Brazilian Journal of Microbiology (2011) 42: 448-458
ISSN 1517-8382

DEVELOPMENT OF CONVENTIONAL AND REAL-TIME MULTIPLEX PCR ASSAYS FOR THE DETECTION OF NOSOCOMIAL PATHOGENS

Deepa Anbazhagan [1], Wang Seok Mui [1], Marzida Mansor [2], Gracie Ong Siok Yan [2], Mohd Yasim Yusof [1], Shamala Devi Sekaran [1]

[1]Department of Medical Microbiology, Faculty of Medicine, University of Malaya, Kuala Lumpur, Malaysia; [2]Department of Anaesthesiology, Faculty of Medicine, University Malaya.

Submitted: January 17, 2010; Returned to authors for corrections: August 30, 2010; Approved: January 13, 2011.

ABSTRACT

Nosocomial infections are major clinical threats to hospitalised patients and represent an important source of morbidity and mortality. It is necessary to develop rapid detection assays of nosocomial pathogens for better prognosis and initiation of antimicrobial therapy in patients. In this study, we present the development of molecular methods for the detection of six common nosocomial pathogens including *Escherichia coli, Staphylococcus aureus, Streptococcus pneumoniae, Klebsiella pneumoniae, Pseudomonas aeruginosa and Acinetobacter spp.* Conventional multiplex PCR and SYBR Green based real time PCR assays were performed using genus and species specific primers. Blind testing with 300 clinical samples was also carried out. The two assays were found to be sensitive and specific. Eubacterial PCR assay exhibited positive results for 46 clinical isolates from which 43 samples were detected by real time PCR assay. The sensitivity of the assay is about 93.7% in blind test isolates. The PCR results were reconfirmed using the conventional culture method. This assay has the potential to be a rapid, accurate and highly sensitive molecular diagnostic tool for simultaneous detection of *Escherichia coli, Staphylococcus aureus, Streptococcus pneumoniae, Klebsiella pneumoniae, Pseudomonas aeruginosa and Acinetobacter spp.* This assay has the potential to detect nosocomial pathogens within 5 to 6 hours, helping to initiate infection control measures and appropriate treatment in paediatric and elderly (old aged) patients, pre-and post surgery patients and organ transplant patients and thus reduces their hospitalization duration .

Key words: Conventional multiplex PCR, SYBR Green Real time PCR, Nosocomial Pathogens

INTRODUCTION

Patients in the intensive care unit (ICU) are more likely to acquire nosocomial infections at a much higher rate than those in other wards in the hospital (12). The frequency and the type of pathogens vary according to the anatomical site and the state of health of patients. This risk can be 5 to 10 times greater and is said to result from three major factors: (a) an intrinsic factors

***Corresponding Author. Mailing address:** Department of Medical Microbiology, Faculty of Medicine, University of Malaya, 50603 Kuala Lumpur, Malaysia.; Tel.; 603-79675759 Fax.: 603-79676672.; E-mail: shamalamy@yahoo.com

relating to requirements for intensive care, such as underlying disease, extremes of age or immunosuppression; (b) invasive medical devices such as endotracheal tubes, mechanical ventilation, intravascular catheters and (c) cross infection due to crowding (10). Some of the nosocomial infections might be due to antibiotic resistant pathogens, namely methicillin-resistant *Staphylococcus aureus* (MRSA), vancomycin-resistant *Enterococcus*, gram-negative *bacilli* especially *Klebsiella Sp., Enterobacter Sp., Pseudomonas aeruginosa* and *Acinetobacters* (10). Nosocomial infection data from surveys of NNIS (National Nosocomial Infections Surveillance) hospitals between 1998 and 1995, demonstrated that there was a significant increase in the number of ICU beds (7, 2). These infections are a great threat in the developing countries and may be contained more effectively by having an infection prevention and control programme. For the war against nosocomial infections to be won, the whole exercise should be handled as a global project with significant inputs from developing countries (26). ICU nosocomial rates associated with usage of medical devices in adult ICUs of three major hospitals in Malaysia were evaluated in a recent study (19).

In 2000, the incidence of sepsis in the USA was 240.4 cases per 100,000 people with an annual increase of 8.7% than the previous year (22). Gram-Positive bacteria are the most frequent causes of blood stream infections with 30-50% of all cases, followed by gram-negative bacteria in 25-30% (1). It is essential for the identification of pathogens in blood stream infections for better prognosis and initiation of adequate antimicrobial therapy (12, 15, 18, 20, 24). Rapid identification of the pathogens and their resistance profile might help in the reduction in the use of broad-spectrum antimicrobials, thus switching to narrow-spectrum antibiotics. This will reduce the cost of therapy in the hospitalized patients (28).

The standard procedure for microbial diagnostics of blood stream infections is the inoculation of blood culture bottles followed by incubation in an automated blood culture system (31, 33). After a positive signal in the automated system (mainly within 1-2 days), the pathogen has to be identified by Gram stain and biochemical tests. This will be followed by the susceptibility determination. The existing methods of detection in microbiological laboratory will take about 2 - 4 days. Molecular medical pathology has since evolved into molecular diagnostics for the rapid and accurate identification of pathogens using DNA microarray (9), RNA-based fluorescence in situ hybridisation probes (16, 30) and PCR-assays (5, 23, 32, 17).

Real time PCR is a technique used to monitor the progress of a PCR reaction in real time. At the same time, a relatively small amount of PCR product (DNA, cDNA or RNA) can be quantified. Real Time PCR is based on the detection of the fluorescence produced by a reporter molecule which increases, as the reaction proceeds. This occurs due to the accumulation of the PCR product with each cycle of amplification. These fluorescent reporter molecules include dyes that bind to the double-stranded DNA (i.e. SYBR® Green) or sequence specific probes (i.e. Molecular Beacons or TaqMan® Probes). Real time PCR facilitates the monitoring of the reaction as it progresses. The present study was envisaged to develop rapid, accurate and highly sensitive molecular diagnostic assays for the detection of bacterial nosocomial pathogens to assist clinicians and clinical pathologists to initiate infection control measures and appropriate treatment in intensive care unit (ICU) patients in the hospital. In this study, a conventional multiplex PCR and a SYBR Green based real time multiplex assays were carried out in two independent experiments to detect six commonly occurring nosocomial pathogens, namely *Escherichia coli, Staphylococcus aureus, Streptococcus pneumoniae, Klebsiella pneumoniae , Pseudomonas aeruginosa and Acinetobacter spp.*

MATERIALS AND METHODS

Culture of nosocomial bacterial pathogens

A total of six nosocomial pathogens, namely *Escherichia*

coli, Staphylococcus aureus, Streptococcus pneumoniae, Klebsiella pneumoniae, Pseudomonas aeruginosa and Acinetobacter spp. were used in the assays standardization. All these isolates were chosen from a library of cultures isolated from University Malaya Medical Centre, Kuala Lumpur during the year 2008-2009. *Streptococcus pneumoniae* was grown on Blood Agar (Oxoid, England) and in Todd-Hewitt broth (Oxoid, England) at 37°C for 24 hours under anaerobic conditions. All other pathogens were cultured on Luria-Bertani (LB) agar (Difco, France) and in LB broth at 37°C for 24 hours.

Genomic DNA Extraction

Genomic DNA was extracted from cultures using modified protocol of the Epicentre Masterpure DNA purification kit (Madison, WI, USA). *Staphylococcus aureus* and *Streptococcus pneumoniae* were grown overnight on Todd Hewitt broth supplemented with 0.2% yeast extract (THY). The bacterial cultures were centrifuged and washed in Tris-EDTA(TE). The cell pellet was incubated with 3µl of lysostaphin (1mg/ml), suspended in TE at 37°C for 1 hour. After this about 150µl of 2× lysis buffer and 1µl of Proteinase K^+ (50µg/µl) were added. The mixture was incubated at 65°C until the clear lysate was obtained. The extraction tubes were then placed on ice for 10 minutes. About 200µl of MasterPure™ Complete (MPC) protein precipitation solution was added to the tubes and were centrifuged at 13,000 rpm (Rotor: FA45-30-11, Eppendorf, 5810R) at room temperature for 10 minutes. The supernatant was then precipitated with 600µl of isopropanol and the pellet was washed twice with 70% ethanol. The pellet was resuspended in 200µl of TE, pH 8.0. *Streptococcus pneumoniae* culture was grown in Todd-Hewitt broth without yeast extract supplement. The same protocol was used for gram-negative bacterial pathogens namely, *Escherichia coli, Klebsiella pneumoniae, Pseudomonas aeruginosa* and *Acinetobacter spp.* except that the lysostaphin step was excluded. The concentration of DNA was measured using a Nanophotometer (Implen, Germany). DNA was stored at -20°C until used for PCR amplification.

Oligonucleotide Primer Selection

The target genes for the six nosocomial pathogens, their oligonucleotide primer pairs and their respective amplicon sizes are shown in Table 1. The target genes selected for each of the organism was very specific for that genera and the primers were designed using NCBI website and were highly specific for each organism. All the primers designed were BLAST analysed before they were synthesized by Sigma Proligo, Singapore.

Conventional multiplex PCR Assay

The conventional multiplex PCR assay was carried out in two sets of three pathogens each, because the amplicon size of 6 genes chosen would not allow their detection in single multiplex reaction. The first set consisted of *Escherichia coli, Staphylococcus aureus and Klebsiella pneumoniae.* The second set consisted of *Streptococcus pneumoniae, Pseudomonas aeruginosa and Acinetobacter spp.* Duplex PCR reactions were also carried out and the PCR amplification conditions were same as that of the multiplex assay. The PCR amplification reaction mixture consisted of 5.0mM PCR buffer, 8.3 mM $MgCl_2$, 200 nM dNTP (MBI Fermentas, Vilnius, Lithuania), 0.3µM of each primer pair, 2 units of TaqDNA polymerase (MBI Fermentas) and 3µl of template DNA in a final volume of 50 µl in a single tube for 3 pathogens. The PCR amplification was performed with Mycycler Thermocycler (BioRad Laboratories, Hercules, California). The protocol used was an initial denaturation at 95°C for 10 minutes followed by 35 cycles of denaturation at 95°C for 45 seconds; primer annealing at 52°C for 45 seconds, primer extension at 72°C for 1 minute and 30 seconds and the final extension at 72°C for 10 minutes. All samples were also initially tested with an in-house developed eubacterial PCR. The primers utilized for the eubacterial 16SrRNA gene were

the forward primer sequence of 5'- CCTAACACATGCAAGT CGA–3' and the reverse sequence of 5'- CCTCTCAGACC AGTTA- 3'. Their amplicon size was 225bp. The primers were designed with slight modification from published eubacterial primer sequence (6).

The PCR product (about 10 µl) was analyzed by electrophoresis on 2% agarose gel with Ethidium Bromide in 1× Tris-acetate-EDTA (TAE) buffer with the final solute concentration of 40mM Tris acetate and 1mM EDTA and the gel image was documented. A 100bp size ladder (MBI Fermentas) was used as marker to indicate the size of amplicons.

SYBR – Green Real Time PCR Assay

The iQ SYBR Green Supermix (BioRad Laboratories, Hercules, CA) was used for the multiplex real time PCR Assay. The reaction was optimized and was carried out in 2 sets of 3 pathogens each depending on melting temperature (*Tm*) of primers. This was done as the *Tm* values of primers were too close for a clear differentiation. The first set consisted of *Staphylococcus aureus, Streptococcus pneumoniae and Acinetobacter spp.* While the second set consisted of *Escherichia coli, Klebsiella pneumoniae* and *Pseudomonas aeruginosa.* The real-time PCR was also carried out as sigleplex reactions. PCR amplification reactions were performed with 12.5µl of SYBR Green Supermix, 1µl of the primers (10 pmol), 3µl of template DNA and deionized water was used to make up the total volume to 25µl. The iCycler iQ Real-time PCR detection system (BioRad Laboratories, Hercules,CA) was programmed for 95°C for 10 minutes followed by 35 cycles of initial denaturation at 95°C for 45 seconds; primer annealing of 52°C for 45 seconds and primer extension of 72°C for 10 minutes with the final holding temperature of 4°C. Fluorescence was measured during the annealing step of each cycle. Individual real time PCR assays were carried out prior to multiplexing for all the six primer pairs to record their exact melt peaks.

Determination of sensitivity of the PCR assays

The sensitivity of the PCR assay was determined using serial dilutions of genomic DNA, from 2×10^{2} to 2×10^{6} genomic molecules.

Investigation of nosocomial pathogens from clinical samples

300 clinical samples were obtained from samples sent for blood culture from the University Malaya Medical Centre. Identification of bacteria was done by standard tests which include Gram stain followed by specific biochemical tests for each organism. DNA was extracted from these blood culture isolates using the Epicentre Masterpure DNA purification kit (Madison, WI, USA) according to the manufacturer's instructions. Eubacterial PCR was performed to verify the presence of bacterial DNA. The multiplex real time PCR was performed on the samples positive for eubacterial PCR to identify the exact bacterial pathogen within the scope of the above developed multiplex assay.

RESULTS

Conventional PCR Assay

Conventional PCR was carried out as a duplex to show the different gene amplicon sizes of the six organisms (Figure 1). *E.coli uid*A gene amplicon has a size of 556bp, *Acinetobacter spp., efp* gene amplicon is 422bp, *Streptococcus pneumoniae ply* gene has amplicon size of 348bp, *Staphylococcus aureus sa442* gene has a amplicon size of 179bp, *Pseudomonas aeruginosa las*A gene amplicon size is 125bp and *Klebsiella pneumoniae ntr*A gene amplicon size is 90bp. Conventional multiplex PCR was then carried out in groups of 3 organisms in 2 sets which showed distinct banding patterns in the gel analysis.The *ntr*A, *uid*A, *sa442* genes were amplified using their respective primers in the first set and they showed three distinct bands. The second set of the reaction tube amplified *efp, ply and las* genes using their respective primers and they are observed as three distinct bands (Figure 2). It was not

possible to have all the six as one group as the molecular weights of the amplicons were too close.

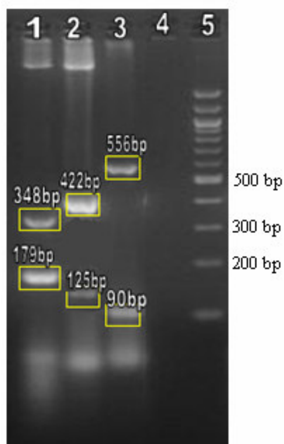


Figure 1. Electrophoretic banding patterns of the amplified products of the six genes by a duplex PCR in a 2% Agarose gel.

Lane 1- *Streptococcus Pneumoniae ply* gene (348bp) & *Staphylococcus aureus sa442* gene (179bp).

Lane 2- *Acinetobacter spp. Efp* gene (422bp) & *Pseudomonas aeroginosa las*A gene (125bp)

Lane 3- *E.coli uid*A gene(556bp) & *Klebsiella pneumoniae ntr*A gene(90bp)

Lane 4- Negative control (PCR product without the DNA template)

Lane 5- 100bp-DNA ladder, the different sizes of the markers are shown on the right side of the gel picture.

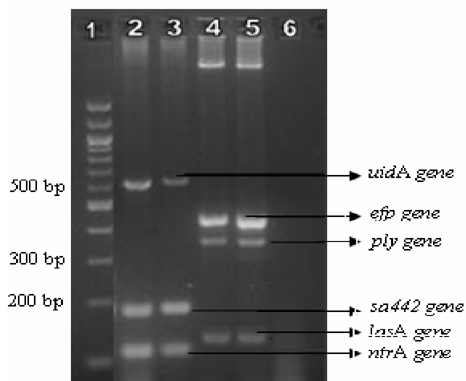


Figure 2. Electrophoretic banding patterns of the amplified products of the six genes by multiplex PCR in 2 sets as groups of 3 organisms in a 2% Agarose gel.

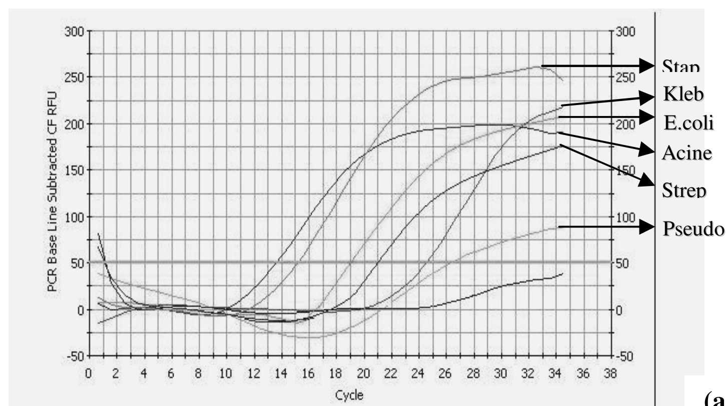
Lane 1- 100bp-DNA ladder, the different sizes of the markers are shown on the left side of the gel picture.

Lanes 2-5- Clinical isolates. The amplified products and their sizes are indicated in the right.

Lane 6 - Negative control (PCR product without the DNA template).

SYBR Green based PCR Assay

SYBR Green Real time PCR was first done as a singleplex and each of the six genes were amplified with their respective primers in separate reaction tubes. The amplification curves and the melt peaks for individual genes are shown in Figure 3 (a & b). *Staphylococcus aureus* showed a melt peak at 79.5°C, *Acinetobacter spp*., at 84°C, *Streptococcus pneumoniae* and *Klebsiella pneumoniae* showed a melt peak at 85°C, *E.coli* at 88°C and *Pseudomonas aeruginosa* at 92°C. The multiplex reaction was carried out in groups of 3 organisms in 2 sets and were identified based on the melt curve analysis. As shown in Figure 4 the respective organisms were detected and further identified by the melt curves.



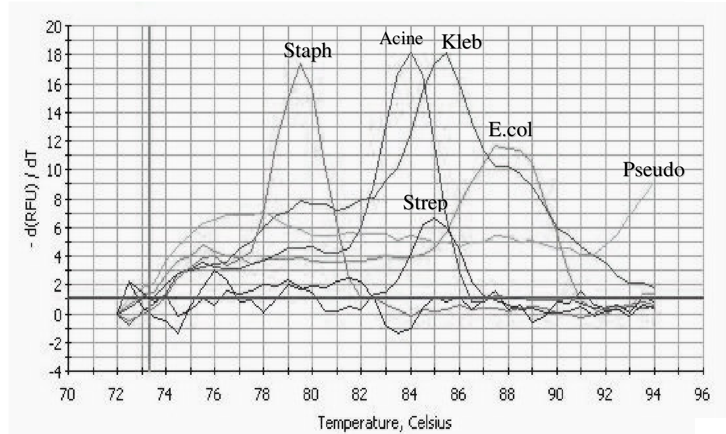


Figure 3. Amplification curves and melt peaks for the selected genes of the 6 nosocomial pathogens in a singleplex PCR reaction:

(a) Amplifications from chromosomal DNA of all the six pathogens (listed in Table 1) as singleplex are shown .Chromosomal DNA (10 ng each) from the six pathogens were used as templates in the SYBR Green PCR & their amplification profiles are represented.

(b) Melting peaks of the 6 genes (Table 1) in clinical pathogens. The primers were designed to detect the genes specific for each of the genus.

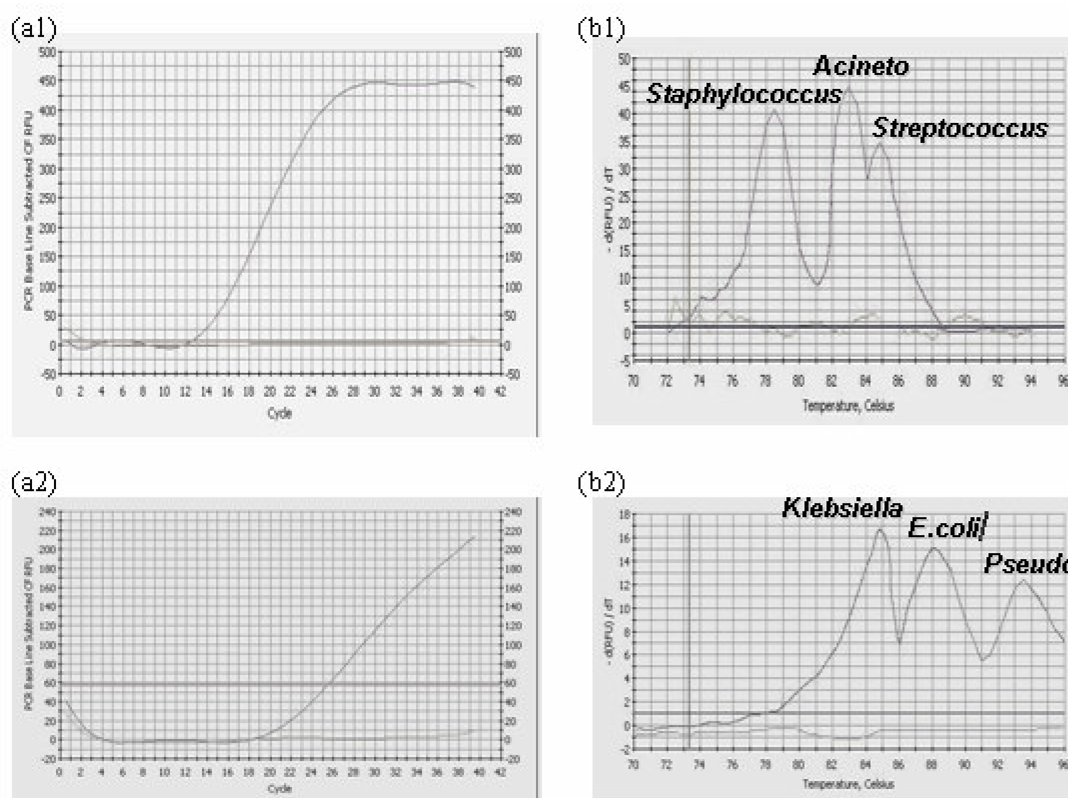


Figure 4. Amplification curves and melt peaks for the selected genes of the 6 nosocomial pathogens in a multiplex PCR reaction:

(a1) Amplifications from chromosomal DNA of *Staphylococcus aureus, Streptococcus pneumoniae and Acinetobacter spp.* Genes in multiplex are shown. Chromosomal DNA (10 ng each) from the six pathogens were used as templates in the SYBR Green PCR & their amplification profiles are represented.

(b1) Melting peaks of the 3 above mentioned genes in clinical pathogens.

(a2) Amplifications from chromosomal DNA of *Escherichia coli, Klebsiella pneumoniae and Pseudomonas aeroginosa* genes in multiplex are shown .Chromosomal DNA (10 ng each) from the six pathogens were used as templates in the SYBR Green PCR & their amplification profiles are represented.

(b2) Melting peaks of the 3 above mentioned genes in clinical pathogens.

Table 1. List of oligonucleotide primers used for conventional multiplex PCR and SYBR Green based real time Multiplex PCR amplification

No	Nosocomial pathogens	Target Gene	Primer pairs	Amplicon Size(bp)
1.	*S.pneumoniae*	*ply*	Forward: 5’-GAATTCCCTGTCTTTTCAAAGTC-3’	348bp
			Reverse: 5’-ATTTCTGTAACAGCTACCAACGA-3’	
2.	*S. aureus*	*sa442*	Forward: 5’-TCGGTACACGATATTCTTCAC-3’	179 bp
			Reverse: 5’- ACTCTCGTATGACCAGCTTC-3’	
3.	*P. aeruginosa*	*lasA*	Forward: 5’-AGTTGTCGCGGCGCTACTAC-3’	125 bp
			Reverse: 5’-GCTCACCTGGATCTGGTCCA-3’	
4.	*Acinetobacter spp.*	*efp*	Forward: 5’-AGCCAGGCCTTAAGGTCATG-3’	422 bp
			Reverse: 5’-GCCAGAAGTATCACCACGTA-3	
5.	*E. coli*	*uidA*	Forward: 5’-CTGGTATCAGCGCGAAGTCT-3’	556 bp
			Reverse: 5’-AGCGGGTAGATATCACACTC-3’	
6.	*K. pneumoniae*	*ntrA*	Forward: 5’-CATCTCGATCTGCTGGCCAA-3’	90 bp
			Reverse: 5’-GCGCGGATCCAGCGATTGGA-3’	

Detection of Nosocomial pathogens in clinical samples

The eubacterial PCR was performed for 300 clinical samples and 46 samples were positive (Figure 5) for the presence of bacterial DNA. To identify the bacterial isolate, multiplex real time PCR was performed on these 46 isolates out of which 43 showed amplification curves and melt peaks (Figure 6). The isolates were identified based on their melt peaks and assigned their genera. The list of organisms identified are shown in Table 2. The identification efficiency using real-time PCR is 93.47 %. This is based on the 46 isolates positive for eubacterial primers and out of this 43 were identified using the developed real time PCR assay. The other 3 isolates may be beyond the scope of identification using these primers as we selected only the six most common nosocomial pathogens. Conventional culture results were comparable with the PCR results (Table 2).

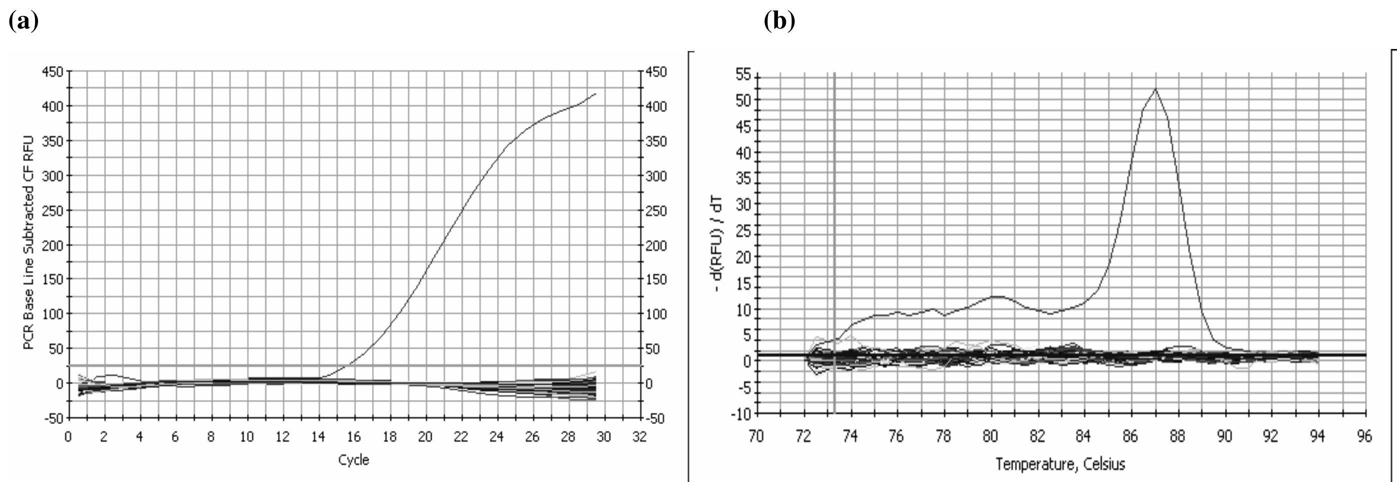


Figure 5. Amplification curve and melt peak for a representative clinical isolate using eubacterial primers:

(a) Amplification chart of the representative clinical isolate that was analysed using the eubacterial primer pairs. Isolates that were positive for this alone were subjected to further multiplex analysis for genus identification.

(b) Chart showing the melt peak of the analysed clinical samples. The melting temperature for the eubacterial primers was found to be 87ºC.

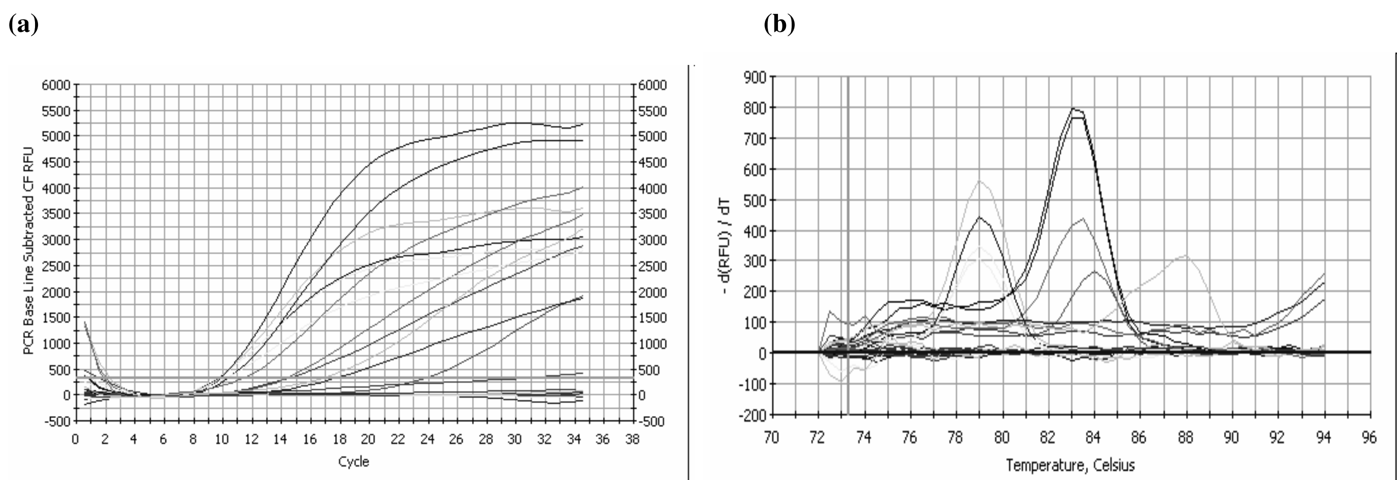


Figure 6. Amplification curves and melt peaks for the representative clinical isolates:

(a) Amplification chart of the representative clinical isolates that were analysed for their genus identification.

(b) Chart showing the melt peaks of the analysed clinical samples. *E.coli* was used as the positive control.

Table 2. List of organisms identified in the Clinical samples by multiplex real time PCR

Nº	Isolates	Organism identified by multiplex PCR (conventional and real time)	Organism identified by culture
1	18b	Staphylococcus	Staphylococcus
2	25b	Streptococcus	Streptococcus
3	27b	Staphylococcus	Staphylococcus
4	37b	Staphylococcus	Staphylococcus
5	45b	Pseudomonas	Pseudomonas
6	75b	Acinetobacter	Acinetobacter
7	83b	Pseudomonas	Pseudomonas
8	89b	Pseudomonas	Pseudomonas
9	93b	Acinetobacter	Acinetobacter
10	100b	Staphylococcus	Staphylococcus
11	108b	Acinetobacter	Acinetobacter
12	117b	Staphylococcus	Staphylococcus
13	118b	Staphylococcus	Staphylococcus
14	119b	Streptococcus	Streptococcus
15	121a	Staphylococcus	Staphylococcus
16	121b	Staphylococcus	Staphylococcus
17	122a	Pseudomonas	Pseudomonas
18	122b	Pseudomonas	Pseudomonas
19	126b	Acinetobacter	Acinetobacter
20	129b	Pseudomonas	Pseudomonas
21	133b	Streptococcus	Streptococcus
22	137b	Negative	Negative
23	141b	Pseudomonas	Pseudomonas
24	142b	Staphylococcus	Staphylococcus
25	144b	Pseudomonas	Pseudomonas
26	145b	Pseudomonas	Pseudomonas
27	147b	Pseudomonas	Pseudomonas
28	149b	Acinetobacter	Acinetobacter
29	150b	Staphylococcus	Staphylococcus
30	151b	Staphylococcus	Staphylococcus
31	153a	Staphylococcus	Staphylococcus
32	153b	Staphylococcus	Staphylococcus
33	154a	Streptococcus	Streptococcus
34	154b	Streptococcus	Streptococcus
35	155a	Pseudomonas	Pseudomonas
36	155b	Pseudomonas	Pseudomonas
37	156b	Staphylococcus	Staphylococcus
38	157a	Pseudomonas	Pseudomonas
39	157b	Pseudomonas	Pseudomonas
40	161b	Staphylococcus	Staphylococcus
41	169b	Staphylococcus	Staphylococcus
42	175b	Acinetobacter	Acinetobacter
43	177b	Negative	Negative
44	214b	Negative	Negative
45	217b	Staphylococcus	Staphylococcus
46	221b	Streptococcus	Streptococcus

DISCUSSION

Multiplex PCR has been used successfully for rapid detection, with high specificity and sensitivity of various pathogenic bacteria from environmental waters (3) and various food products (4, 29, 13, 21, 25, 11, 8). Although the classical microbiological techniques currently in use for nosocomial pathogen detection and identification are satisfactory in most situations and remain necessary for drug susceptibility testing, more rapid tests may be useful in some specific situations. These include diagnosis in critically ill patients such as patients with burns, patients at risk for nosocomial pneumonia and patients at risk for chronic infections with *Pseudomonas aeruginosa*, for whom early diagnosis of initial colonization could be essential. There is previous report on the real time PCR for the detection of *Pseudomonas aeruginosa* (27). Eventhough this is a real time PCR, it is not a multiplex reaction. Conventional multiplex PCR has been employed to detect the presence of *Escherichia coli, Pseudomonas aeruginosa, and Salmonella spp.,* directly in waters (14). The conventional PCR is time consuming and only three common pathogens are detected.

In our study, multiplex PCR was used to detect nosocomial pathogens from clinical samples. Selection of appropriate target genes, oligonucleotide primers, PCR reaction and cycling parameters resulted in the simultaneous amplification of these target genes in two reaction tubes with high sensitivity and specificity of detection. The specificity of the multiplex PCR assay was determined with 300 clinical samples and the results were that the multiplex PCR yielded a detectable DNA fragment of expected molecular weight only in the presence of their respective DNA template and gave negative results when tested with other bacteria. The sensitivity of the multiplex PCR assay with purified DNA was shown to be 1 ng for all the organisms which is considerably good. The reliability of the test is comparable to existing culture based techniques. The major benefit of this study was the development of a PCR detection method for more than one bacterial species in the same reaction vessel.

In addition to its use in clinical diagnostics, the multiplex assay may be of value for the detection of these bacterial strains in the environmental samples. The multiplex feature of this assay is optional; if so preferred it can be used as singleplex assay as shown here. This makes the assay adaptable to circumstances that may not require the simultaneous detection of all the six organisms for a diagnostic decision. We have proved that it can detect the organisms with high sensitivity and specificity as we have compared the PCR results with conventional culture techniques and have shown it as a reliable method of accurately identifying the pathogens. It can be used as an alternative method for the routine microbiological analysis and the treatment can be fastened as this assay has only a short turnaround time. The result obtained is the confirmatory result and this does not require the unique expertise involved in morphology and biochemical characteristics-based tests and can be performed in any laboratory with adequate infrastructure for real-time PCR testing. Further studies can be done to design new primers specific for a wider range of pathogens whose early detection is beneficial for prognosis.

ACKNOWLEDGEMENTS

This work was supported by e-Science grant No 12-02-03-2004 and 12-02-03-2058 and carried out at the Dept of Medical Microbiology, Faculty of Medicine, University Malaya.

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