products of *Staphylococcus* spp. L-100 bp ladder, Lane 1-13 Positive samples, P- Positive control](image)

![Fig 4: Agarose gel electrophoresis of the PCR amplified products of *Staphylococcus aureus* isolates. L- 250 bp ladder, Lane 2-13 samples, P- Positive control, N- Negative control](image)

Conclusion

*S. aureus* is a potent opportunistic pathogen causing serious illness in humans and animals. Nasal carriage of pigs is a major concern as they can act as a source of *S. aureus* infection in humans. The present study confirmed the occurrence of *S. aureus* among healthy pigs and the prevalence was found to be 42.86 percent by molecular detection techniques. This finding of *S. aureus* colonization in healthy pigs warrants further studies to be conducted to detect the presence of MRSA. Screening of pig farm workers and other persons engaged in farm activities for nasal carriage is another area to be explored. Continuous surveillance of persons in close contact with pigs should be carried out and stringent preventive measures such as adoption of hygienic management practices should be practiced to avoid the risk of humans getting exposed and colonized with these potentially pathogenic bacteria.

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References