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Rapid Detection of Bacterial Pathogens in Clinical Body Fluids by Nested PCR

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Abstract: Nested PCR method was used in the present study for detection of pathogenic bacteria in clinical body fluids. This method permitted a reliable amplification of 16S rRNA of common bacterial pathogens in body fluids with 1 CFU mL^{-1} sensitivity threshold in detection. A total of 36 clinical fluid specimens (9 peritoneal, 9 synovial, 9 pleural and 9 cerebrospinal fluids) were analyzed. Bacterial pathogens in 26 specimens were successfully detected by culture-based method and by nested PCR method. Whereas, DNA extracts from seven fluid specimens which showed no bacterial growth on conventional media were positively amplified by nested PCR. Thus, nested PCR method proved to be more rapid and more sensitive than culturing method in detection of bacterial pathogens, including fastidious and low levels of bacteria, in clinical body fluids. In conclusion, nested PCR method is worth considering in detection protocols of bacterial pathogens in clinical body fluids.

Key words: Bacterial pathogens, clinical body fluids, CSF, nested PCR, 16S rRNA

INTRODUCTION

Rapid detection of the low levels bacterial pathogens in clinical fluid specimens is necessary for successful treatment of patients with bacterial infections. There are several conventional methods have been used for detection of bacterial pathogens in clinical samples of body fluids. The conventional methods depend on culturing of clinical specimens for detection of bacterial pathogens. However, this method of detection is laborious, time-consuming, not always enough to detect very small amounts of bacterial pathogens occurring in fluids or due to antibiotic treatment (Trampuz *et al*., 2003) and may prolong diagnosis of the causative agent of disease and treatment of the patient. Consequently, there is a need to develop and perform a rapid method to improve the routine procedure of detecting bacterial pathogens with low colony-forming units (CFU) in clinical specimens, in particular clinical specimens of body fluids. Polymerase Chain Reaction (PCR) is widely accepted as a rapid and sensitive method in detection and identification of bacteria (Jimenez *et al*., 1999). Nested PCR method would provide a rapid approach and highly specific detection of bacterial pathogens with low CFU in few hours (Jimenez *et al*., 2000). In general, this method employs two PCR reactions using amplification a target region of DNA with an outer primer pair in an initial reaction, followed by a second amplification using an internal primer pair (Apfalter *et al*., 2002). It is useful for detection of pathogenic bacteria with low CFU in clinical specimens because of its enhanced sensitivity over a single amplification (Apfalter *et al*., 2002). Nested PCR has also been used for the detection of bacteria, especially if bacteria are difficult to isolate by conventional methods, by amplification of the 16S rRNA genes or the 16S-23S rRNA gene interspacer region (Patel, 2001; Kiratisin *et al*., 2003; Sato *et al*., 2003; Chen *et al*., 2004). Moreover, nested PCR provides multiple overlapping amplicons for accurate sequencing of 16S and 23S rRNA genes (Fischer *et al*., 2004).

The aim of the current study was to evaluate the efficiency and sensitivity of nested PCR method, compared with culture method, in detection of bacterial pathogens in clinical body fluids.

MATERIALS AND METHODS

Collection of clinical fluid specimens: Thirty six clinical fluid specimens were collected from 36 patients suffering from possible bacterial infections in their sterile fluids; they included 9 peritoneal, 9 synovial, 9 pleural and 9 cerebrospinal fluid (CSF) specimens. Those specimens were kindly obtained from eight medical institutes (Jordan University Hospital, Jordan Hospital, Prince Hamzah Hospital, Specialty Hospital, Islamic Hospital, Al-Essra Hospital, Arab Medical Center and Medlabs Medical laboratories) in Amman, Jordan (Table 1). The developed bacterial cultures of clinical fluid specimens on several traditional media were identified by conventional methods which based on morphological, biochemical and physiological properties.

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DNA extraction from clinical fluid specimens: About 0.5 mL from each clinical fluid specimen were centrifuged at 14000 rpm for 4 min, pellets were washed four times with distilled water, then used for DNA isolation using Wizard® Genomic DNA purification kit (Promega, USA, part No. A1120) according to the manufacturer's instructions. Because the application of PCR to clinical fluid specimens has many potential pitfalls due to the susceptibility of PCR to inhibitors and contamination, phenol-chloroform purification step was repeated four times.

To evaluate the efficacy and sensitivity of nested PCR in detection of pathogenic bacteria in body fluids, eight reference strains of most common pathogenic bacteria in body fluids were chosen (*Streptococcus pneumoniae* ATCC 49619, *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 27253, *Neisseria meningitidis* ATCC 53414, *Enterococcus faecalis* ATCC 51299, *Haemophilus influenzae* ATCC 49766, *Klebsiella pneumoniae* ATCC 13883 and *Staphylococcus aureus* ATCC 25923). Genomic DNA from reference bacteria was extracted from 1 CFU mL^{-1} bacterial concentration, one colony was picked up by a sterile loop from 24 h culture and inoculated into 1 mL sterile distilled water, using Wizard® Genomic DNA purification kit.

16S rRNA amplification by nested PCR: Conservative sequences of 16S rRNA of common bacterial pathogens were amplified as previously described (Sauer *et al*., 2005) by universal oligonucleotide primer pairs: outer forward UNI_OL 5'-GTGTAGCGGTGAAATGCG-3', outer reverse UNI_OR 5'-ACGGGCGGTGTGTACAA-3', inner forward UNI_IL 5'-GGTGGAGCATGTGGTTTA-3' and inner reverse UNI_IR 5'-CCATTGTAGCACGTGTGT-3'. Amplicons with 709 and 287 bp for outer primer pair amplification and inner primer pair amplification were assumed to produce, respectively. PCR master mix but with no added DNA, were also run as negative control.

The amplification of 16S rRNA with outer primer pair (first PCR reaction) and with inner primer pair (second PCR reaction) was carried out in a DNA MyCycler (Bio-Rad, USA) for 30 reaction cycles for first and second PCR reactions. Each PCR reaction was performed according to Sauer *et al*., 2005) in a total volume of 25 μL. A 50 bp, 100 bp and 1 kb DNA ladder markers (GeneDirex Medical Co., USA) were used to estimate the approximate molecular weight of the amplified products. Generated bands were screened and digitally photographed under UV light (Alphaimager™ 2200, Alpha Innotech, USA).

Sequencing analysis: Nested PCR products (287 bp band) of the seven clinical fluid specimens (specimens number 12, 15, 21 and 29-32) with negative growth bacterial cultures were recovered from agarose gel (Sambrook *et al*., 1989). The sequences of 16S rDNA of the recovered bands were determined with an Applied Biosystems model 373A DNA sequencer by using the ABI PRISM cycle sequencing kit (Macrogen, Korea). The sequences were compared with those contained within GenBank (Benson *et al*., 1999) by using a BLAST search (Altschul *et al*., 1990) and retrieved from the database.

RESULTS

A total of 36 clinical specimens of four body fluid types (peritoneal, synovial, pleural and CSF) were investigated in the present study. It was observed that pathogenic bacteria were isolated by cultural methods from 26 specimens (Table 1). Whereas, the remaining 10 specimens (3 synovial, 2 pleural and 5 CSF) showed no any bacterial growth on culture media. The pure cultures of isolated bacteria from clinical fluid specimens were identified by conventional methods (data not shown).

The ability of nested PCR primers to amplify 16S rRNA gene sequence of most common bacterial

Table 1: Isolation and characterization of pathogenic bacteria from 36 clinical fluid specimens collected from 36 patients by conventional methods

Clinical fluid type	Specimen No.	Isolated bacteria[a]
Peritoneal fluid	1	*Enterobacter* sp.
	2	*Klebsiella pneumoniae*
	3	*Enterobacter* sp.
	4	*Pseudomonas* sp.
	5	*Klebsiella pneumoniae*
	6	*Klebsiella pneumoniae*
	7	*Escherichia coli*
	8	*Enterococcus faecalis*
	9	*Enterococcus faecalis*
Synovial fluid	10	*Staphylococcus aureus*
	11	*Klebsiella pneumoniae*
	12	-
	13	*Staphylococcus aureus*
	14	*Klebsiella pneumoniae*
	15	-
	16	*Staphylococcus aureus*
	17	-
	18	*Klebsiella pneumoniae*
Pleural fluid	19	*Streptococcus pneumoniae*
	20	*Escherichia coli*
	21	-
	22	*Streptococcus pneumoniae*
	23	*Escherichia coli*
	24	*Streptococcus pneumoniae*
	25	-
	26	*Escherichia coli*
	27	*Streptococcus pneumoniae*
Cerebrospinal fluid	28	*Streptococcus pneumoniae*
	29	-
	30	-
	31	-
	32	-
	33	*Streptococcus pneumoniae*
	34	-
	35	*Streptococcus pneumoniae*
	36	*Streptococcus pneumoniae*

[a]Bacteria were isolated from cultures of fluid specimens on conventional media and identified by cultural, morphological, physiological and biochemical properties

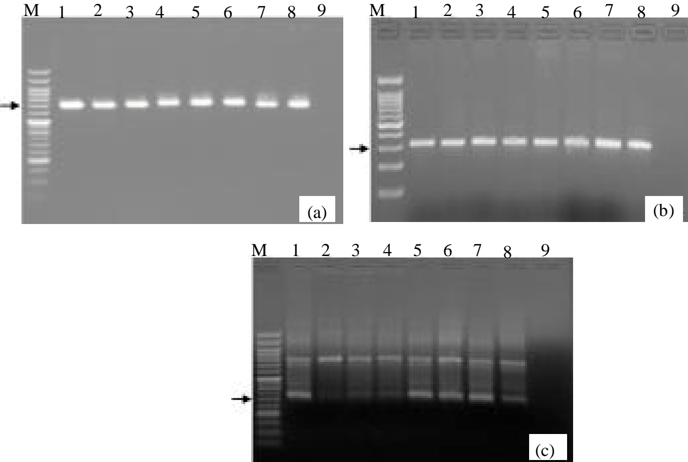

Fig. 1(a-c): Agarose gel electrophoresis of 16S rRNA amplification: (a) With outer oligonucleotide primers UNI_OL and UNI_OR (50 bp DNA ladder, arrow indicated band is 700 bp in size), (b) With direct inner oligonucleotide primers UNI_IL and UNI_IR (100 bp DNA ladder, arrow indicated band is 300 bp in size) and (c) With nested (second PCR reaction) inner oligonucleotide primers (50 bp DNA ladder, arrow indicated band is 300 bp in size) of reference strains. Lanes 1-8: reference strains *Streptococcus pneumoniae* ATCC 49619, *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 27253, *Neisseria meningitidis* ATCC 53414, *Enterococcus faecalis* ATCC 51299, *Haemophilus influenzae* ATCC 49766, *Klebsiella pneumoniae* ATCC 13883 and *Staphylococcus aureus* ATCC 25923, respectively. Lane 9: Negative control composed from PCR master mix without DNA

Table 2: The comparison of the 16S rDNA sequences obtained from the seven clinical fluid specimens, which showed no bacterial growth on culture media, with the 16S rDNA sequences in the GenBank

	Sequence		
Specimen No.[a]	No. of nucleotides[b]	% identity[c]	Closest phylogenetic relative[d]
12	281	99	*Haemophilus influenzae* M10030 (AY613585)
15	277	100	*Staphylococcus aureus* MSHR1132 (FR821777)
21	269	99	*Streptococcus pneumoniae* D39 (CP000410)
29	287	99	*Streptococcus pneumoniae* R6 (AE007317)
30	283	100	*Streptococcus pneumoniae* R6 AE007317)
31	274	100	*Neisseria meningitides* Z2491 (AL157959)
32	279	99	*Streptococcus pneumoniae* R6 (AE007317)

[a]No. 12 and 15 are synovial fluid specimens, No. 21 is pleural fluid specimen and No. 29-32 are cerebrospinal fluid specimens, [b]The number of 16S rDNA nucleotides used for the alignment, [c]The percentage identity with the 16S rDNA sequence of the closest phylogenetic relative of bacteria, [d]The code between parenthesis is GenBank accession number

pathogens in body fluids was evaluated by preparing 1 CFU mL^{-1} concentration of eight reference bacterial strains (Fig. 1). Genomic DNA of All reference strains was successfully amplified with outer primer pair (Fig. 1a) and with inner primer pair in a single direct PCR reaction (Fig. 1b). This direct PCR amplification produced about 700 bp PCR band with outer primer pair and about 300 bp PCR band with inner primer pair. Furthermore, nested PCR amplification of 1 CFU mL^{-1} of the reference strains was found reproducible in the second PCR reaction and produced about 300 bp amplicon (Fig. 1c).

The presence of bacterial pathogens in clinical fluids was directly determined by nested PCR amplification of 16S rRNA gene sequence of bacteria (Fig. 2). The genomic DNA of the 26 specimens that showed bacterial growth on culture media was successfully amplified by nested PCR (Fig. 2). Interestingly, it was observed that genomic DNA extract of seven fluid specimens, out of 10 specimens with negative bacterial growth on culture media, was amplified by nested PCR [2 synovial specimens (lanes 12 and 15; Fig. 2a and 2c), 1 pleural specimen (lane 21; Fig. 2b and 2d) and 4 CSF specimens (lanes 29-32; Fig. 2b and 2d)]. The 16S rDNA sequences obtained from those seven specimens were found to be closely related to *Haemophilus influenzae* (specimen No. 12), *Staphylococcus aureus* (specimen No. 15), *Neisseria meningitidis* (specimen No. 31) and *Streptococcus pneumoniae* (specimens No. 21, 29, 30 and 32) with 99-100% identity (Table 2).

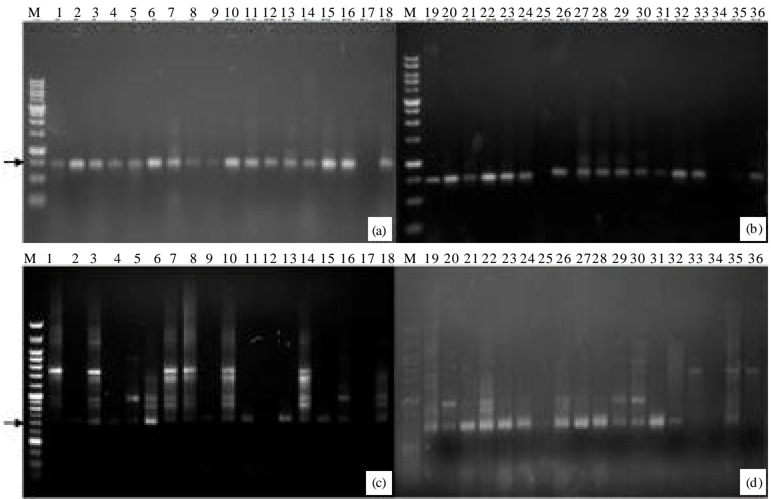

Fig. 2(a-d): Agarose gel electrophoresis of 16S rRNA amplification: (a, b) With outer oligonucleotide primers UNI_OL and UNI_OR (1 kb DNA ladder, arrow indicated band is 750 bp in size), (c, d) With nested (second PCR reaction) inner oligonucleotide primers UNI_IL and UNI_IR (50 bp DNA ladder, arrow indicated band is 300 bp in size) of pathogenic bacteria in clinical fluid specimens. Lanes 1-9: Peritoneal fluid specimens number 1 to 9, Lanes 10-18: Synovial fluid specimens number 10 to 18, Lanes 19-27: Pleural fluid specimens number 19 to 27 and Lanes 28-36: Cerebrospinal fluid specimens number 28 to 36

DISCUSSION

Screening of clinical body fluid specimens for pathogenic bacteria such as *E. coli*, *S. aureus*, *P. aeruginosa*, *N. meningitidis* and pneumonia-causing bacteria is an important and a critical step in infectious disease diagnosis and therapy. Detection of such bacterial pathogens by traditional methods is still a crucial element of the diagnostic process. However, methods such as culturing clinical specimens can be laborious and time-consuming and may prolong definitive diagnoses and treatment of the patient. Therefore, there is an urgent need for rapid and sensitive detection of pathogenic bacteria in clinical fluids. The current study presents simultaneous detection of low levels or fastidious pathogenic bacteria in four clinical fluids, including; peritoneal, synovial, pleural and CSF.

The results of this study indicated that the time required for detection of bacterial pathogens in clinical fluids is at least three times (about 8 h) less than that time required for detection of bacterial pathogens by culture methods. In comparison, nested PCR provides a simultaneous detection in a short time. This could reduce the cost of labor and media employed.

It was observed that nested PCR has the ability to amplify DNA from 1 CFU mL^{-1} most common pathogenic Gram positive and Gram negative bacteria in body fluids. Several previous studies (Radstrom *et al*., 1994; Wilkinson *et al*., 1999; Carroll *et al*., 2000) demonstrated that nested PCR assay is efficient in DNA amplification of such pathogens. Moreover, this study illustrated that nested PCR is a very sensitive method in detection of low levels (1 CFU mL^{-1}) of bacteria.

Interestingly, DNA extract from seven clinical specimens with negative bacterial cultures were successfully amplified by nested PCR (Fig. 2) and produced 16S rDNA sequences related to the most common bacterial pathogens in body fluids. This successful amplification could be related to the ability of PCR method to detect fastidious or low levels of bacteria in the specimens and to the sensitivity of nested PCR in detection of bacteria when compared with direct PCR. This finding is in agreement with Jimenez *et al*. (2000) who reported that PCR method is effective in detection of less than 10 CFU mL^{-1} of bacteria and with Sato *et al*. (2003) who showed that nested PCR in detection of bacteria was more sensitive than direct PCR. Therefore, the culture method is not as sensitive as nested PCR method in detection of low levels of bacteria in clinical fluid specimens. This is another advantage of PCR methods that is worth considering in detection protocols of bacterial pathogens.

Nested PCR amplification produced several non-specific DNA bands in the second reaction (Fig. 1c, 2c and 2d). The non-specific DNA bands produced due to carryover contamination from the first reaction to the second. This result is in agreement with Apfalter *et al.* (2002) who reported that production of non-specific bands in the second PCR reaction of nested PCR was due to contamination from the first reaction. However, nested PCR is still a valuable technique for detection of microbes such as viruses (Poggio *et al.*, 2000; Abdalla *et al.*, 2006; Gonzalez-Losa *et al.*, 2006; Lam *et al.*, 2007; Chauhan *et al.*, 2009), bacteria (Sorouri *et al.*, 2009; Giloteaux *et al.*, 2010; Sichani *et al.*, 2011; Rahimi and Doosti, 2012), fungi (Zhao *et al.*, 2001; Tsay *et al.*, 2006) and protozoa (Altay *et al.*, 2005; Fallah *et al.*, 2008; Sabry *et al.*, 2009; Hassanain *et al.*, 2011).

In conclusion, nested PCR provides a rapid method for detection of fastidious or low levels of bacterial pathogens in clinical body fluid specimens, especially CSF, because of its enhanced sensitivity over conventional methods.

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