Malaysian Journal of Microbiology, Vol 18(3) 2022, pp. 242-250
DOI: http://dx.doi.org/10.21161/mjm.211331

Malaysian Journal of Microbiology
Published by Malaysian Society for Microbiology
(In SCOPUS since 2011)

# Comparing multiplex and multiplex real-time polymerase chain reaction with traditional blood culture in bacterial detection among patients with septicemia

**Loan Thi Nguyen[1,2], Linh Trong Nguyen[3], Trang Thu Nguyen[3], Hien Minh Nguyen[4], Thuy Thi Pham[5] and Huyen Thi La[1,3]***

[1]Graduate University of Sciences and Technology, Vietnam Academy of Science and Technology, Hanoi, Vietnam.
[2]High School Pedagogy Department, Hoa Lu University, Hanoi, Vietnam.
[3]Institute of Biotechnology, Vietnam Academy of Science and Technology, Hanoi, Vietnam.
[4]Thanh Nhan Hospital, Hanoi, Vietnam.
[5]19-8 Hospital, Ministry of Public Security, Hanoi, Vietnam.
Email: lthuyen@ibt.ac.vn

Received 18 October 2021; Received in revised form 15 February 2022; Accepted 28 February 2022

## ABSTRACT

**Aims:** This study was aimed to test the specificity of primers and probes with target genes by using multiplex PCR and multiplex real-time PCR methods. These methods were compared with traditional blood culture methods in detecting five bacteria causing sepsis, including *Acinetorbacter baumannii*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *Escherichia coli* and *Staphylococcus aureus*.
**Methodology and results:** A total of 587 blood samples from patients diagnosed with sepsis and septic shock were collected at Thanh Nhan Hospital, Hanoi, Vietnam. Each sample was divided into three parts for bacterial culture, multiplex PCR and multiplex real-time PCR to detect the similarity of the two PCR methods with the bacterial culture method. Conditions in multiplex PCR and multiplex real-time PCR were optimized to ensure the successful amplification of target genes. Results showed that the primers and probes were tested completely specific to the target genes and using multiplex PCR and multiplex real-time PCR techniques could detect five pathogens causing sepsis, including *A. baumannii*, *K. pneumoniae*, *P. aeruginosa*, *E. coli* and *S. aureus*.
**Conclusion, significance and impact of study:** Both multiplex PCR and multiplex real-time PCR methods have high similarities with the culture method, showing potential in the application of bacteria detection in sepsis.

*Keywords:* Multiplex PCR, multiplex real-time PCR, sepsis, bacteria

## INTRODUCTION

Sepsis is an acute infection caused by bacteria infecting the bloodstream. Patients with sepsis often have clinical symptoms such as high fever, hepatosplenomegaly, tachycardia, cholecystitis, hepatitis or pneumonia (Gyawali *et al.*, 2019). Some patients with sepsis develop liver failure, kidney failure and then develop complications into septic shock, which may lead to death (Chong *et al.*, 2015). Septic shock is a severe manifestation of sepsis with a high risk of mortality (Winters *et al.*, 2010). According to the World Health Organization (WHO), the burden of sepsis in the world is still high despite a decrease from 60.2 million cases in 1990 to 48.9 million cases in 2017 (Rudd *et al.*, 2020). There were 11 million deaths due to sepsis, accounting for 16.5% of all-cause mortality, which was predominant in low- and low-middle income countries (Rudd *et al.*, 2020). The mean rate of sepsis in intensive care units was 29.5% (13.6%-39.3%), with a mortality rate of 25.8% (Sakr *et al.*, 2018). Bacteria that cause sepsis enter the patient's bloodstream directly or indirectly from the foci of infection in tissues and organs such as skin, soft tissues, muscles, bones, joints, respiratory or digestive tracts. The main Gram-negative bacteria causing sepsis include *Acinetobacter baumannii*, *Klebsiella pneumoniae*, *Escherichia coli* and *Pseudomonas aeruginosa.* Meanwhile, the Gram-positive bacterium that mainly causes sepsis is *Staphylococcus aureus*. In China, a previous study showed that 14.5% of cases with sepsis were caused by the Gram-positive bacterium such as methicillin-resistant *S. aureus* and 62.5% of cases were caused by Gram-negative bacteria such as *P. aeruginosa*, *A. baumannii*, *E. coli* and *K. pneumoniae* (Zhou *et al.*, 2014). In Vietnam, the most common bacteria causing sepsis included *K. pneumoniae* (17.5%), *E. coli* (17.3%), *S. aureus* (14.9%), *Stenotrophomonas maltophilia* (9.6%) and *S. suis* (7.6%) (Dat *et al.*, 2017).

*Corresponding author


ISSN (print): 1823-8262, ISSN (online): 2231-7538

Malays. J. Microbiol. Vol 18(3) 2022, pp. 242-250
DOI: http://dx.doi.org/10.21161/mjm.211331

Patients with sepsis often have a rapid disease progression, especially in older adults and children. Therefore, early detection of pathogens is essential for timely treatment and contributes to limiting bacterial denaturation, thereby reducing the risk of mortality in patients with sepsis. Currently, blood cultures and biochemical tests are common methods for detecting pathogens (Gyawali *et al*., 2019). However, these methods have limitations, including time-consuming and low sensitivity while requiring a blood volume of 20 mL to 30 mL per culture, becoming a great challenge when the patients are children. For the early detection of infectious microorganisms in the body, recent studies have suggested the verification of bacterial-specific DNA through multiplex Polymerase Chain Reaction (PCR) and multiplex real-time PCR (Ozkaya-Parlakay *et al*., 2014; Trung *et al*., 2018). These two methods use multiple primer pairs in the same reaction, allowing the detection of many types of bacteremia agents in a short time with high sensitivity and accuracy, suggesting great significance in the diagnosis of sepsis. This study aimed to test the specificity of primers and probes with target genes through multiplex PCR and multiplex real-time PCR methods, compared with traditional blood culture methods, to detect five bacteria causing sepsis, including *A. baumannii*, *K. pneumoniae*, *P. aeruginosa*, *E. coli* and *S. aureus*.

## MATERIALS AND METHODS

### Blood specimens and materials

A total of 587 blood samples from 587 patients diagnosed with sepsis treated at the Intensive Care Unit at Thanh Nhan Hospital, Hanoi were used in this study. Each sample was divided into 3 parts for three methods: 1) culture; 2) multiplex PCR and 3) multiplex real-time PCR.

For testing equipment, Nanodrop 1000, Aligent's AriaMx real-time PCR System, Eppendorf centrifuge and Bio-Rad DNA electrophoresis kit were used; real-time master mix (Integrated DNA Technologies-IDT); primers and probes (IDT), EDTA, SDS, EtBr, agarose and other chemicals were purchased from Merck, Invitrogen, Promega and Bioline.

### Bacterial culture method

Blood samples were cultured on agar plates including Columbia blood agar, chocolate agar and Biomerieux's aerobic culture bottles (using BacT/ALERT 3D automatic blood culture machine). Bacteria were identified based on morphology and biochemical properties by using Api20E, Api20 NE, Api Stap identifiers (BioMerieux company, France).

### Extracting DNA from blood samples of patients with suspected sepsis, healthy human and bacterial culture by QIAgen DNA extraction kit

A total of 1 mL of blood samples of patients with suspected sepsis and healthy human extracted according to the manufacturer's instructions (QIAamp@ DNA Mini Kit of QIAgen, Germany). After extracting, DNA products were stored at –20 °C for the next experiments.

Bacteria including *A. baumannii*, *K. pneumoniae*, *P. aeruginosa*, *E. coli* and *S. aureus* were cultured in lysogeny broth medium at 37 °C within 16 h and centrifuged to obtain biomass and isolated genomic DNA of the bacteria according to the instructions of the kit. DNA was then used for the study to verify primer and probe pairs as well as become positive controls in PCR and real-time PCR reactions.

### Primer test with genomic DNA by PCR method

The specific of primer sets was confirm by single PCR with extraction genomic DNA from 5 bacteria namely *A. baumannii*, *K. pneumoniae*, *P. aeruginosa*, *E. coli* and *S. aureus*. Single PCR was performed in 25 µL reaction mixture using PCR machine (PCR System 9700, Applied Biosystem, USA). The reaction mixture included 1 µL forward/reverse primers (Table 1), 12.5 µL master mix (Promega), 3 µL DNA template (genomic DNA of each *A. baumannii*, *K. pneumoniae*, *P. aeruginosa*, *E. coli* and *S. aureus*) and 7.5 µL $H_2O$. PCR negative control comprising ultrapure water instead of genomic DNA were prepared. The PCR reaction program included: 1) denaturation at 94 °C for 3 min; 2) repeating 30 cycles (94 °C for 45 sec, 57 °C for 45 sec and 72 °C for 1 min); and 3) finalizing the product at 72 °C for 8 min.

### Multiplex PCR with genomic DNA

The multiplex PCR was performed with 5 pairs of primers to examine (i) whether each species could be specifically detected and (ii) whether both species could be simultaneously detected. The total volume of the reaction was 25 µL with the composition including: 12.5 µL master mix, 0.5 µL SaF 10 pM; 0.5 µL SaR 10 pM; 0.3 µL AbF 10 pM; 0.3 µL AbR 10 pM, 0.3 µL EcF 10 pM; 0.3 µL EcR 10 pM; 0.3 µL PaF 10 pM; 0.3 µL PaR 10 pM; 0.3 µL KpF 10 pM, 0.3 µL KpR 10 pM; 3 µL DNA template (single genomic DNA target to examine specifically detected and mixture of 5 genomic DNA targets to examine simultaneously detection) and 6.1 µL $H_20$. The PCR thermal conditions were as follows: denaturation at 95 °C for 3 min; 30 cycles at 94 °C for 45 sec, 57 °C for 45 sec and 72 °C for 1 min; finalizing the product at 72 °C for 8 min and cooling of the PCR products at 10 °C for 30 min.

### Primer probe test with genomic DNA by real-time PCR

Primers and probes were newly designed and synthesized by IDT and tested using the BLAST tool (Boratyn *et al*., 2013), the detection threshold of primer

 ISSN (print): 1823-8262, ISSN (online): 2231-7538

Malays. J. Microbiol. Vol 18(3) 2022, pp. 242-250
DOI: http://dx.doi.org/10.21161/mjm.211331

**Table 1:** Primer sequences and PCR product size according to theoretical calculation.

| Target bacteria | Target gene | Primers | Melting temperature (°C) | References |
|---|---|---|---|---|
| *Acintobacter baumannii* | *GltA* (722 bp) | F: 5'-ATTTACAGTGGCACATTAGGT-3'<br>R: 5'-GCAGAGATACCAGCAGAGAT-3' | 51.9<br>53.8 | (Thong *et al.*, 2011),<br>(Bartual *et al.*, 2005) |
| *Klebsiella pneumoniae* | *Mdh* (364 bp) | F: 5'-GCGTGGCGGTAGATCTAAGTCA-3'<br>R: 5'-TTCAGCTCCGCCACAAAGGTA-3' | 58.7<br>58.9 | (Thong *et al.*, 2011) |
| *Pseudomonas aeruginosa* | *Oprl* (504 bp) | F: 5'-ATGGAATAGCTGAAATTCGG-3'<br>R: 5'-CTTCTTCAGCTCGACGCGA-3' | 51.3<br>57.4 | (Thong *et al.*, 2011),<br>(De Vos *et al.*, 1997) |
| *E. coli* | *PhoA* (890 bp) | F: 5'-AAGCCCGGACACCATAAATGC-3'<br>R: 5'-TCATTACGTTGCGGATTTGGC-3' | 58.2<br>56.3 | (Thong *et al.*, 2011) |
| *Staphylococcus aureus* | *FemA* (296 bp) | F: 5'-CTCTTGCTGGTTTCTTCTTTATC-3'<br>R: 5'-GTGCGGTATATGCTGCGTAA-3' | 54.6<br>55.7 | (Thong *et al.*, 2011),<br>(Mehrotra *et al.*, 2000) |

**Table 2:** Sequences of primers and probes for real-time PCR.

| Target bacteria | Target gene | Primers and probes | Sequence (5'-3') | Melting temperature (°C) |
|---|---|---|---|---|
| *Acinetobacter baumannii* | *bla*$_{Oxa-51-like}$ | AcOxa_F | 5'-GAAGTGAAGCGTGTTGGTTAT-3' | 53.3 |
| | | AcOxa_R | 5'-GCCTCTTGCTGAGGAGTAAT-3' | 54.3 |
| | | AcOxa_probe | /5HEX/CG ACT TGG G/ZEN/T ACC GAT ATC TGC ATT GC/3IABkFQ/ | 61.1 |
| *Klebsiella pneumonia* | Citrate synthase (Cyt) | KpCy_F | 5'-CCAGTTAGCGACCGAATCTAAT-3' | 54.4 |
| | | KpCy_R | 5'-CGGGTGATCTGCTCATGAAT-3' | 54.9 |
| | | KpCy_probe | 5'-/56-FAM/CGACCCAG/ZEN/CCGAATATGACGAAT/3IABkFQ/-3' | 61.0 |
| *Pseudomonas aeruginosa* | DNA gyrase subunit B (*gyrB*) | PaGyr_F | 5'-CACCCTGCTGTTGACCTTCTT-3' | 57.5 |
| | | PaGyr_R | 5'-CTGGTCGTCCTTGATGTACTG-3' | 55.0 |
| | | PaGyr_probe | 5'-/5Cy5/CCCACATGG/TAO/CCTCCAAGGAGTAAG/3IABkRQSp/3' | 61.5 |
| *Escherichia coli* | *yccT* | EcYcc_F | 5'-CCAATTCCGGTAAAGCTGGATA-3' | 54.8 |
| | | EcYcc_R | 5'-GCGTTTCGCGGATTTGTTATAG-3' | 54.7 |
| | | EcYcc_probe | /5HEX/TCTCGCCATTACCTCAACGGCAAA/3IABkfQ/ | 60.9 |
| *Staphylococcus aureus* | *nuc* | SaNucF | 5'-AGGGATGGCTATCAGTAATGTTT-3' | 53.8 |
| | | SaNucR | 5'-CGCCGTTATCTGTTTGTGATG-3' | 54.3 |
| | | SaNuc-probe | 5'/56-FAM/CGAAAGGGC/ZEN/AATACGCAAAGAGGT/3IABkFQ/3 | 59.8 |
| *Homo sapiens* | *GAPDH* | GAPDH-F | 5'-AGGGTGGTGGACCTCAT-3' | 55.6 |
| | | GAPDH-R | 5'-CTCTCTTCCTCTTGTGCTCTTG-3' | 55.0 |
| | | GAPDH-Probe | 5'/5Cy5/CCCACATGG/TAO/CCTCCAAGGAGTAAG/3IABkRQSp/3' | 61.3 |

 ISSN (print): 1823-8262, ISSN (online): 2231-7538

Malays. J. Microbiol. Vol 18(3) 2022, pp. 242-250
DOI: http://dx.doi.org/10.21161/mjm.211331

**Table 3:** Components of two sets of reactions for multiplex real-time PCR with genomic DNA.

| Set 1 | | Set 2 | |
|---|---|---|---|
| Component | Volume per reaction | Component | Volume per reaction |
| Master mix | 10 µL | Master mix | 10 µL |
| Primer SaNuc_F | 0.5 µL | Primer KpCy_F | 0.5 µL |
| Primer SaNuc_R | 0.5 µL | Primer KpCy_R | 0.5 µL |
| SaNuc_probe | 1 µL | KpCy_probe | 1 µL |
| Primer EcYcc_F | 0.5 µL | Primer AcOxa_F | 0.5 µL |
| Primer EcYcc_R | 0.5 µL | Primer AcOxa_R | 0.5 µL |
| EcYcc_probe | 1 µL | AcOxa_probe | 1 µL |
| Primer PaGyr_F | 0.5 µL | GAPDH_F primer | 0.5 µL |
| Primer PaGyr_R | 0.5 µL | GAPDH_R primer | 0.5 µL |
| PaGyr_probe | 1 µL | GAPDH_probe | 1 µL |
| Target DNA template | 1.5 µL | Target DNA | 1.5 µL |
| $H_2O$ | up to 20 µL | $H_2O$ | up to 20 µL |

and probe pairs was $10^3$ CFU/mL. The sequences of primers and probes are shown in Table 2.

The single real-time PCR reaction was used to check the activity and specificity of primers after design using Aligent's AriaMx real-time PCR System. Target genes were amplified by real-time PCR with specific primers and probes. Each pair of primers and probes for each gene was tested in three different reaction tubes: 1) Tube 1: All necessary reagents were added and the DNA template was a mixture of 6 DNA targets (5 bacterial genomic DNA and human genomic DNA); 2) Tube 2: All necessary reagents were added and the DNA template was the target DNA of that primer and probe; and 3) Tube 3: All necessary reagents were added, but no DNA template was utilized. The volume of a reaction was 20 µL, including 10 µL real-time master mix (IDT), 0.5 µL forward primer (10 pmol/µL), 0.5 µL reverse primer (10 pmol/µL), 1 µL probe (2.5 pmol/µL), 1 µL DNA template and $H_2O$ added up to 20 µL. Real-time PCR thermal conditions were as follows denaturation at 95 °C for 3 min; 40 cycles at 95 °C for 15 sec and 60 °C for 60 sec.

### Multiplex real-time PCR with genomic DNA

Six pairs of primers and 6 probes were divided into two sets of reactions. The first set included primers and probes for detecting DNA of *S. aureus*, *E. coli* and *P. aeruginosa*, while the second set included primers and probes to detect DNA of *K. pneumoniae*, *A. baumannii* and human DNA. Each set was used to test the combination of 3 primer pairs and 3 probes in detecting single and mixed DNA samples and negative control samples without DNA. The total reaction volume was 20 µL with components of each set as followed (Table 3). Kappa coefficient was calculated to measure the similarity among these three methods. A higher coefficient indicated a higher level of similarity.

### Ethical approval

The study protocol was granted by the Institutional Review Board of Thanh Nhan Hospital. All patients were asked to give written informed consent before collecting their blood for the experiment.

**Table 4:** Profiles of bacteria caused sepsis in blood samples.

| Bacteria name | n | % |
|---|---|---|
| Gram-negative bacteria | 42 | 75.0 |
| *Acinetobacter baumannii* | 3 | 5.4 |
| *Acinetobacter* spp. | 1 | 1.7 |
| *Pseudomonas aeruginosa* | 1 | 1.7 |
| *Klebsiella pneumoniae* | 11 | 19.7 |
| *Escherichia coli* | 9 | 16.1 |
| Other bacilli | 17 | 30.4 |
| Gram-positive bacteria | 11 | 19.6 |
| *Staphylococcus aureus* | 9 | 16.1 |
| *Enterococcus* sp. | 2 | 3.5 |
| Fungus | 3 | 5.4 |
| Total | 56 | 100 |

## RESULTS

After culturing blood samples of 587 patients with sepsis, 56 samples (9.5%) had positive results. Gram-negative bacteria predominated with the rate of 75.0%, of which *K. pneumoniae* accounted for 19.7%, followed by *E. coli* (16.1%). *S. aureus* was the most common Gram-positive bacterium at 16.1% (Table 4).

We conducted a PCR reaction with the corresponding target gene-specific primer pairs. The product was electrophoresed on 1% agarose gel and shown in Figure 1. The results of agarose gel electrophoresis showed that in all wells, there were clear bands with the corresponding size of about 296 bp (lanes 1-3), 364 bp (lanes 4-6), 504 bp (lanes 7-9), 722 bp (lanes 10-12) and 890 bp lanes 13-15) fit the size of *femA* gene of *S. aureus*, *mdh* gene of *K. pneumonia*, *oprL* gene of *P. aeruginosa*, *gltA* gene of *A. baumannii* and *phoA* gene of *E. coli*. All tested primer pairs were specific to the target organisms because the predicted genes were amplified.

The multiplex PCR reaction was designed based on a standard PCR reaction with a total volume of 25 µL of

 ISSN (print): 1823-8262, ISSN (online): 2231-7538

Malays. J. Microbiol. Vol 18(3) 2022, pp. 242-250
DOI: http://dx.doi.org/10.21161/mjm.211331

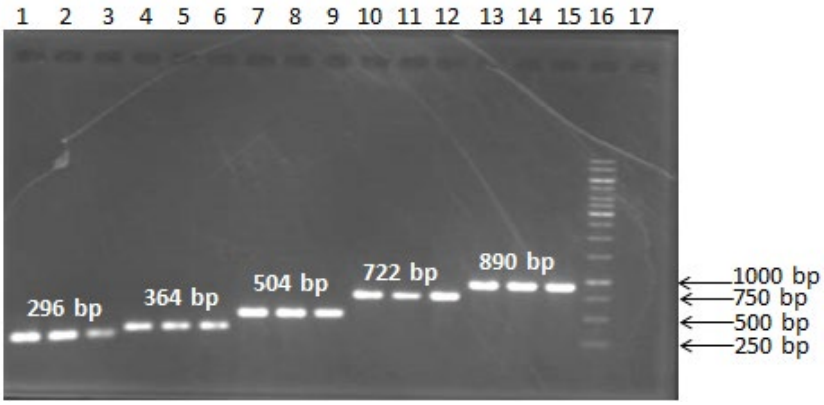


**Figure 1:** Agarose gel electrophoresis (1% agarose) of the PCR amplified products. Lanes 1-3: *femA* gene PCR product (296 bp theoretically), 4-6: *mdh* gene PCR product (364 bp theoretically), 7-9: *oprL* gene PCR product (504 bp theoretically), 10-12: PCR product *gtlA* gene (722 bp theoretically), 13-15: PCR product *phoA* gene (890 bp theoretically), 16: Marker 1 kb (Fermentas), 17: Negative control.

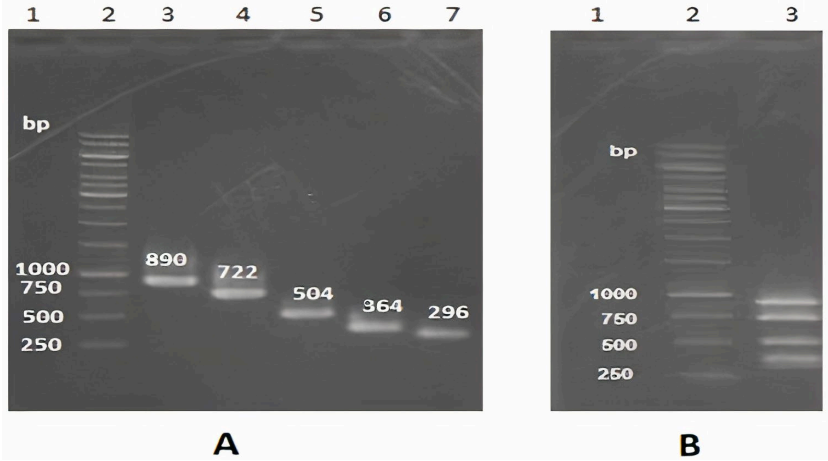


**Figure 2:** Agarose gel electrophoresis (1% agarose) of the multiplex PCR amplified products. (A) Multiplex PCR with single target, 1: Negative control (no DNA template); 2: Marker 1 kb; 3: PCR product of *phoA* gene (890 bp); 4: PCR product of *gltA* gene (722 bp); 5: *oprL* gene PCR product (504 bp); 6: *mdh* gene PCR product (364 bp); 7: *femA* gene PCR product (296 bp). (B) Multiplex PCR multiple with mixed targets, 1: Ngative control (no DNA template); 2: 1 kb marker; 3: Multiplex PCR product mix of *phoA*, *gltA*, *oprL*, *mdh* and *femA* genes.

each reaction including mastermix, primer, DNA template and deionized water. However, in the multiplex PCR reaction, there were additional primer pairs to detect different genes simultaneously. This addition caused the changes in components compared to the standard PCR reaction and at the same time, the primers cross-pair might have been inhibited, leading to the undetectable target gene. Therefore, we conducted multiplex PCR to check the specificity of primer pairs and corresponding target genes. The results of electrophoresis on 1% agarose gel are presented in Figure 2A. Results of electrophoresis on agarose gel showed that the obtained multiplex PCR products were specific, and consistent with the size of the target genes. It suggested that in the multiplex PCR reaction, the primer pairs were specific to the target genes, and not inhibited and cross-paired each other. To confirm the specificity of primer pairs with target genes in the same multiplex PCR reaction, we mixed the DNA of 5 different bacterial strains to form a template DNA mixture and carried out a multiplex PCR reaction. As a result, the product of this multiplex PCR reaction appeared in all 5 sharp, bold bands corresponding to the size of the 5 target genes and no extra bands existed (Figure 2B).

Primer and probe pairs were checked for specificity with target DNA through real-time PCR reaction. We tested each pair of primer and probe with each sample of the total DNA of the target bacteria and with a mixture of the total DNA of other bacteria. Figures 3-5 shows the primers and probes that are specific to their target DNA, for example, Figure 3 shows the primer pair SaNuc-F/R and probe SaNuc-probe specific to the genomic DNA of

 ISSN (print): 1823-8262, ISSN (online): 2231-7538

Malays. J. Microbiol. Vol 18(3) 2022, pp. 242-250
DOI: http://dx.doi.org/10.21161/mjm.211331

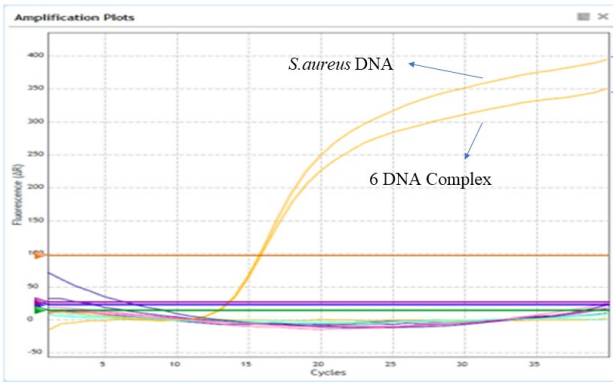


**Figure 3:** Real-time PCR amplification curves for *bla*$_{oxa-51}$ from *Staphylococcus aureus* sample and DNA complex sample (5 bacteria strains and human).

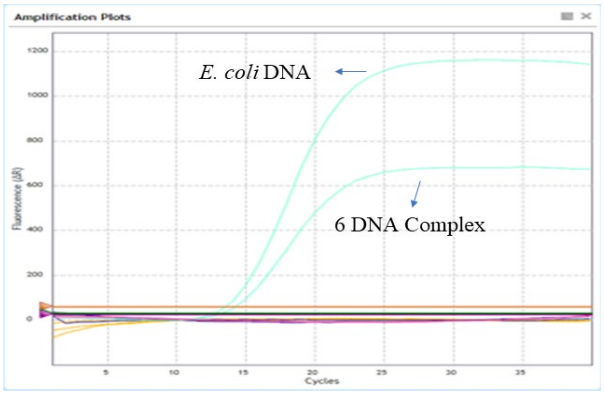


**Figure 4:** Real-time PCR amplification curves for *yccT* from *Escherichia coli* sample and DNA complex sample (5 bacteria strains and human).

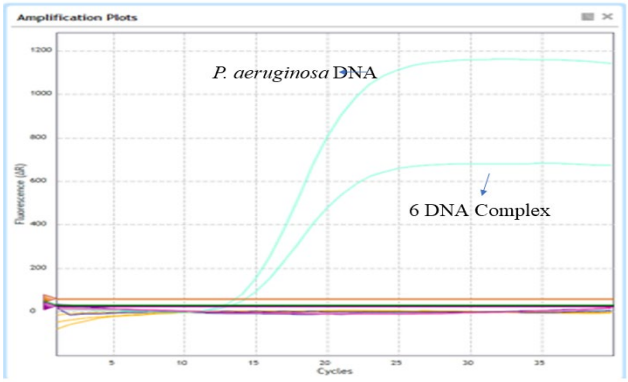


**Figure 5:** Real-time PCR amplification curves for *gyrB* from *P. aeruginosa* sample and DNA complex sample (5 bacteria strains and human).

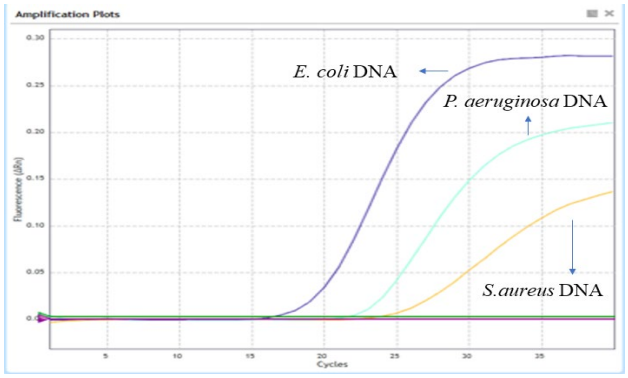


**Figure 6:** Real-time PCR amplification curves for *S. aureus*, *P. aeruginosa* and *E. coli* with 6 DNA targets complex.

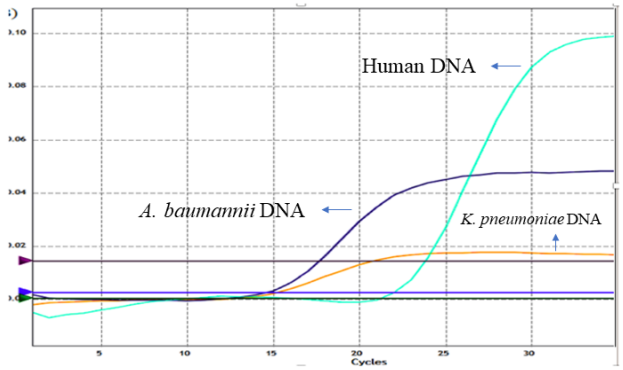


**Figure 7:** Real-time PCR amplification curves for human, *A. baumannii* and *K. pneumoniae* with 6 DNA targets complex.

*S. aureus*, Figure 4 shows the primer pair EcYcc-F/R and probe EcYcc-probe which are specific for the genomic DNA of *E. coli* and Figure 5 shows the probe and primer pair which was specific for *P. aeruginosa*.

Table 5 shows that the samples with the target DNA of each probe showed fluorescence signal and no fluorescence signal appeared in the samples without the target DNA. The negative control sample did not have a Ct value. The *P. aeruginosa* sample had a Ct value of 36.13 in the tube without DNA. Because the Ct value was >35, the real-time reaction condition was still selected, which was acceptable when the condition for real-time PCR reaction was optimal. Thus, the pairing of each pair of primers, probes and target DNA of each bacterial strain was specific and had good cloning ability.

The multiplex real-time PCR reaction was designed based on a single-primer PCR reaction with a total reaction volume of 20 µL, including master mix, primer, probe, DNA template and deionized water. However, in the multiplex real-time PCR reaction, there were additional pairs of primers and probes to simultaneously detect different genes. This addition could inhibit the primer pairs and cross-pair each other leading to undetected of the target genes. Therefore, we carried out multiplex real-time PCR to check the specificity of primers and probes and target genes corresponding to the

 ISSN (print): 1823-8262, ISSN (online): 2231-7538

Malays. J. Microbiol. Vol 18(3) 2022, pp. 242-250
DOI: http://dx.doi.org/10.21161/mjm.211331

**Table 5:** Ct values of real-time PCR reaction to check the specificity of bacterial primers and probes.

| PCR reaction | | Sample | Ct value |
|---|---|---|---|
| 1 | *S. aureus* | No DNA | No signal detected |
| 2 | | Total DNA *S. aureus* | 13.65 |
| 3 | | Total DNA *S. aureus*, *P. aeruginosa*, *E. coli*, *A. baumannii*, *K. pneumoniae*, *H. sapiens* | 13.55 |
| 4 | *E. coli* | No DNA | No signal detected |
| 5 | | Total DNA *E. coli* | 12.98 |
| 6 | | Total DNA *S. aureus*, *P. aeruginosa*, *E. coli*, *A. baumannii*, *K. pneumoniae*, *H. sapiens* | 13.61 |
| 7 | *P. aeruginosa* | No DNA | 36.13 (Ct>35) |
| 8 | | Total DNA *P. aeruginosa* | 15.25 |
| 9 | | Total DNA *S. aureus*, *P. aeruginosa*, *E. coli*, *A. baumannii*, *K. pneumoniae*, *H. sapiens* | 15.67 |

**Table 6:** Testing results of primer and probe kit to detect *E. coli*, *S. aureus* and *P. aeruginosa*.

| Pipe | Sample | Ct value | | |
|---|---|---|---|---|
| | | HEX (*E. coli* signal probe) | FAM (*S. aureus* signal probe) | CY5 (*P. aeruginosa* signal probe) |
| 1 | No target DNA | No signal | No signal | No signal |
| 2 | *E. coli* DNA | 17.88 | No signal | No signal |
| 3 | *S. aureus* DNA | No signal | 22.48 | No signal |
| 4 | *P. aeruginosa* DNA | No signal | No signal | 20.99 |
| 5 | Mixture of DNA targets | 17.00 | 23.36 | 21.17 |

**Table 7:** Testing results of primer and probe kit to detect *A. baumannii*, *K. pneumoniae* and *H. sapiens*.

| Pipe | Sample | Ct value | | |
|---|---|---|---|---|
| | | HEX (*A. baumannii* probe signal) | FAM (*K. pneumoniae* probe signal) | CY5 (*H. sapiens* probe signal) |
| 1 | No target DNA | No signal | No signal | No signal |
| 2 | *A. baumannii* DNA | 12.93 | No signal | No signal |
| 3 | *K. pneumoniae* DNA | No signal | 10.93 | No signal |
| 4 | Human DNA | No signal | No signal | 20.99 |
| 5 | Mixture of DNA targets | 14.91 | 18.48 | 23.92 |

composition and concentration as described above. Results are shown in Table 6 and Figure 6. Samples with *E. coli*, *S. aureus* DNA and *P. aeruginosa* had a fluorescence signal that exceeded the background fluorescence signal compared with Ct values is 17.88, 22.48 and 20.99, respectively. Similarly, we mixed 5 DNA of all 5 different bacterial strains to form a DNA template mixture and perform the reaction. The product of the reaction showed all 3 fluorescence signal lines and no by-products (Figure 6).

With primers and probes of bacteria *A. baumannii*, *K. pneumoniae* and human internal standard genes, we also obtained similar results. Thus, with 5 pairs of primers and 5 bacterial probes and 1 pair of primers and probes of the human *GADPH* internal standard gene were suitable to combine in a reaction to detect 5 pathogens, including *A. baumannii*, *K. pneumoniae*, *E. coli*, *S. aureus* and *P. aeruginosa*. After confirming the specificity of the primers and probes, the multiplex real-time PCR kit was used to analyze the presence of target genes in the genomic DNA isolated from the patient blood samples (Table 7 and Figure 7).

The results of the similarity assessment between the three methods of blood culture, multiplex PCR and multiplex real-time PCR are presented in Table 8. After 7 days, it shows that all three methods had high sensitivity and specificity in all 5 types of bacterial strains.

## DISCUSSION

Early diagnosis and prognosis of patients with sepsis and appropriate treatment regimens within the first few hours play an important role in reducing mortality risk and duration of treatment. This study showed that the detection rate of microorganisms in the study was 9.5%, lower than some other studies (Zhou *et al.*, 2014; Dat *et al.*, 2017) because the patients in our study were in the early stage of sepsis.

We performed multiplex PCR and multiplex real-time PCR simultaneously with the blood culture method.

 ISSN (print): 1823-8262, ISSN (online): 2231-7538

Malays. J. Microbiol. Vol 18(3) 2022, pp. 242-250
DOI: http://dx.doi.org/10.21161/mjm.211331

**Table 8:** Comparison of PCR and real-time PCR results with the results of blood culture method.

| Results | Blood culture (n=587) | PCR (n=587) | Real-time PCR (n=587) |
|---|---|---|---|
| Positive samples | 33 | 33 | 33 |
| *Acinetobacter baumannii* | 3 | 3 | 3 |
| *Klebsiella pneumoniae* | 11 | 12 | 10 |
| *Escherichia coli* | 9 | 9 | 10 |
| *Staphylococcus aureus* | 9 | 9 | 9 |
| *Pseudomonas aeruginosa* | 1 | 1 | 1 |
| Other bacteria | 23 | 0 | 0 |
| Negative samples | 531 | 554 | 554 |
| Samples with >1 bacteria | 0 | 1 | 0 |
| Kappa (ƙ) | | 0.99 ($p$<0.05) | 0.96 ($p$<0.05) |

During the process, we found that it is necessary to dissolve the cells in 180 µL of lysis buffer containing lysozyme enzyme 1 mg/mL and incubate at 37 °C for 30 min to ensure that the cell walls of *S. aureus* could dissolve. With multiplex real-time PCR, we selected different primers and probes and performed tests on primer and probe specificity as well as optimizing reaction conditions. Although primers and probes were designed at different genes, but the results of multiplex PCR and multiplex real-time PCR in this study were suitable for detecting five bacteria causing sepsis. We evaluated the specificity of these methods on samples containing target bacterial DNA, samples containing other bacterial DNA and samples without bacterial DNA. The results showed that both PCR methods had 100% sensitivity and specificity, suggesting that these results are specific for target bacteria, consistent with previous studies (Wang *et al.*, 2014; Weiss *et al.*, 2019). Another study used multiplex real-time PCR to rapidly detect antibiotic resistance of *Acinetobacter baumannii*. The authors used the *ompA* gene on 48 patients and when compared with the culture results, the authors showed the similarity reached 95.8%, specificity 97.5%, sensitivity 92.9%, positive predictive value 95.6% and negative predictive value 96.0%. This finding suggested that the multiplex real-time PCR results were reliable and could distinguish between resistant and non-resistant strains (Martín-Peña *et al.*, 2013). Nomanpour *et al.* (2011) used multiplex real-time PCR to identify *A. baumannii* causing pneumonia. The authors used multiplex real-time TaqMan PCR method based on the sequence of $bla_{OXA-51}$ which was designed and used for direct detection of *A. baumannii* from 361 respiratory specimens of pneumonia patients. All samples were examined in parallel with the conventional blood culture method. The results showed that the multiplex real-time PCR method could detect the bacteria with less than 200 CFU/mL in the sample and the results were like the blood culture method (kappa value 1.0, $p$-value<0.001). The sensitivity, specificity, and predictive value of the real-time PCR method was 100% (Nomanpour *et al.*, 2011). Liu *et al.* (2018) showed a detection rate of 96% at 100 CFU/mL and detection rate of 100% at 1000 CFU/mL.

Results of the two PCR methods showed that compared with the results after 7 days of blood culture, the samples performed with molecular biology techniques were 100% consistent with the positive and negative characteristics of blood cultures. There was a sample containing *Klebsiella pneumoniae* which could be detected by the PCR method but not the culture method. We had also confirmed by sequenced and blasted (data not shown). Meanwhile, the results of 32/33 samples of the real-time PCR method were consistent with blood culture. There was one sample that could not detect *K. pneumoniae* when using the real-time PCR method. We assumed that in that sample, the number of microorganisms was lower than $10^4$ CFU, which was the minimum number of copies for multiplex real-time PCR to detect infectious microorganisms. There was one sample of real-time PCR method showing the presence of *E. coli*, but the results of blood culture and PCR method were negative. This patient had a blood infection via the urinary tract; hence, the urine culture showed the presence of *E. coli*. Thus, the real-time PCR method was able to detect microorganisms that the culture and PCR method was unable to detect. Yang and Rui (2016) applied multiplex real-time PCR method and detected *Acinetobacter baumannii* and *Acinetobacter* spp with 100% sensitivity and specificity on sputum samples. Another prior study showed that using the real-time PCR method to detect *Staphylococcus aureus* in the blood had a sensitivity and specificity of 99.5% and 100%, respectively. Besides, the real-time PCR method took three hours to get results, while the culture method took 48-72 h (Wang *et al.*, 2016). This suggested that multiplex real-time PCR was a method of detecting bacteria in blood with high sensitivity and specificity, as well as short detection time.

## CONCLUSION

This study used multiplex PCR and multiplex real-time PCR methods to detect five common bacterial species causing sepsis including *A. baumannii*, *K. pneumoniae*, *P. aeruginosa*, *E. coli* and *S. aureus*. Both multiplex PCR and multiplex real-time PCR methods have high similarities with the culture method, showing potential in the application of bacteria detection in sepsis.

## ACKNOWLEDGEMENTS

This work was supported by the Vietnam Academy of Science and Technology.

 ISSN (print): 1823-8262, ISSN (online): 2231-7538

## REFERENCES

**Bartual, S. G., Seifert, H., Hippler, C., Luzon, M. A. D., Wisplinghoff, H. and Rodríguez-Valera, F. (2005).** Development of a multilocus sequence typing scheme for characterization of clinical isolates of *Acinetobacter baumannii*. *Journal of Clinical Microbiology* **43(9), 4382-4390.**

**Boratyn, G. M., Camacho, C., Cooper, P. S., Coulouris, G., Fong, A., Ma, N., Madden, T. L., Matten, W. T., McGinnis, S. D., Merezhuk, Y., Raytselis, Y., Sayers, E. W., Tao, T., Ye, J. and Zaretskaya, I. (2013).** BLAST: A more efficient report with usability improvements. *Nucleic Acids Research* **41, W29-W33.**

**Chong, J., Dumont, T., Francis-Frank, L. and Balaan, M. (2015).** Sepsis and septic shock: A review. *Critical Care Nursing Quarterly* **38(2), 111-120.**

**Dat, V. Q., Vu, H. N., Nguyen The, H., Nguyen, H. T., Hoang, L. B., Viet, D. V. T., Bui, C. L., Nguyen, K. V., Nguyen, T. V., Trinh, D. T., Torre, A., van Doorn, H. R., Nadjm, B. and Wertheim, H. F. L. (2017).** Bacterial bloodstream infections in a tertiary infectious diseases hospital in Northern Vietnam: Aetiology, drug resistance, and treatment outcome. *BMC Infectious Diseases* **17(1), 493.**

**De Vos, D., Lim, A., Jr., Pirnay, J. P., Struelens, M., Vandenvelde, C., Duinslaeger, L., Vanderkelen, A. and Cornelis, P. (1997).** Direct detection and identification of *Pseudomonas aeruginosa* in clinical samples such as skin biopsy specimens and expectorations by multiplex PCR based on two outer membrane lipoprotein genes, *oprI* and *oprL*. *Journal of Clinical Microbiology* **35(6), 1295-1299.**

**Gyawali, B., Ramakrishna, K. and Dhamoon, A. S. (2019).** Sepsis: The evolution in definition, pathophysiology, and management. *SAGE Open Medicine* **7, 2050312119835043.**

**Liu, C. F., Shi, X. P., Chen, Y., Jin, Y. and Zhang, B. (2018).** Rapid diagnosis of sepsis with TaqMan-based multiplex real-time PCR. *Journal of Clinical Laboratory Analysis* **32(2), e22256.**

**Martín-Peña, R., Domínguez-Herrera, J., Pachón, J. and McConnell, M. J. (2013).** Rapid detection of antibiotic resistance in *Acinetobacter baumannii* using quantitative real-time PCR. *Journal of Antimicrobial Chemotherapy* **68(7), 1572-1575.**

**Mehrotra, M., Wang, G. and Johnson, W. M. (2000).** Multiplex PCR for detection of genes for *Staphylococcus aureus* enterotoxins, exfoliative toxins, toxic shock syndrome toxin 1, and methicillin resistance. *Journal of Clinical Microbiology* **38(3), 1032-1035.**

**Nomanpour, B., Ghodousi, A., Babaei, A., Abtahi, H. R., Tabrizi, M. and Feizabadi, M. M. (2011).** Rapid, cost-effective, sensitive and quantitative detection of *Acinetobacter baumannii* from pneumonia patients. *Iranian Journal of Microbiology* **3(4), 162-169.**

**Ozkaya-Parlakay, A., Cengiz, A. B., Ceyhan, M., Hascelik, G., Kara, A., Celik, M., Ozsurekci, Y. and Karadag-Oncel, E. (2014).** Evaluation of multiplex real time polymerase chain reaction and procalcitonin in the diagnosis of sepsis. *Clinical Laboratory* **60(7), 1075-1081.**

**Rudd, K. E., Johnson, S. C., Agesa, K. M., Shackelford, K. A., Tsoi, D., Kievlan, D. R. *et al.* (2020).** Global, regional, and national sepsis incidence and mortality, 1990-2017: Analysis for the global burden of disease study. *Lancet* **395, 200-211.**

**Sakr, Y., Jaschinski, U., Wittebole, X., Szakmany, T., Lipman, J., Ñamendys-Silva, S. A., Martin-Loeches, I., Leone, M., Lupu, M. and Vincent, J. (2018).** Sepsis in intensive care unit patients: Worldwide data from the intensive care over nations audit. *Open Forum Infectious Diseases* **5(12), ofy313.**

**Thong, K. L., Lai, M. Y., Teh, C. S. and Chua, K. H. (2011).** Simultaneous detection of methicillin-resistant *Staphylococcus aureus*, *Acinetobacter baumannii*, *Escherichia coli*, *Klebsiella pneumoniae* and *Pseudomonas aeruginosa* by multiplex PCR. *Tropical Biomedicine* **28(1), 21-31.**

**Trung, N. T., Tong, H. V., Lien, T. T., Son, T. V., Thanh Huyen, T. T., Quyen, D. T., Hoan, P. Q., Meyer, C. G. and Song, L. H. (2018).** Clinical utility of an optimised multiplex real-time PCR assay for the identification of pathogens causing sepsis in Vietnamese patients. *International Journal of Infectious Diseases* **67, 122-128.**

**Wang, H. Y., Kim, S., Kim, J., Park, S. D., Kim, H. Y., Uh, Y. and Lee, H. (2016).** Comparison of multiplex real-time PCR and PCR-reverse blot hybridization assay for the direct and rapid detection of bacteria and antibiotic resistance determinants in positive culture bottles. *Journal of Medical Microbiology* **65, 962-974.**

**Wang, H. Y., Kim, S., Kim, J., Park, S. D., Uh, Y. and Lee, H. (2014).** Multiplex real-time PCR assay for rapid detection of methicillin-resistant staphylococci directly from positive blood cultures. *Journal of Clinical Microbiology* **52(6), 1911-1920.**

**Weiss, D., Gawlik, D., Hotzel, H., Engelmann, I., Mueller, E., Slickers, P., Braun, S. D., Monecke, S. and Ehricht, R. (2019).** Fast, economic and simultaneous identification of clinically relevant Gram-negative species with multiplex real-time PCR. *Future Microbiology* **14, 23-32.**

**Winters, B. D., Eberlein, M., Leung, J., Needham, D. M., Pronovost, P. J. and Sevransky, J. E. (2010).** Long-term mortality and quality of life in sepsis: A systematic review. *Critical Care Medicine* **38(5), 1276-1283.**

**Yang, Q. and Rui, Y. (2016).** Two multiplex real-time PCR assays to detect and differentiate *Acinetobacter baumannii* and non- *baumannii Acinetobacter* spp. carrying *bla*$_{NDM}$, *bla*$_{OXA-23-Like}$, *bla*$_{OXA-40-Like}$, *bla*$_{OXA-51-Like}$, and *bla*$_{OXA-58-Like}$ genes. *PLoS ONE* **11(7), e0158958.**

**Zhou, J., Qian, C., Zhao, M., Yu, X., Kang, Y., Ma, X. *et al.* (2014).** Epidemiology and outcome of severe sepsis and septic shock in intensive care units in mainland China. *PLoS ONE* **9(9), e107181.**

 ISSN (print): 1823-8262, ISSN (online): 2231-7538