Development Of PCR-Hybridization For The Identification Of Major Gram Positive Bacteria Causing Bacteremia

A Mahayotha, R Wongvilairat, S Dejsirilert, T Sumpradit, A Kerdsin

Abstract
Blood culture is considered to be a gold standard for diagnosis of bacterial septicemia. However, this method has some limitations. Molecular techniques for detection of DNA of bacteria causing septicemia such as PCR and hybridization are sensitive, specific and more rapid compared with blood culture. The aims of this project are to develop PCR – slot blot hybridization technique for detection and identification of Gram positive bacteria causing bacteremia.

16S rRNA gene of gram positive cocci (11 isolates) were extracted and amplified by using universal primer. These fragments of each species were cloned in plasmids and preserved as clone libraries. Plasmid DNA was amplified by using M13 primer and PCR product used as DNA template to blot on a nylon membrane. V6 region in 16S rRNA gene of Streptococcus pneumoniae was amplified by PCR using digoxigenin-11-dUTP. PCR product was an initial experimental probe to optimize conditions of the hybridization with the reference DNA blotted on nylon membrane. Various anneal temperature and concentration of SSC buffer were tested to determine optimum hybridization conditions based on visual observation by using anti-DIG-AP conjugate, CDP-StarTM.

This study found that the hybridize temperature at 65oC exhibited more specific for probe of S. aureus, S. epidermidis and E. faecalis and unspecific for Streptococci. At 67oC, hybridization exhibited more intense signal when used probe of all streptococcal species without any cross hybridization. The optimal condition of PCR-Slot blot Hybridization that was chosen as 67oC of the hybridize temperature using for method validation. The lowest of pure DNA concentration of S. aureus ATCC 25923 which the PCR-Slot blot hybridization was given as positive results, was 0.7ng/µl. Sensitivity and specificity of PCR-Slot blot hybridization were highly value (100%). The results revealed that probe design and the optimized conditions were successful in the detection of gram positive septicemia-bacteria.

INTRODUCTION
Septicemia is the presence of bacteria in blood (bacteremia) and is often associated with severe disease. This disease is a serious, life-threatening infection that gets worse very quickly. In case of severe sepsis is a serious condition that requires a hospital stay and an intensive care unit (ICU) for admission (Amersfoort et al., 2003; Cunha, 2003; Abraham, 1999; Ammerlaan et al., 2009). Early diagnosis of this disease is a key for prevention of septic shock progression and associated with the correctly treatment of clinicians. Physical examination and laboratory confirmation are used as a tool in septicemia diagnosis. Especially laboratory tests play an important role in identifying the infectious agent causing the infections (Agnihotri, 2004). Blood culture is considered to be the gold standard for diagnosing bacterial septicemia (Baron et al., 2005; Chen et al., 2008). However, this method has some limitations, including; a long time for growth of an organism(48

MATERIALS AND METHODS
Bacterial strains and culture. 31 Reference septicemia bacteria were obtained from the Culture Collection at the National Institute of Health, Department of Medical Sciences, Ministry of Public Health (NIH, Nontaburi, Thailand). All strains were cultured on agars and incubated 1-3 days depend on strains and species. Pure colonies are selected and sub-cultured again for DNA extraction.

Extraction of bacterial DNA and DNA amplification. Bacterial cells of each strains were harvested in sterile DW. DNA were extracted by using High pure PCR Template preparation kit (Roche Diagnostic?, USA) according to the manufacturer.
RESULTS
In the step of DNA template preparation comprised of reference bacterial cultures, extraction of bacterial nucleic acids, DNA target amplification by using universal primer and DNA target cloning. 1,446 bps- DNA target-template of all gram bacteria blotted on membrane were synthesized by using M13 primers via PCR, shown in Fig. 1.

Figure 1
A = PCR products from 16S rRNA gene amplification of reference bacteria by universal primer (27F, 1492R), B = PCR products from plasmid DNA amplification by using M13 primer as DNA template and blotted on a nylon membrane.

Polynucleotide probe-labeled sized 125 bps was synthesized by using a primer pair (V6F, V6R) and DIG-11-dUTPs via PCR, shown in Fig. 2.

Figure 2
Polynucleotide probe-labeled sized 125 bps was synthesized by using a primer pair (V6F, V6R) and DIG-11-dUTPs via PCR

The result of initial experimental probe to optimize conditions of the hybridization, showed that all gram positive bacteria were positive under the hybridized temperatures varied from 58 to 62°C and were negative at hybridized temperature at 70°C (Fig. 3). Interesting, only S. pneumoniae was discriminated from other gram positive bacteria at the optimized temperatures varied from 65 to 68°C (Fig. 3).

Table 3

<table>
<thead>
<tr>
<th>Specific probes</th>
<th>Temperature of Hybridization</th>
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<tbody>
<tr>
<td></td>
<td>65°C</td>
</tr>
<tr>
<td>S. pyogenes</td>
<td>Cross hybridization</td>
</tr>
<tr>
<td>S. agalactiae</td>
<td>Cross hybridization</td>
</tr>
<tr>
<td>S. equi</td>
<td>Cross hybridization</td>
</tr>
<tr>
<td>S. mitis</td>
<td>Cross hybridization</td>
</tr>
<tr>
<td>S. bovis</td>
<td>Cross hybridization</td>
</tr>
<tr>
<td>S. dysgalactiae</td>
<td>Cross hybridization</td>
</tr>
<tr>
<td>S. mutans</td>
<td>Cross hybridization</td>
</tr>
<tr>
<td>S. aureus</td>
<td>Specific hybridization</td>
</tr>
<tr>
<td>S. epidermidis</td>
<td>Specific hybridization</td>
</tr>
<tr>
<td>E. faecalis</td>
<td>Specific hybridization</td>
</tr>
</tbody>
</table>

The hybridize temperature varied from 65 to 67°C was tested again with the other probes of gram positive bacteria included S. pyogenes, S. agalactiae, S. equi, S. mitis, S. bovis, S. dysgalactiae, S. mutans, S. aureus, S. epidermidis and E. faecalis. These results showed that all gram positive bacteria were positive at hybridized temperature at 67°C (Table 3).

Table 3
Cross hybridization and Specific hybridization of gram positive septicemia-bacteria probes with gram positive septicemia target bacteria at temperature hybridization 65°C and 67°C.

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<td>Specific hybridization</td>
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<td>E. faecalis</td>
<td>Specific hybridization</td>
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Method validation
The optimal hybridization temperature for the PCR-Slot blot Hybridization was at 67°C, positive blood culture in clinical
specimens are tested by this technique to determine method validation (Alonzo & Pepe 1999): limit of detection, sensitivity, specificity in comparison with conventional blood culture results.

The limit of detection, pure DNA concentration of S. aureus ATCC 25923 which the PCR-Slot blot Hybridization was detected as positive results, was 0.7 ng/μl.

DISCUSSION

The results of this study showed that gram positive bacteria causing frequently septicemia were positive under the hybridize temperature varied from 58 to 62°C and were negative at hybridize temperature at 70°C. Interestingly, only S. pneumoniae was discriminated from other gram positive septicemia-bacteria at the optimized temperature varied from 65 to 68°C. But the detection signal of hybridization at 68°C was light signal as a consequence that this temperature was higher than the melting temperature (Table 4) and may have substantial effects on weaker hydrogen bonding between probe-target sequences (DIG Application Manual, Roche applied science, 2009). This polynucleotide probe will benefit for the identification S. pneumoniae in hema-cultures samples by using the specific optimized conditions (Davis & Fuller 1991; Zhang et al., 1995; Chadravorty et al., 2007).

The hybridize temperature varied from 65 to 67°C was tested again with the other specific probes of gram positive septicemia-bacteria included S. pyogenes, S. agalactiae, S. equi, S. mitis, S. bovis, S. dysgalactiae, S. mutans, S. aureus, S. epidermidis and E. faecalis. The hybridize temperature at 65°C exhibited more specific for S. aureus, S. epidermidis and E. faecalis and unspecific for all Streptococcus spp.

From the similarity matrix of multiple sequence alignments of V6 region (961-1085) among S. aureus strain ATCC 14458, S. epidermidis strain ATCC 12228, E. faecalis strain ATCC 29212, S. pneumonia strain ATCC 49619 and S. dysgalactiae subsp. equisimilis ATCC 12394. Within Streptococci group, the results of V6 region sequences alignments had shown the aligned score between 80-100% similar sequences (Fig. 5). When hybridize temperature was increased to 67°C, each specific probe hybridized to each specific Streptococci within Streptococci group with intense signal without any cross hybridization. The finding indicated that there were many factors that influenced on specificity of probe hybridization such as hybridize temperature, the differentiation of sequence, % GC contents, sequence length, hybrid buffer, also probe affinity.

Table 4

<table>
<thead>
<tr>
<th>V6-Probe type</th>
<th>Length (bp)</th>
<th>Tm (°C)</th>
<th>Log[10]</th>
<th>Stringency (%GC)</th>
<th>Stringency (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>S. aureus</td>
<td>123</td>
<td>59</td>
<td>0.51</td>
<td>69</td>
<td>60</td>
</tr>
<tr>
<td>S. epidermidis</td>
<td>123</td>
<td>59</td>
<td>0.51</td>
<td>69</td>
<td>60</td>
</tr>
<tr>
<td>S. pneumonia</td>
<td>123</td>
<td>59</td>
<td>0.51</td>
<td>69</td>
<td>60</td>
</tr>
<tr>
<td>S. dysgalactiae</td>
<td>123</td>
<td>59</td>
<td>0.51</td>
<td>69</td>
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<td>E. faecalis</td>
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**Figure 4**

Similarity matrix of multiple sequence alignments of V6 region sequences (961-1085) among S. aureus strain ATCC 14458, S. epidermidis strain ATCC 12228, E. faecalis strain ATCC 29212, S. pneumonia strain ATCC 49619 and S. dysgalactiae subsp. equisimilis ATCC 12394.
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concentration (Brown 1993; Greisen et al., 1994; Heuer et al., 1999; Clarridge 2004).

Figure 5
Similarity matrix of multiple sequence alignments of V6 region sequences (961-1085) within Streptococci composed S. pneumonia strain ATCC 49619, S. dysgalactiae subsp. equisimilis ATCC 12394, S. pyogenes strain ATCC 12344, S. mutans strain ATCC 25175, S. equi subsp. zooepidemicus ATCC 35246, S. bovis strain ATCC 33317, S. agalactiae strain ATCC 13813 and S. mitis strain ATCC 49456

The optimal condition of PCR-Slot blot Hybridization that was chosen as 67°C of the hybridize temperature using for method validation due to this condition could classified all reference strains of gram positive septicemia-bacteria by using only one primer pair for probe synthesis (Greisen et al., 1994; Rodr

CONCLUSION
The results revealed that probe design and the optimized conditions were successful in the detection of gram positive septicemia-bacteria. From these results, polynucleotide probes targeting a region of the V6 of 16S rRNA gene probe, and the specific optimized hybridization conditions at 67°C proved to be more specific for the identification each septicemia- gram positive bacteria. This finding is a major progress; however further investigation of the gram negative septicemia-bacteria would be required for a broader identification of septicemia bacteria.

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We would like to thank you the department of Medical Sciences, Ministry of Public Health, Thailand to support the finance of this project and really thank you the faculty of Medical Science, Naresuan University to give us about introduction, equipment for all of the project processes. And thank you of officer of the Khon Kaen hospital and Regional Medical Science Center, Khon Kaen to help me to collect blood culture samples and sample

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Author Information

Athicha Mahayotha
Department of Microbiology and Parasitology, Faculty of Medical Science, Naresuan University
Phitsanulok, Thailand
aroon59@hotmail.com

Rosarin Wongvilairat
Department of Microbiology and Parasitology, Faculty of Medical Science, Naresuan University
Phitsanulok, Thailand

Surang Dejsirilert
National Institute of Health, Department of Medical Sciences, Ministry of Public Health
Thailand

Tawatchai Sumpradit
Department of Microbiology and Parasitology, Faculty of Medical Science, Naresuan University
Phitsanulok, Thailand

Anusak Kerdsin
National Institute of Health, Department of Medical Sciences, Ministry of Public Health
Thailand