Development Of V6-16S DNA Probe-Hybridization As A Rapid Method For Detecting Bacterial Pathogens In Blood Culture Samples

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Citation

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Abstract

To develop a rapid technique for detecting the pathogenic bacteria, V6 regions of 16S rRNA gene were carried out for PCR probe-hybridization. We used eight bacterial strains; Cardiobacterium hominis, Haemophilus influenzae, Psuedomonas aeruginosa, Legionella pneumophila, Francisella tularensis, Kingella kingae, Acinetobacter baumannii, and Enterobacter aerogines in this study. Forty-seven isolates collected from 139 standard haemo-cultures, which were collected routinely from three laboratories, were identified by the development method. We found that all results were not differently significant ($p \leq 0.001$, kappa agreement = 1.0) when compared to the standard culture. Specificity and sensitivity of this technique were as high as 100%. In conclusion, the V6-16S DNA probe- hybridization was able to detect 8 pathogenic bacterial species within 2 days which was more rapid than a week of the routine culture. Our method is a very useful and might be the alternative tool for detecting these bacterial pathogens simultaneously. Rapid detection will benefit to patient treatment and reduce unnecessary antibiotic usage.

List of Abbreviations

2X of SSC; double times of standard saline-citrate buffer concentration;
16S rRNA, 16S ribosomal RNA; A, adenine; ATCC, American Type Culture Collection; BM blue POD substrate, 3,3□-5,5□-Tetramethylbenzidine; C, cytosine; dATP, 2'-Deoxyadenosine 5'triphosphate; dCTP, 2'-Deoxycytidine 5'-triphosphate; DEPC, Diethyl pryrocarbonate; dGTP, 2'-Deoxyguanosine 5'-triphosphate; DIG-11-dUTP, Digoxigenin-11-deoxyuridine triphosphate; dNTP, 2´-Deoxynucleoside 5´-triphosphates; DMST, Department of Medical Sciences Thailand; dTTP, 2'-Deoxythymidine 5'-triphosphate; G, guanine ; MALDI-TOF MS, matrix-assisted laser desorption/Ionization mass spectrometer; MDR, multidrug-resistant; PCR, polymerase chain reaction; PCR-ELISA, polymerase chain reaction-Enzyme-linked immunosorbent assay; SDS, sodium dodecyl sulphate; SPSS, Statistical Package for the Social Sciences; T, thymine; Taq, Thermus aquaticus; UV, ultraviolet; V6-16S DNA, V6 region of 16S DNA; XDR, extensively drug-resistant

INTRODUCTION

Bacterial pathogens, which caused different symptoms and crisis of human organs, composed of many bacterial species both Gram positive and Gram negative bacteria [Angus and Poll, 2013; Fan et al., 2016; Hotchkiss et al., 2016]. The early report of bacterial identification from laboratory is one of key factors for effective antibiotic usages and reducing unnecessary antimicrobial treatment. However, the traditional identification of pathogenic bacteria such as cultivation and biochemistry testing are being mainly important and used as the gold standard in this filed, particularly in bacterial growth in culture media [Weinstein et al., 1997; Vandamme, 2011]. For other bacteria growth on artificial media/cell lines, non-cultural pathogens and slow growing groups, the traditional culture and other rapid method are recommended to characterize these bacteria [Doern, 2000; Houpikian and Raoult, 2000; Brouqui and Raoult, 2001; Urbána et al., 2001]. More than two decades, genetic approaches have been proved to use for the bacterial identification based on the broad-range polymerase chain reaction (PCR). Due to the PCR technique is highly sensitive and reliable, thus it is capable to detect specifically the bacterial DNA in a small volume of specimens. Furthermore, the multiplex PCR is applied widely for more specific

detection of various genomic sequences [Yamamoto, 2003; Onori et al., 2014]. The general analysis of PCR products is the gel electrophoresis which amplicons need to be stained with radioactive agent or carcinogens to render visible the specific DNA band and take long time in overall processes [Sambrook and Russel, 2001]. Consequently, alternative techniques avoiding carcinogens in the DNA analysis have developed to identify these bacterial pathogens or new bacterial species, such as PCR-Hybridization, PCR-ELISA, Real time PCR, Microarray and MALDI-TOF MS [Nilsson et al., 2000; Ge et al., 2002; Espy et al., 2006; Peplies et al., 2003; Wieser et al., 2012]. Indeed, those techniques need expertise persons who understand in genetic basis of organisms and have been more experienced in molecular practices.

New approach technology including automatic machine of genotype assay is powerfully available currently and easy to use for any person who require the skill improvement and practices. DNA sequencing is one of more recent techniques combining to broad-range PCR techniques that a specific primer is designed to determine species-specific sequences [Drancourt et al., 2005; Metzker, 2005; Shendure and Ji, 2008]. The goals of this research are to develop the rapid methods for the bacterial detection and not concerned only the success of development but also plan to target users and the cost effectiveness. Hence, this study aimed to modify two steps; PCR amplification by using a V6 primer pairs and the PCR product typing with the optimized hybridization for identification of 8 bacterial strains, such as Cardiobacterium hominis, Haemophilus influenzae, Psuedomonas aeruginosa, Legionella pneumophila, Francisella tularensis, Kingella kingae, Acinetobacter baumannii, and Enterobacter aerogines. This developed technique was rapid detection (within 2 days), earlier than the routine-testing (approximately 2-7 days). Moreover, the accuracy, sensitivity, and specificity of V6-16S DNA probe-hybridization was excellent and could be the one of molecular methods for detecting bacterial pathogens rapidly.

MATERIALS AND METHODS

Bacteria strains

Eight reference strains were used in this study including C. hominis DMST 17290, H. influenzae ATCC 49247 DMST 7943, P. aeruginosa ATCC27859 DMST 4739, L. pneumophila ATCC 33152 DMST 12800, A. baumannii ATCC 19606 DMST 10437, E. aerogenes ATCC 13048 DMST 8841, F. tularensis, and K. kingae supported by Culture collection, National Institute of Health (Department of Medical Sciences, Ministry of Public Health, Thailand). 16S fragments of these bacteria were prepared by cloning DNA from 16S rRNA gene in the previously published study [Mahayotha et al., 2013] and stored at -80 °C as reference DNA template.

V6 fragment amplification and coating on membranes

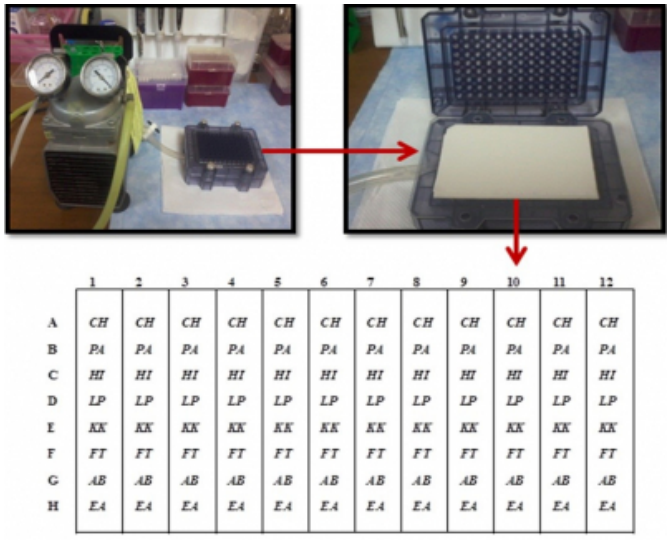
V6 fragments of each reference strains were constructed as short DNA templates by using a primer pair as V6-Forward; 5'- TCG ATG CAA CGC GAA GAA - 3' and V6-Reverse; 5'- ACA TTT CAC AAC ACG AGC TGA CGA - 3'. The amplifying step was applied from the previous study [Mahayotha et al., 2013] in a final volume of 50 µl PCR reaction containing: 5.0 µl of 10X PCR reaction buffer (160 mM (NH4)2SO4, 500 mM Tris-HCl pH 9.2, 17.5 mM MgCl2 and 0.1% TritonTM X-100), 2.0 µl of 50 mM MgCl2, 4.0 µl of 10 mM dNTP mixtures, 2.5 µl of each of 10 µM of forward and reverse primer, 0.4 µl of 5 unit/µl of Taq DNA polymerase (vivantis-Taq), 5.0 µl of 16S fragments, and 28.6 µl of double sterile distilled water. The PCR condition was operated as follows: pre-denaturation at 95 °C for 3 min, followed by 35 cycles of denaturation at 95 °C for 1 min, annealing at 58 °C for 45 sec, extension at 72 °C for 1 min, and final extension at 72 °C for 7 min. The PCR products were analyzed by 2.8% agarose gel electrophoresis. DNA concentration was determined by UV-Spectrophotometer (NanoDrop 2000, Thermo Fisher Scientific, USA) and stored at -20 °C. 50 ng/ml of V6 fragments of each reference strain was diluted with diethylpyrocarbonate-treated water (DEPC-treated water, OMEGA, Bio-tek, Inc. USA) in a 1.5-ml tube. Each V6 fragment-diluted tube was boiled on heating box at 100 °C for 10 min and then placed on ice immediately. Then 100 µl of each diluted fragment was applied on a nylon membrane by using the dot blot machine (Bio-RAD, USA). The position of each strain starting from row 1 at position A to H vertically as C. hominis, P. aeruginosa, H. influenzae, L. pneumophila, K. kingae, F. tularensis, A. baumannii, and E. aerogines, repeated the fragment coating in row 2-12, shown in Figure 1. Each membrane was fixed by the ultraviolet light from UV-Transluminator for 1 min. V6 fragment-coated membranes were placed in plastic bags and stored at 2-8 °C. These membranes were used for setting in hybridized conditions and testing in the method validation.

V6 probe synthesis and labeling

V6 probes were derived from 16S rRNA gene from 8 reference strains. PCR reaction was performed in a total volume of 25 ml containing: 3.0 ml of 10X PCR reaction buffer, 1.0 ml of 20 mM MgCl2, 0.5 ml of 10 mM dNTP mixtures with digoxigenin-11-dUTP, 1.25 ml of each of 10 pmol of V6-Forward and V6-Reverse primer, 0.2 ml of 5 unit/ml of Taq DNA polymerase (vivantis-Taq), 3.0 ml of DNA template solution, and 14.8 ml of DEPC water. The PCR condition was carried out as follows: pre-denaturation at 95 °C for 3 min, followed by 35 cycles of denaturation at 95 °C for 1 min, annealing at 58 °C for 45 sec, extension at 72 °C for 1 min, and final extension at 72 °C for 7 min. V6-probe concentration was determined by UV-Spectrophotometer.

Figure 1

V6 fragments of each strain had been applied vertically on a nylon membrane by using the dot blot machine (Bio-RAD, USA). The position of each strain starting from row1 at position A to H vertically as C. hominis (CH), P. aeruginosa (PA), H. influenza (HI), L. pneumophila (LP), K. kingae (KK), F. tularensis (FT), A. baumannii (AB), and E. aerogines(EA), repeated the fragment coating in row 2-12.



	1	2	3	4	5	6	7	8	9	10	11	12
A	CH	CH	CH	CH	CH	CH	CH	CH	CH	CH	CH	CH
B	PA	PA	PA	PA	PA	PA	PA	PA	PA	PA	PA	PA
C	HI	HI	HI	HI	HI	HI	HI	HI	HI	HI	HI	HI
D	LP	LP	LP	LP	LP	LP	LP	LP	LP	LP	LP	LP
E	KK	KK	KK	KK	KK	KK	KK	KK	KK	KK	KK	KK
F	FT	FT	FT	FT	FT	FT	FT	FT	FT	FT	FT	FT
G	AB	AB	AB	AB	AB	AB	AB	AB	AB	AB	AB	AB
H	EA	EA	EA	EA	EA	EA	EA	EA	EA	EA	EA	EA

The hybridized condition setting

The V6-coated membrane was dried in the ambient conditions. A piece of dried membrane was cut vertically following position A to H, divided to 12 pieces per probe sample and labeled the probe type depending on the testing number of probes. A piece of membrane was transferred into a plastic bag contained 1.5 ml of DIG Easy Hybridization (Roche DiagnosticÔ, USA) and closed by heat-sealing maker. The membrane was pre-hybridized by putting in the rolling bottle and placing in the rotation position of hybridization oven at 65 °C for 1 hour. V6 probes of eight strains were used to optimize the conditions of the hybridization. 2.5 ml of labeled probe was added in a 1.5-ml tube containing 250 ml of DIG Easy Hybridization. Each mixture solution tube was heat on heating box for denaturing probes at 100 °C for 5 min. 5 ml of denatured probes of each stain was pipetted into the plastic bag contained a piece of nylon membrane and incubated in the rotation position of hybridization oven for 12 to 18 hours (overnight) with three different condition settings at 65, 67, and 69 °C, respectively. Each hybridized membrane was washed three time by standard saline-citrate buffer, SSC (Roche DiagnosticÔ, USA) at 68 °C for 15 min per time by starting the variable buffer concentration from 2X, 0.5X and 0.1X, respectively. All hybridized membranes were placed on the absorbent paper at room temperature for 15 min.

Then they were soaked in plastic box containing the maleic acid buffer, decanted, added 40 ml of blocking reagent and mixed gently on a shaking rocker at room temperature for 30 min. 20 ml of Anti-DIG-POD conjugate (Roche DiagnosticÔ, USA) was pipetted into 10 ml of blocking reagent gently and poured to the hybridized membranes in plastic box. The plastic box contained hybridized membranes was incubated on the shaking rocker gently at room temperature for 1 hour. Then, each membrane was placed in a plastic box containing 2X of SSC buffer plus 0.1% SDS and soaked on the shaking rocker at room temperature for 10 min, three times. 2X of SSC buffer was used as the last washing step in the same plastic box and soaked on the shaking rocker for 5 min, repeated this step, twice. Membranes were placed on the absorbent paper at room temperature for 15 min. In the visualization step, each hybridized membrane was placed in a plastic box containing BM blue POD substrate (Roche DiagnosticÔ, USA) and soaked for 1 min. Hybridized signals appeared as blue dots on membranes. Enzyme reaction on membranes was stopped by washing membranes twice with sterile DEPC-treated water for 5 min on shaking rocker.

The titration of DIG-11-dUTP concentrations in PCR reactions

The 7 DIG-11-dUTP dilutions of dNTP mixtures were prepared by using increasing 125 nmol DIG-11-dUTP in mixed dNTPS solutions composed of 0, 0.0147, 0.0294, 0.0588, 0.0882, 0.1176 and 0.1764 nmol/ml. The ingredients of each solution including of dATP, dTTP, dGTP, dCTP, DIG-11-dUTP and DEPC-treated water. The final

concentrations in PCR reaction were 0.0, 0.000294, 0.000588, 0.001176, 0.001764, 0.002352, and 0.003528 nmol/ul. V6 probes of each reference template were amplified separately by using each dilution of 7-diluted series of dNTP mixtures in PCR reaction. V6 Probes of all dilutions were tested by the optimized condition of PCR Probe-Hybridization.

The accuracy testing

34 DNA spiked samples were collected from known DNA stored at -80 °C at the Clinical Pathology of Regional Medical Sciences Center 7, Khon Kaen, Khon Kaen Province, Thailand. These samples were tested the specific hybridization and cross hybridization of this developing method [Ramisse et al., 2003; Al-Khaldi et al., 2004]. There were three sample types including 10 negative bacterial sample, 8 target strains; C. hominis, P. aeruginosa, H. influenza, L. pneumophila, K. kingae, F. tularensis, A. baumannii, and E. aerogenes and 16 other species; Staphylococcus aureus, S. epidermidis, Bacillus cereus, B. thuringenis, B. subtilis, Listeria monocytogennes, Clostridium perfringens, Streptococcus suis, Enterococcus faecalis, Escherichia coli, Salmonella spp., Burkholderai pseudomallei, Proteus mirabillis, Klebsiella pneumoniae, Aeromonas sobria, and A. hydrophila.

Method validation

This experiment composed of two steps, limit of detection (LOD) and reliable testing. DNA extracts of L. pneumophila ATCC 33152 DMST 12800 were used for LOD study. 5-singled colonies of L. pneumophila were suspended separately in 1.0 ml of sterile DW and mixed thoroughly. 500 ml of the cell suspension was transferred in to 2.0 ml-sample tube and DNA extraction was performed by using the automatic DNA extraction as QIAsymphonySP machine (QIAGEN, Germany) with QIAsymphony DNA Mini Kit according to the manufacturing protocol. L. pneumophila DNA concentration was determined by UV-Spectrophotometer. 6-diluted series of DNA extract were prepared by diluting in DEPC-treated water started at 1:100, 1:200, 1:400, 1:800, 1:1,600, and 1:3,200. Each diluted sample was amplified and typed by the developing PCR Probe-Hybridization.

For the steps of reliable testing, samples in our study were 139 haemo-culture samples collected between January, 2017 to October, 2018 from three routine laboratories, the Clinical Microbiology unit, Pathology department, Songklanagarind hospital, Songkla Province, two microbiology laboratories from Mahasarakham hospital, Mahasarakham Province, and Khon Kaen hospital, Khon Kaen Province, Thailand. These samples were divided into three groups including 47 samples of positive bacterial strains in target groups, 30 samples of negative bacterial strains and 62 samples of positive bacterial strains in other groups. 0.5 ml of each sample was pipetted into 1.5-ml tube and centrifuged at 13,000 rpm for 10 min. The supernatant was discarded by pipetting. The pellet were suspended with 1.0 ml of DEPC-treated water, mixed thoroughly and centrifuged at the same speed, repeat this step twice. 100 µl of DEPC-treated water was added to each pellet sample and mixed thoroughly. 20 µl of 50 mg/ml Lysozyme was pipetted into the mixture solution, mixed gently and incubated at 37 °C for 30 min.

Each mixture solution was centrifuged at 10,200 rpm for 3 min. The supernatant was discarded by pipetting up gently and 200 µl of DEPC-treated water was added to each pellet, mixed thoroughly by using vortex mixer. 20 µl of 20 mg/ml Proteinase K was pipetted into the mixture sample and mixed gently. Then the mixture sample was incubated at 65 °C for 40 min and transferred the mixture solution to a 2.0-ml sample tube for DNA extraction by using QIAsymphonySP machine with QIAsymphony DNA Mini Kit according to the manufacture protocol. 3 µl of each DNA sample was use for V6 probe amplification by PCR following step of V6 probe synthesis and labeling. PCR products were hybridized with V6 fragment -coated membranes and typed by the optimized condition of PCR Probe-Hybridization following the steps of the hybridize condition setting. Results of the developing method were compared with the standard culture findings to determine the method sensitivity, specificity and accuracy. The Chi-square test at $p< 0.05$ and Cohen's kappa coefficient at 0.70 were used as the statistic criteria [McHugh, 2013; Sim and Wright, 2005].

RESULTS

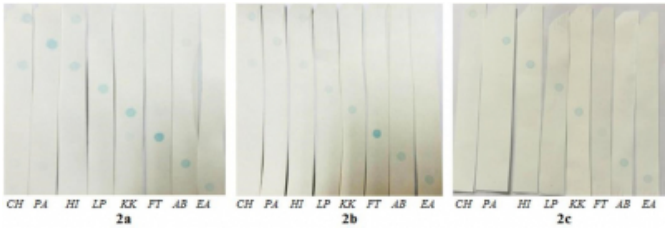
The optimised condition of V6-16S probe hybridization

The V6-16S probe hybridized condition was set and tested at the variable temperatures at 65, 67 and 69 °C. The signals of blue dots were different among these bacterial pathogens. There were three pairs of V6 probes and fragments, C. hominis - H. influenza, P. aeruginosa - A. baumannii and K. kingae - F. tularensis showed crossed reaction at 65 °C. One pair of them, C. hominis - H.

influenza was still appeared the light signals when the temperature was increased to 67 °C. In the other hand, V6-16S probes and fragments of all 8 species were reacted with their own targets and had clearly shown blue signals without any cross hybridization at 69 °C (shown in Figure 2).

Figure 2

The V6-16S probe hybridized condition were set and tested at the variable temperatures at 65, 67 and 69 C. Figure 2a, 2b and 2c had shown hybridization results betweenV6-DIG labeled probes and V6 fragments on membranes at 65, 67 and 69 C respectively. Each V6-coated membrane was hybridized separately with V6-DIG labeled probes of reference strains composed of C. hominis (CH), P. aeruginosa (PA), H. influenza (HI), L. pneumophila (LP), K. kingae (KK), F. tularensis (FT), A. baumannii (AB), and E. aerogines (EA).The signals of blue dots were different among these bacterial pathogens. There were three pairs of V6 probes and fragments, C. hominis - H. influenza, P. aeruginosa - A. baumannii and K. kingae - F. tularensis showed crossed reaction at 65 C. One pair of them, C. hominis - H. influenza was still appeared the light signals when the temperature was increased to 67 C. In the other hand, V6-16S probes and fragments of all 8 species were reacted with their own targets and had shown clear the blue signals without any cross hybridization at 69 C.



DIG-11-dUTP concentrations in PCR reaction

The titration of DIG-11-dUTP levels in PCR reactions was performed to determine the lowest concentration which all study strains has been detected and appeared the blue dots on the V6 coated-membranes. This titration found that lowest concentration of the DIG-11-dUTP in PCR reaction is equal to 0.001764 nmol/ul showing clearly signals with 6 strains and light signals with 2 strains (shown in Table 1). Moreover, these signals of those study strains were increased following the amount of DIG-11-dUTP in PCR reaction. They were more intensive when 0.003528 nmol/µl of DIG-11-dUTP used in PCR reaction. As a result, this concentration was optimal for using in PCR amplification and showing clear signals among these bacterial pathogens.

Table 1

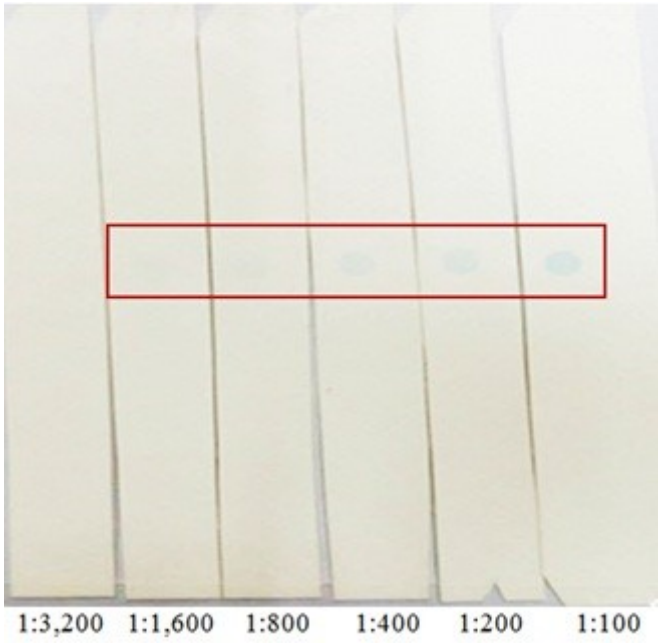
Results of the DIG-11-dUTP titration in PCR reactions.

DIG-11-dUTP[a] (nmol/ul)	Signal detection (blue dot) on membranes by V6-16S Probe-Hybridization[b]							
	C. hominis	*P. aeruginosa*	*H. influenzae*	*L. pneumophila*	*K. kingae*	*F. tularensis*	*A. baumannii*	*E. aerogines*
0.003528	strong signal	strong signal	strong signal	strong signal	strong signal	strong signal	strong signal	strong signal
0.002352	strong signal	strong signal	strong signal	strong signal	clear signal	clear signal	strong signal	strong signal
0.001764	clear signal	clear signal	clear signal	clear signal	light signal	light signal	clear signal	clear signal
0.001176	light signal	light signal	light signal	light signal	light signal	-	light signal	light signal
0.000588	light signal	light signal	light signal	light signal	-	-	light signal	light signal
0.000294	-[c]	-	-	-	-	-	-	-
0	-	-	-	-	-	-	-	-

a =The final concentration of DIG-11-dUTP used for PCR amplification.
b = Results of V6-16S Probe-Hybridization were observed on each V6-hybridized membrane depending on amount of DIG-11-dUTP levels via PCR reaction and probe types. The lowest concentration of the DIG-11-dUTP in PCR reaction was 0.001764 nmol/ul showing clearly signals with 6 strains and light signals with 2 strains and these signals were more intensive when 0.003528 nmol/µl of DIG-11-dUTP used in PCR reaction.
c = No signal was observed on a nylon membrane.

Figure 3

6-serial dilutions were prepared from the pure DNA concentration of L. pneumophila ATCC 33152 DMST 12800 as 7.3 ng/µl. These DNA-diluted samples composed of 1:100, 1:200, 1:400, 1:800, 1:1600 and 1:3200. The last dilution showing light signals was 1:1600. The final concentration of DNA was roughly 0.00456 ng/µl. The intensive appearance of blue dot signals was decreased depending on the DNA concentrations from 1:100 to 1:1600. No signal was appeared when probes conducted from the DNA dilution equal 1:3200 were tested.



The accuracy of V6-16S PCR Probe-Hybridization

The accuracy of the developed method was performed by testing three groups of known 34 DNA spiked samples in the optimized condition. We found that V6-16S probes reacted directly and showed clearly positive signals in each 8 target species. For others non target species, 10 negative samples and 16 other species did not appear any signals on each hybridized nylon membrane (shown in Table 2). These results indicated that this method was highly

accurate and able to classify 8 species simultaneously.

Table 2

The accuracy testing of V6-16S Probe-Hybridization performed with known DNA samples.

Known DNA Samples[a]	Signal detection (blue dot) on membranes by V6-*16S* Probe-Hybridization[b]							
	C. hominis	*P. aeruginosa*	*H. influenzae*	*L. pneumophila*	*K. kingae*	*F. tularensis*	*A. baumannii*	*E. aerogenes*
Negative samples N = 10	-[c]	-	-	-	-	-	-	-
Target species N = 8								
C. hominis	clear signal	-	-	-	-	-	-	-
P. aeruginosa	-	clear signal	-	-	-	-	-	-
H. influenzae	-	-	clear signal	-	-	-	-	-
L. pneumophila	-	-	-	clear signal	-	-	-	-
K. kingae	-	-	-	-	clear signal	-	-	-
F. tularensis	-	-	-	-	-	clear signal	-	-
A. baumannii	-	-	-	-	-	-	clear signal	-
E. aerogenes	-	-	-	-	-	-	-	clear signal
Other species N = 16	-	-	-	-	-	-	-	-

a = The accuracy of the developed method was performed by testing three groups of known 34 DNA spiked samples in the optimized condition.
b = Results of V6-*16S* Probe-Hybridization observed on each V6-hybridized membrane were divided into 3 groups of known DNA samples, negative samples, target species and other species. V6-16S probes of target species reacted directly and showed clearly positive signals in each 8 target species. For others non target species, 10 negative samples and 16 other species were not seen any signals on each hybridized nylon membrane
c = No signal was observed on a nylon membrane.

Method validation of V6-16S PCR Probe-Hybridization

This developed method had been tested with the last step for the method validation; LOD and reliable testing. For results of LOD value, 6-serial dilutions were prepared from the pure DNA concentration of L. pneumophila ATCC 33152 DMST 12800. The undiluted concentration of DNA extracts was 7.3 ng/µl. The last dilution showing light signals was 1:1,600 (shown in Figure. 3). The final concentration of DNA was roughly 0.00456 ng/µl equal to approximately 1,140 cells/ml. The reliable testing of this method was the last experiment. 139 haemo-culture samples were processed and performed amplification by using V6-16S probe and typing by hybridization on each V6-fragment coated membrane in the optimized condition. 47 positive haemo-culture samples containing target species were seen positive signals directly at their species specific positions on coated membranes (100 % sensitivity). For other groups, 30 negative samples and 62 other isolates were not seen any signals on each coated membrane (shown in Table 3). Those detecting results were in concordance to the standard culture findings and no cross reaction both target species and other groups (100% specificity and accuracy). All data were analyzed to compare between the findings results of developed method and the standard cultivation by using SPSS program version SPSS Statistic 17.0. There is no significantly difference between standard culture and PCR Probe-Hybridization method when 30 negative and 47 positive target samples were compared ($\chi^2 = 77.00$, $p \leq 0.001$, kappa correlation = 1.0 or 100% Agreement).

Table 3

The reliable testing of the developed method determined in the routine samples.

Haemo-culture Samples[a]	Standard culture findings		PCR probe typing by V6-*16S* Probe-Hybridization[b]	
	Positive	Negative	Positive	Negative
Positive group				
C. hominis (2)	2	0	2	0
P. aeruginosa (20)	20	0	20	0
H. influenza (2)	2	0	2	0
A. baumannii (20)	20	0	20	0
E. aerogenes (3)	3	0	3	0
Negative group Negative samples (30)	0	30	0	30
Other species				
S. aureus (12)	12	0	0	12
K. pneumoniae (10)	10	0	0	10
B. pseudomallei (10)	10	0	0	10
E. coli (10)	10	0	0	10
Salmonella spp. (10)	10	0	0	10
P. mirabillis (5)	5	0	0	5
A. sobria (2)	2	0	0	2
A. hydrophila (3)	3	0	0	3

a = There were 3 groups of haemo-culture samples that were performed for the reliable testing of the developed method
b = Results of PCR probe typing by using V6-16S probe- hybridization were compared with the standard culture method. 47 positive haemo-culture samples containing target species were seen positive signals directly at their species specific positions on coated membranes. For other groups, 30 negative samples and 62 other isolates were not seen any signals on each coated membrane

DISCUSSION

No cross hybridization between V6 probes and fragments of each reference strain had appeared when the hybridized temperature was increased to 69 °C, which was higher than the previous study [Mahayotha et al., 2013]. The increased temperature may be effective from using different strains in this study and the differently visualized techniques in the signal detection step. However, this hybridized result at the high temperature closely to DNA melting point was obviously depended on the complementary of DNA sequences between reference templates coated on a nylon membrane and PCR probes; Tm [Wetmur, 1991; Ehrmann et al., 1994; Chan et al., 1995; Goris et al., 2007]. Results of DIG-11-dUTP titration had indicated that the DIG-11-dUTP concentration in PCR reactions was directly influenced to visible the blue dot signals on the coated membranes. This DIG-labeled base played an important role in the visual detection step of PCR probe amplifications [Lion and Haas, 1990; An et al., 1992; Ying et al., 1999]. Shading levels of blue dot signal were more intensive depending on the DIG-11-dUTP concentration. Clear signals of all tested probes were appeared at the concentrations of DIG-11-dUTP from 0.002352 to 0.003528 nmol/ml of PCR reaction. Hence, the optimized concentration should be adjusted and considered both the accuracy and cost-effectiveness.

The LOD of developed method was 0.00456 ng/µl of DNA-concentration equal to approximately 1,140 cells/ml. This were studies in diluted DNA series which were prepared from the pure colonies so we can estimate LOD value. In case of direct specimens, which numbers of bacterial cell are less than 1,140 cells/ml, may not be

detected by this method. Therefore, the enrichment step should be performed initially before processing the DNA extraction. The limitation of this method was found in previously published papers in developing DNA-DNA hybridization [Fitts et al., 1983; Siqueira et al., 2001; Socransky et al., 2004, do Nascimento et al., 2012]. On the other hand, this reverse dot blot study had shown yields for detecting levels of bacterial-cell greater than similar principle techniques which detecting levels were 104-106 cells/ml [Siqueira et al., 2001; Zhou et al., 2011; Cao et al., 2013]. However, its level was quite higher than other advanced techniques such as Real time PCR which detection limit ranges between 10-100 cells/ml [Bej et al., 1991; Levi et al., 2003; Khanna et al., 2005]. In fact, primer and probe of the Real time PCR were designed from conserved regions of specific genes for individual microbe.

The reliable testing of V6-16S Probe-Hybridization had been carried out with 139-enriched blood culture bottles. Their results were in concordance to the standard culture findings as 100% of specificity and sensitivity with χ^2 = 77.00, $p \leq 0.001$, k = 1.0 and 100% Agreement [McHugh, 2013; Sim and Wright, 2005]. Hence, the developed method was able to identify 8 pathogenic species based on V6 regions of 16S rRNA gene. From the multiple sequence alignments of V6 region derived from following reference strains; C. hominis strain ATCC 15826 (GenBank: M35014.1), P. aeruginosa strain ATCC 27853 (GenBank: CP015117.1), H. influenzae strain ATCC 10810 (NC_016809.1), L. pneumophila strain ATCC 33152 (GenBank: X73402.1), K. kingae strain ATCC 23330 (NR_042976.1), F. tularensis strain ATCC 6223 (NR_029362.1) , A. baumanni strain ATCC 19606T (GenBank: Z93435.1), and E. aerogenes strain ATCC 13048 (NR_118556.1), their similarity of V6 regions were 78-91 %. Their alignment results could show the analysis range of detection which there were no cross hybridization among these bacterial species. Although, this study had already been proved to classify those 8 strains, closed species which V6 sequences different less than 9 % are very challenged and advanced for our next studies in order to create a broader identification of bacterial pathogens. Indeed, this method is able to apply for detection of the resistance gene in MDR or XDR groups.

The PCR Probe-Hybridization based on amplifying in a short target as V6 region of 16S rRNA gene, typing in the optimized temperature and washing buffer in the hybridization steps could identify all target species in this study simultaneously. This developed molecular method has been demonstrated high specificity and sensitivity (100 %) and feasible to provide the one of rapid identification for 8 bacterial pathogens with LOD approximately 1,140 cells/ml. A V6 primer pair was the only one primer pair used for amplifying the V6 probes of all strains; C. hominis, P. aeruginosa, H. influenzae, K. kingae, L. pneumophila, F. tularensis, A. baumannii, and E. aerogines, so this benefit to reduce both cost and time of detection. This advantage is more cost-effective than other molecular method as using only one V6 primer to amplify all 8 strains in the same testing. Although, the cost of this developed method was more expensive than the routine culture method, the advantage of rapid and accurate identification reports is very important to the patient treatment and reducing non-reasonable antibiotics usage.

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Disclosure

The authors declare that there is no conflict of interest regarding the publication of this paper. For this study, blood culture samples collected from the routine laboratories were used for reliable testing and classified as rep specimen. The full study protocol had been proved as an excepted protocol by the Naresuan University Institutional Review Board of Human Ethics since November, 2017. The criteria of this Board Committee is performed following the Declaration of Helsinki, The Belmont Report. ClOMS Guideline International Conference on Harmonization in Good Clinical Practice or ICH-GCP and 45CFR 46.101(b). Hence, the authors have no ethical conflicts to disclose.

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