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

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Citation

A Mahayotha, R Wongvilairat, S Dejsirilert, T Sumpradit, A Kerdsin. *Development Of PCR-Hybridization For The Identification Of Major Gram Positive Bacteria Causing Bacteremia*. The Internet Journal of Microbiology. 2013 Volume 12 Number 1.

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 bacterimia.

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.

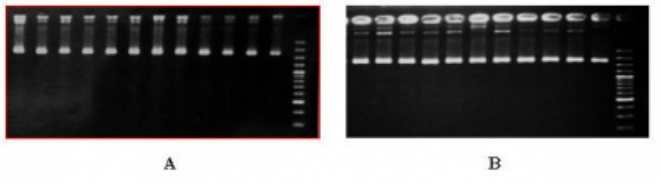
Extraction of bacterial DNA and DNA amplification. Bacterial cells of each strains were harvested in sterile DW. DNA were extracted by using High pure PCR Template preparation kit (Roche Diagnostic?, USA) according to the manufacturer

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

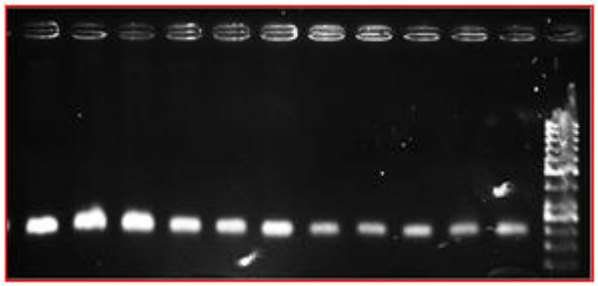
A = PCR products from 16S rRNA gene amplification of reference bacteria by universal primer (27F, 1492R), B = PCR products from plasmid DNA amplification by using M13 primer as DNA template and blotted on a nylon membrane.



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

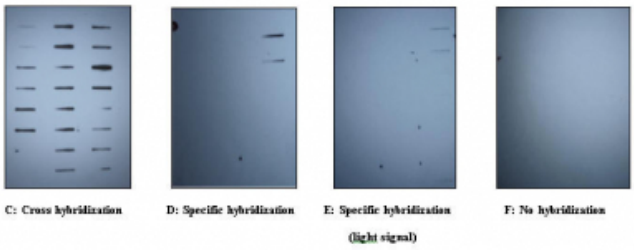
Polynucleotide probe-labeled sized 125 bps was synthesized by using a primer pair (V6F, V6R) and DIG-11-dUTPs via PCR



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 62oC and were negative at hybridized temperature at 70oC (Fig. 3). Interesting, only S. pneumoniae was discriminated from other gram positive bacteria at the optimized temperatures varied from 65 to 68oC (Fig. 3).

Figure 3

C=Cross hybridization of S. pneumoniae probe with gram positive septicemia target bacteria at temperature hybridization 58 oC , D = Specific hybridization of S. pneumoniae probe with S. pneumoniae target bacteria at temperature hybridization 65 oC, E = Specific hybridization of S. pneumoniae probe with S. pneumoniae target bacteria at temperature hybridization 68oC , and F = No hybridization of S. pneumonia probe with gram positive septicemia target bacteria temperature hybridization 70 oC.



The hybridize temperature varied from 65 to 67oC 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 bactertia were positive at hybridized temperature at 67oC (Table 3).

Table 3

Cross hybridization and Specific hybridization of gram positive septicemia-bacteria probes with gram positive septicemia target bacteria at temperature hybridization 65oC and 67oC.

Specific probes	Temperature of Hybridization	
	65°C	67°C
S. pyogenes	Cross hybridization	Specific hybridization
S. agalactiae	Cross hybridization	Specific hybridization
S. equi	Cross hybridization	Specific hybridization
S. mitis	Cross hybridization	Specific hybridization
S. bovis	Cross hybridization	Specific hybridization
S. dysgalactiae	Cross hybridization	Specific hybridization
S. mutans	Cross hybridization	Specific hybridization
S. aureus	Specific hybridization	Specific hybridization
S. epidermidis	Specific hybridization	Specific hybridization
E. faecalis	Specific hybridization	Specific hybridization (light signal)

Method validation

The optimal hybridization temperature for the PCR-Slot blot Hybridization was at 67oC, 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/

DISCUSSION

The results of this study showed that gram positive bacteria causing frequently septicemia were positive under the hybridize temperature varied from 58 to 62oC and were negative at hybridize temperature at 70oC. Interesting, only S. pneumoniae was discriminated from other gram positive septicemia-bacteria at the optimized temperature varied from 65 to 68oC. But the detection signal of hybridization at 68oC 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 heamo-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 67oC 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 65oC 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) including S. aureus strain ATCC 14458 (GenBank: DQ269498), S. epidermidis strain ATCC 12228 (NC_004461), E. faecalis strain ATCC 29212 (GenBank: GU585587), S. pneumonia strain ATCC 49619 (GenBank: AY281082) and S. dysgalactiae subsp. equisimilis ATCC 12394 (NC_017567), found that differentiation of Streptococci from Enterococci and Staphylococci varying between 15- 30% in comparative sequence alignments and direct effect on specific hybridization with Enterococci and Staphylococci (Fig. 4).

Table 4

The melting temperature (Tm) of V6- Probes which were calculated from reference bacterial strains.

V6 - Probe type (961-1085)*	length (bp)	%GC	Tm (basic) °C **	Tm(DIG Easy) °C***	Thyb(DIG Easy) °C	Reference Sequence
E. faecalis strain ATCC29212	125	50	80	65	40-45	GU585587
S. aureus strain ATCC14458	127	49	80	65	40-45	DQ269498
S. epidermidis strain ATCC12228	127	50	80	66	41-46	NC_004461
S. pneumonia strain ATCC49619	125	51	81	66	41-46	AY281082
S. dysgalactiae subsp. equisimilis ATCC12394	125	54	81	67	42-48	NC_017567
S. pyogenes strain ATCC12344	125	50	80	66	41-46	AB002521
S. mutans strain ATCC25175	125	48	79	65	40-45	DQ303189
S. equi subsp. zooepidemicus ATCC 35246	125	46	79	64	39- 44	NC_017582
S. bovis strain ATCC 33317	125	49	80	65	40-45	AB002482
S. agalactiae strain ATCC 13813	125	51	81	66	41-46	NR_040821
S. mitis strain ATCC 49456	125	51	81	66	41-46	AF003929

* = 16S ribosomal RNA gene position of *E. coli* (Chadravorty *et al.*, 2007)
** = BioMath Calculators, Tm Calculations for Oligos
*** = The optimum hybridization temperature (Thyb) in DIG Easy Hyb buffer
Tm = 49.82 + 0.41 (% G + C) - 600/L
Thyb = Tm – (20° to 25°C)

Figure 4

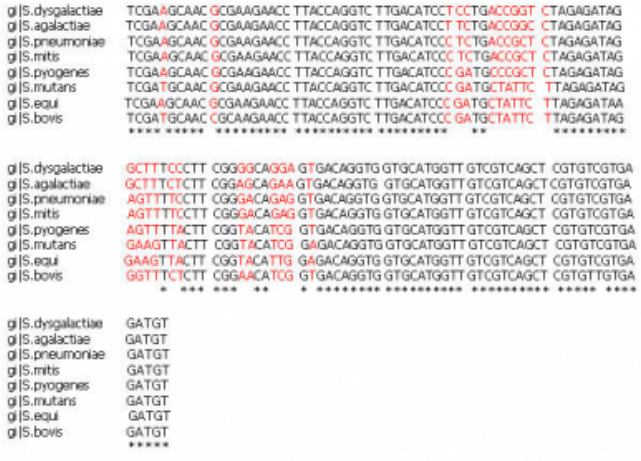
Similarity matrix of multiple sequence alignments of V6 region sequences (961-1085) among S. aureus strain ATCC 14458, S. epidermidis strain ATCC 12228, E. faecalis strain ATCC 29212, S. pneumonia strain ATCC 49619 and S. dysgalactiae subsp. equisimilis ATCC 12394

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 67oC, each specific probe hybridized to each specific Streptococci within Streptococci group with intense signal without any cross hybridization. The hybridized temperature at 67 oC, results in probe specific hybridize with S. pneumonia strain ATCC 49619 and S. mitis strain ATCC 49456, eventhough both of them were 100% similarity and 51% GC content (Fig.5). 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

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 67oC 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 67oC 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.

ACKNOWLEDGMENTS

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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