MEDIA
Kedokteran Hewan
Veterinary Medicine Journal


Species Identification of Coagulase Positive Staphylococci (CPS) by Multiplex Polymerase Chain Reaction (PCR)

Mustofa Helmi Effendi

Department of Veterinary Public Health, Faculty of Veterinary Medicine, Airlangga University,
“C Campus”, Jl. Mulyorejo Surabaya 60115; e-mail: mheffendi@yahoo.com

Abstract

The aim of this work was to identify the species specific by using multiplex PCR for the identification of two species of coagulase positive staphylococci (CPS): Staphylococcus aureus, and Staphylococcus intermedius. Twenty eight staphylococcus isolates, previously characterized as CPS through the coagulase, and catalase production tests and Gram staining, were identified at genus level. At the same time, the DNA of these isolates was extracted and amplified by multiplex PCR, using primers for the nuc gene, specific for S. aureus, and S. intermedius. It can be concluded that nuc gene can be used for differentiation between coagulase positive staphylococci.

Key words: CPS, Staphylococcus aureus, Staphylococcus intermedius, nuc gene, multiplex PCR

Introduction

Bacteria of the genus Staphylococcus represent the most significant and widespread bacterial pathogens in human and animal infections (el-Zubair et al., 2007). However, in field genus Staphylococcus especially coagulase positive staphylococci (CPS) face a problem to identify on species level with high sensitivity. In the last few years, much research has concentrated on the determination of the minimum number of phenotypical tests, which can be used, reliably to identify a species of microorganism. Consequently, several authors investigated different tests to identify and differentiate S. aureus and S. intermedius. In strains previously classified as belonging to the coagulase positive staphylococcus group (by the tests of free coagulase, thermonuclease, catalase and Gram) the beta-galactosidase production test and sensitivity to aminocillin are those that best discriminate these species (Loutz et al., 2006).

Staphylococcus aureus is recognized worldwide as a frequent cause of subclinical intramammary infections in dairy cows. The main reservoir of S. aureus seems to be the infected quarter, and transmission between cows usually occurs during milking. S. aureus produces a spectrum of extracellular protein toxins and virulence factors which are thought to contribute to the pathogenicity of the organism. Staphylococcus aureus is a major cause of intramammary infection in ruminants and is a causative agent of a range of human and animal diseases. S. aureus mastitis tends to commence with an acute clinical episode which subsequently develops to become a chronic infection (Akineden et al., 2001). The cure rate after antibiotic therapy is low. The chronic nature of bovine staphylococcal mastitis and the ability of the bacteria to withstand strong inflammatory responses may be associated with an impairment of the immune response mediated by factors secreted by S. aureus. The staphylococcal enterotoxins (SEs) are recognized agents of the staphylococcal food poisoning syndrome and may be involved in other types of infections with cause shock in humans and animals (Akineden et al., 2001).

Staphylococcus intermedius is a coagulase-positive zoonotic organism found in pigeons, dogs, foxes, mink, and horses (Potumbarthy et al., 2004). Initially, all coagulase-positive staphylococci were identified as Staphylococcus aureus, until Hajek in 1976 established the unique identity of a group of organisms, originally identified as S. aureus biotypes E and F, as S. intermedius. The name of this species reflects the fact that while the organisms possess some phenotypic properties of S. aureus, they also exhibit some properties of Staphylococcus epidermidis. S. intermedius is a common commensal of oral, nasal, and skin flora in healthy dogs, where it can also cause invasive disease. In humans, it is recognized as an invasive zoonotic pathogen and has been isolated from 18% of canine infected wounds (Pottumbarthy et al., 2004).

More recently, it has been suggested that techniques based on the Polymerase Chain Reaction (PCR) could be used for the discrimination of bacterial species. However, little has been done regarding the...
differentiation of *S. aureus* and *S. intermedius*. The aim of this work was to compare and evaluate a molecular technique based on PCR, using as targets the *nic* gene to identify *S. aureus* and *S. intermedius*.

**Materials and Method**

**Bacterial isolates**

Twenty three isolates from subclinical bovine mastitis and five isolates were taken from dogs wound infection which previously identified as coagulase positive staphylococci (CPS) through the tests of Gram staining (Gram+), catalase production (catalase+), thermonuclease production (TNase+), and coagulase production (coagulase+) were used. All isolates were maintained on TSA (Tryptic Soy Agar – Acumedia) until required for analysis.

**Molecular identification of *S. aureus* and *S. intermedius***

A molecular identification was performed by amplifying the *nic* gene of *S. intermedius* and *S. aureus* using species-specific oligonucleotide primers respectively. The oligonucleotide primer sequences and PCR programs were summarized in Table 1. For DNA preparation, three colonies of freshly subcultured strains to be investigated were suspended in 100 μl TE buffer (10 mm Tris-HCl, 1 mm of ethylene diamine tetraacetic acid (EDTA)/l, pH 8 and 5 μl lysostaphin (1.8 U/μl; Sigma, Steinheim, Germany). After incubation for 1 h at 37°C, 10 μl proteinase K (Qiagen, Hilden, Germany) was added and the suspension was incubated for 2 h at 56°C. Finally, the proteinase K was inactivated through boiling of the mixture for 10 min followed by 2 min cooling on ice. After centrifugation at 13 000 g for 3 min the supernatant was used for PCR. For some of the strains the DNA was isolated by suspending 3-4 colonies in 180 μl TE buffer containing 8 μl lysostaphin. After incubation for 1 h at 37°C 20 μl proteinase K was added. The suspension was then reincubated as described above and the DNA was isolated with DNeasy Tissue-Kit (Qiagen) according to the manufacturer’s recommendations. The PCR reaction mixture (20 μl) contained 1.8 μl, 1.7 μl, and 0.4 μl of SlnuC, Slnuc and 16S primers (10 pmol/μl respectively, 0.8 μl dNTP (10 mmol; Genecraft, Germany), 2 μl of 10× biobuffer with a final concentration of 8 mM MgCl₂, 0.3 μl biobuffer polymerase (Genecraft). PCR 0.7 μl of each primer (10 pmol/μl) and 0.3 μl Expand High Fidelity PCR system (Roche Diagnostics, Mannheim, Germany) and 0.9 μl H₂O. Finally 5 μl DNA preparation was added to each reaction tube. The tubes were then subjected to thermal cycling (Gene Amp PCR System 2400, Perkin Elmer, Germany) with programs described in Table 1. The presence of PCR products was determined by electrophoresis of 10 μl of reaction product in an 1.5% agarose gel (Geno BRL, Karlsruhe, Germany) with Tris-acetate electrophoresis buffer (TAE, 40 mmol/l Tris-HCl 1 mmol/l EDTA, pH 8.0) visualized under UV light and visualized under UV light (Image Master VDS, Pharmacia Biotech, Freiburg, Germany) (Lautz et al., 2006).

**Results and Discussion**

All strains investigated in the present study could be preliminary identified as coagulase positive Staphylococci (CPS) based upon their properties in culture, b-hemolysis on sheep blood agar and a positive coagulase showed in Fig 2 and also microscopic structure by Gram staining showed in Fig 1. The species identity could be confirmed by *nic* and 165 rDNA multiplex PCR yielding positive reactions with the *S. intermedius* *nic* gene specific oligonucleotide primer and *S. aureus* *nic* specific oligonucleotide primer.

<table>
<thead>
<tr>
<th>Table 1. Sequences and Thermal Cycler Programs of the Oligonucleotide Primers Used in the Present Study</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Oligonucleotide</strong></td>
</tr>
<tr>
<td>1/SlnuC</td>
</tr>
<tr>
<td>2/SlnuC</td>
</tr>
<tr>
<td>3/Slnuc</td>
</tr>
<tr>
<td>4/Slnuc</td>
</tr>
<tr>
<td>5/16S1</td>
</tr>
<tr>
<td>6/16S2</td>
</tr>
</tbody>
</table>

Reference: Baron et al. (2004); PCR program: 1x (95°C, 240 s), 30x (95°C, 30 s; 55°C, 30 s; 72°C, 30 s), and 1x (72°C, 420 s).
Figure 1. Microscopic structure of coagulase positive Staphylococci (CPS) with Gram staining.

Figure 2. Colonies of coagulase positive Staphylococci (CPS) from wounded skin dog on Blood Agar.

In the last decade, several genes, e.g. 16S rRNA, 23S rRNA, nuc, coa and tuf, have been used for identification of S. aureus and other clinically important CPS using PCR-based methods. For example, a PCR targeting the nuc gene is now becoming established in clinical microbiology laboratories for detection of S. aureus in blood cultures in humans (Lautz et al., 2006). Similarly, Akineden et al. (2001) developed a PCR test that allows specific detection of low concentrations of S. aureus in bovine milk samples. However, less encouraging results have been achieved when trying to identify CPS other than S. aureus (Becker et al., 2005; Devriese et al., 2005).

Nucleic acid amplification by PCR has applications in many fields of biology and medicine, including the detection of viruses, bacteria, and other infectious
agents. In the present study, author used oligonucleotide primer set which recognized sequences of the S. aureus nuc gene and S. intermedius nuc gene, which encodes the Tnase produced by these bacteria. This strategy was chosen since earlier data obtained by using polyclonal or monoclonal antibodies to detect the S. aureus Tnase and S. intermedius Tnase indicated that these protein has species-specific sequences and that DNA hybridization-based methods corroborated the assumption that these nuclease species specific (Braskstad et al., 1992; Lautz et al., 2006). On the other hand, some non-CPS, streptococci, and possibly other bacteria (el Zubeir et al., 2007) may produce nucleases with enzymatic activity that mimics that of these proteins. A simple method of lysing the bacteria was applied in this study. The primer set determined the generation of a PCR product of approximately 125 bp, and 420 bp refer to S. intermedius nuc gene and S. aureus nuc gene respectively (Baron et al., 2004).

The nuc PCR detected >20 viable coagulase positive staphylococci cells or correspondingly low levels (0.69 pg) of extracted DNA in saline. The sensitivity accords with that described for PCR with other bacteria, being between 1 and 20 CFU (Braskstad et al., 1992) or between 1 and 100 pg for DNA extracted from coagulase positive staphylococci. An increase in sensitivity may be achieved by nested PCR amplification. The nuc primer set recognized all staphylococci identified as coagulase positive staphylococci, but it did not recognize the other bacteria tested (da Silva et al., 2003).

A multiplex PCR assay allows simultaneous amplification of several genes in one reaction mixture. The multiplex PCR which could successfully identify and differentiate S. aureus and S. intermedius. This multiplex PCR was based on the nuc genes of both species and an universal segment of 16S rDNA as targets. Becker et al. (2005). In the present study, the multiplex PCR described by Baron et al. (2004). According to the present results, 23 strains could be identified as S. aureus that isolates were used from subclinical bovine mastitis and five strains as S. intermedius that indicating the predominant role of S. intermedius plays in infections of dogs showed in Fig. 3. This PCR systems used in the present study appeared to be highly specific. Comparable to this, the 16S rRNA gene of S. intermedius and S. aureus displayed a similarity of more than 99% (Devries et al., 2005). Author observed that the 16S rRNA can be used as a tool to separate the Staphylococcus genus from other bacteria genus.

These results substantiate those obtained by other methodological approaches (Lautz, et al., 2006), which have suggested that the nuc gene and its product have sequences which, on the one hand, are found in all coagulase positive staphylococci isolates and, on the other hand, are unique to bacteria of that species. Recently, PCR amplification of the nuc gene was reported by da Silva et al. (2003), who used a primer set different from that used in the present study. Those investigators amplified a 431-bp segment of the gene. However, very few data of this amplification were described. The combination of the nuc PCR for species identification of coagulase positive staphylococci and PCR for the identification of genes encoding defined phenotypic characteristics of the bacteria is therefore possible. DNA amplification by PCR has applicability in the diagnosis of infectious diseases provided that the tests have adequate sensitivity and specificity.

All of the 28 isolates which contained coagulase positive staphylococci were positive by direct testing in the nuc PCR for the 125-bp and 420-bp PCR product refer to genes for thermonuclease of S. intermedius and S. aureus respectively, whereas coagulase negative staphylococci (CNS) such as Staphylococcus epidermidis were negative. These findings substantiate the prospects of the rapid diagnosis of coagulase positive staphylococci infections by the nuc PCR described in this report, for instance, when a rapid diagnosis is important in order to initiate adequate therapy immediately (Lautz et al., 2006). Author observed that the quantity of the 125-bp and 420-bp PCR product varied with the number of coagulase positive staphylococci CFU isolated from the specimen. This method and other techniques that may increase the sensitivity of the nuc PCR for the detection of coagulase positive staphylococci in clinical specimens need to be investigated further.

Conclusions

The primer sets proposed in this study showed their potential to be used in the differentiation of the species studied, constituting a very useful tool for epidemiological identification as well as coagulase positive staphylococci (CPS) species identification involved in outbreaks of staphylococcus infection. Therefore, it can be concluded that isolates of CPS from wound dogs were Staphylococcus intermedius and isolates of CPS from subclinical bovine mastitis were Staphylococcus aureus.

Acknowledgements

This work was supported by DAAD Foundation, Germany. The author wish to thank Talah Kanbar for providing isolates of coagulase positive staphylococci from wounded dogs and thank Claudya Hesse for providing isolates of coagulase positive staphylococci from subclinical bovine mastitis.
Figure 3. Detection of thermolabile encoding gene nuc of Staphylococcus intermedius (125 bp) and S. aureus (420 bp), and a highly conserved region of 16S rDNA (252 bp) as internal positive control by multiplex PCR; typical amplicons of S. aureus (lanes 1, 2, 3); S. intermedius (lanes 4, 5) and a staphyloccocal control strain (lane 6); M, Gene Ruler DNA Ladder Mix by Fermentas.

References


