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Rapid detection and identification of pathogens in patients with continuous ambulatory peritoneal dialysis (CAPD) associated peritonitis by 16s rRNA gene sequencing

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Abstract. Peritonitis still remains a serious complication with high rate of morbidity and mortality in patients on CAPD. Rapid and accurate identification of pathogens causing peritonitis in a CAPD patient is essential for early and optimal treatment. The aim of this study was to use 16S rRNA and ITS gene sequencing to identify common bacterial and fungal pathogens directly from the peritoneal fluid without culturing. Ninety one peritoneal fluids obtained from 91 different patients on CAPD suspected for peritonitis were investigated for etiological agents by 16S rRNA and ITS gene sequencing. Data obtained by molecular method was compared with the results obtained by culture method. Among the 45 patients confirmed for peritonitis based on international society of peritoneal dialysis (ISPD) guidelines, the etiological agents were identified in 37(82.2%) samples by culture method, while molecular method identified the etiological agents in 40(88.9%) samples. Despite the high potential application of the 16S rRNA and ITS gene sequencing in comparison to culture method to detect the vast majority of etiological agents directly from peritoneal fluids; it could not be used as a standalone test as it lacks sensitivity to identify some bacterial species due to high genetic similarity in some cases and inadequate database in Gene Bank. However, it could be used as a supplementary test to the culture method especially in the diagnosis of culture negative peritonitis.

INTRODUCTION

Continuous ambulatory peritoneal dialysis (CAPD) is a common, safe and cost effective method to dialyze metabolic waste in patients with end stage renal failure. Peritonitis is the most frequent complication of CAPD, leading to increased morbidity and mortality in these patients (Troidle *et al.*, 2003). Although bacteria, virus and fungi could be the etiological agents for peritonitis, bacterial peritonitis is most common in patients on CAPD. The rapid and accurate identification of pathogens causing peritonitis in a CAPD patient is crucial for early and optimal treatment of the patient. In routine procedure, different culture methods such as concentration, white blood cell lysis and BAC/TEC alert system are used in the clinical microbiology laboratories for the identification of the etiological agents of peritonitis; however this method takes 2 to 3 days to obtain the final identification (Kim *et al.*, 2012). Apart from longer hours, identification through culture method is a challenge when bacterial numbers are small, unculturable or fastidious and from patients who have recently received antibiotic therapy (Rantakokko & Jalava, 2002; Yoo *et al.*, 2006).

16S rRNA gene sequencing is a well established tool for rapid and accurate identification of bacterial species. 16S rRNA gene which is highly conserved between

different species of bacteria have hyper variable regions that provide species specific signature sequence useful for bacterial identification, moreover 16S rRNA gene is not prone to mutation (Patel, 2001). The 16S rRNA gene sequence of almost all common bacterial pathogens found in body fluids are available in the gene bank (Clarridge, 2004; Reller *et al.*, 2007). The availability of several commercial kits for DNA extraction directly from biological samples improves the application of molecular technique to detect and identify the bacterial species directly from peritoneal fluid faster than the routine culture method.

Therefore in this study, we aimed to apply 16S rRNA and ITS gene sequencing to identify the etiological agents directly from the peritoneal fluid collected from patients suspected for peritonitis.

MATERIAL AND METHOD

Ninety one peritoneal fluids obtained from 91 patients suspected for peritonitis admitted to Serdang Hospital, Malaysia from June 2011 to May 2012. A patient is suspected for peritonitis when any two of the following three criteria recommended by international society of peritoneal dialysis (ISPD) guideline is seen: (1) symptoms and signs of peritonitis such as fever and abdominal pain; (2) cloudy dialysate fluid with white blood cells more than 100/mm^3 with more than 50% neutrophils; and (3) detection of the pathogens in the peritoneal fluid (Piraino *et al.*, 2005).

In the concentration process, a large volume (30 ml) of peritoneal fluid was centrifuged at 3000 RPM for 15 min. Then a loopful of sediment was plated onto 5% sheep blood agar in aerobic and anaerobic condition, chocolate agar (in CO_2 jar), MacConkey agar and Sabouraud's dextrose agar. The cultured plates were incubated at 37ºC for 48 hours and the isolated colonies were identified by standard bacterial identification techniques.

All samples were investigated for bacteria peritonitis by 16S rRNA gene sequencing using the universal primers described by Lu *et al.* (2000) and for fungal peritonitis by using internal transcribed spacer (ITS1 & ITS4) region primers described by Gardes & Bruns (1993) (Table 1). The following bacteria and fungi were used as controls: *Staphylococcus aureus* ATCC 25923, *Staphylococcus epidermidis* ATCC 12228, *Escherichia coli* ATCC 25922, *Klebsiella pneumoniae* ATCC 13883, *Pseudomonas aeruginosa* ATCC 27853, *Mycobacterium tuberculosis* ATCC 25618 and *Candida albicans* ATCC 10231. Briefly 10 ml of peritoneal fluid collected from CAPD patients suspected for peritonitis was centrifuged at 3000 RPM for 15 min. The pellet (200 µl) was used as a template for DNA extraction using the QIAamp DNA Mini kit (QIAGEN, Germany) according to the manufacturer's instruction. The PCR cocktail for amplification was prepared in a final volume of 50 µl containing 10 µl of master mix (iDNA Biotechnology Sdn. Bhd, Malaysia), 0.2 µl of U1 and 0.2 µl of U2 primer, 38.6 µl of distill water and 1 µl of DNA template. The PCR cycling condition was as follows: 10 min initial preincubation at 94ºC, followed by 35 cycles consisting of denaturation at 94ºC for 1 min, annealing at 55ºC for 1 min, and extension at 72ºC for 2 min, with a final extension at 72ºC for 10 min. The amplified products were purified by using Gel/PCR DNA fragments purification

Table 1. PCR primers and cycling parameters for16S and 5.8S rRNA gene amplification

Gene	Primer/Sequence	PCR condition	Expected amplicon size	Reference
U1 U2	52-CCAGCAGCCGCGGTAATACG-32 52-ATCGG(C/T)TACCTTGTTACGACTTC-32	Cycling of 1 min 94°C, 1 min 55°C, 2 min 72°C	960bp	(Lu *et al.*, 2000)
ITS1 ITS4	5'-TCCGTAGGTGAACCTGCGG-3' 5'-TCCTCCGCTTATTGATATGC-3'	Cycling of 1 min 94°C, 1 min 55°C, 2 min 72°C	600bp	(Gardes & Bruns, 1993)

kit (Geneaid Sdn Bhd, Malaysia) and sequenced commercially (1ST Base). The sequences were analyzed through the NCBI Blast search to identify the organisms. Identification of genus and species was presumed with high score similarity and sequence identities based on CLSI recommendation guidelines using DNA target for identification of bacteria and fungi (Wayne, 2008).

RESULTS

Of the 91 patients suspected for peritonitis, 45(49.4%) patients confirmed as peritonitis based on ISPD guidelines with clinical symptoms of peritonitis such as abdominal pain and fever with cloudy peritoneal fluid and WBC>100/mm^3, while 46 (50.6%) although had clinical symptoms such as pain and fever but the peritoneal WBC counts were less than100/mm^3 and negative culture. Among the 45 patients with true characterization for peritonitis, 35 showed positive results for bacteria and 2 for fungi by the culture method (37/45). By 16S rRNA gene sequencing bacterial DNA was detected in 38 samples with a PCR product size of approximately 996 bp (Figure 1) and fungal DNA was detected in 2 samples with a PCR product size of approximately 600bp (Figure 2). Both culture method and rRNA gene sequencing showed similar identification for the 37 samples (35 for bacteria and 2 for fungi) which include *Staphylococcus* spp. (11/37), *Streptococcus* spp. (6/37), *Enterococcus* spp. (2/37), *Micrococcus* spp. (1/37), *Escherichia* spp. (5/37), *Klebsiella* spp. (4/37), *Pseudomonas* spp. (3/37), *Acinetobacter* spp. (1/37), *Neisseria* spp. (1/37), *Burkholderia* spp. (1/37) and *Candida* spp. (2/37). Results obtained from this study showed that in this CAPD center, *Escherichia* species and *Staphylococcus* species were the most common causes for bacterial peritonitis.

The percentage of gram positive bacterial peritonitis (44.4%; 20/45) were higher than gram negative bacterial peritonitis (40%; 18/45) and fungal peritonitis (4.4%; 2/45). An increase in the absolute lymphocyte count as an indicator for viral infection was not seen in any of the remaining 5 samples. In this study, the presence of culture negative peritonitis was 11.1% (5/45) which is much lower than the recommended 20% by international society peritoneal dialysis (ISPD) guidelines (Table 2).

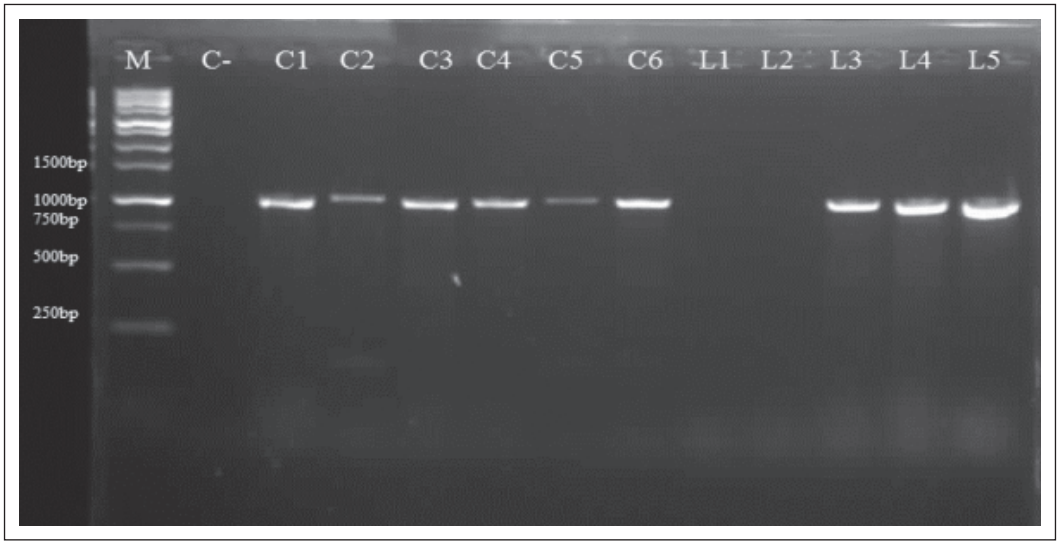

Figure 1. Lane M: 1 Kb DNA Ladder. C: control negative. C1,C2,C3,C4,C5,C6: ATCC control strain (*Staphylococcus aureus* ATCC 25923, *Staphylococcus epidermidis* ATCC 12228, *Escherichia coli* ATCC 25922, *Klebsiella pneumoniae* ATCC 13883, *Pseudomonas aeruginosa* ATCC 27853 and *Mycobacterium tuberculosis* ATCC 25618). L1 and L2 were negative samples. L3, L4 and L5 were positive samples

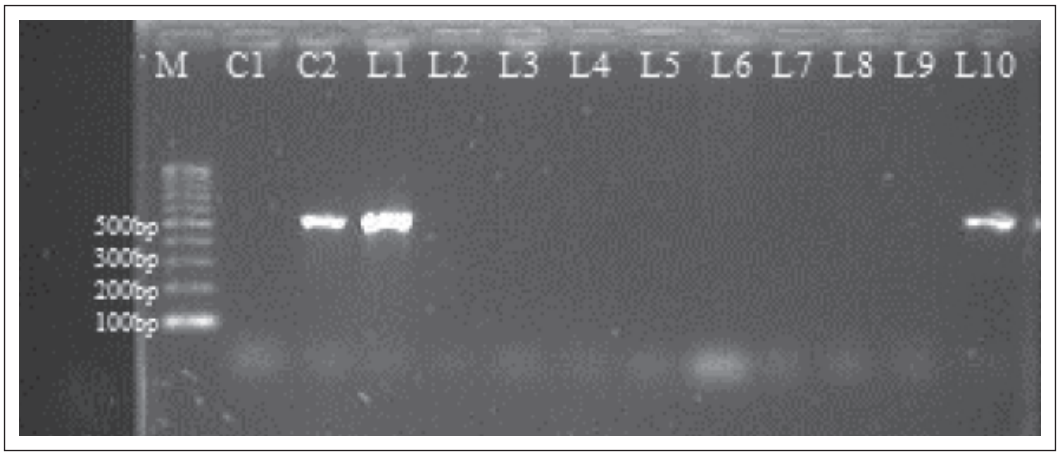

Figure 2. Lane M: 100 bp DNA Ladder. C1: Control negative. C2 ATCC control strain (*Candida albicans* ATCC 10231). L1 and L10 positive samples. L2, L3, L4, L5, L6, L7, L8, L9 negative samples

Table 2. Genus and species identification and unidentifiable by 16S rRNA typing compared to culturing result in peritoneal fluids

Organism	Species identification by culturing method	N	Species identification, by 16S rRNA gene sequencing	N
Staphylococcus spp.	*Staphylococcus aureus*	7	*Staphylococcus aureus*	7
	Staphylococcus epidermidis	4	*Staphylococcus epidermidis*	4
Streptococcus spp.	*Streptococcus viridans*	3	*Streptococcus viridans*	3
	Streptococcus salivarius	2	*Streptococcus salivarius / Streptococcus vestibularis*	2
	Streptococcus bovis	1	*Streptococcus bovis*	1
Enterococcus spp.	*Enterococcus faecium*	1	*Enterococcus faecium / Enterococcus ratti*	1
	Enterococcus faecalis	1	*Enterococcus faecalis / Enterococcus hirae*	1
Micrococcus spp.	*Micrococcus luteus*	1	*Micrococcus luteus*	1
Escherichia / Shigella spp.	*Escherichia coli*	5	*Escherichia fergusomii / Escherichia coli / Shigella flexneri*	7
Klebsiella spp.	*Klebsiella pneumoniae*	4	*Klebsiella pneumoniae*	4
Pseudomonas spp.	*Pseudomonas aeruginosa*	3	*Pseudomonas aeruginosa*	3
Acinetobacter spp.	*Acinetobacter baumannii*	1	*Acinetobacter baumannii*	1
Neisseria spp.	*Neisseria* spp.	1	*Neisseria elongata / Neisseria subflava*	1
Burkholderia spp.	*Burkholderia cepacia*	1	*Burkholderia cepacia / Burkholderia gladioli*	1
Citrobacter spp.	————————	0	*Citrobacter freundii*	1
Candida spp.	*Candida parapsilosis*	1	Candida parapsilosis	1
	Candida tropicalis	1	*Candida tropicalis*	1
Total		**37**		**40**

DISCUSSION

In the current study, 16S rRNA gene sequencing provides genus identification >82.5% in all positive cases and species identified in 67.5% cases. Mignard & Flandrois (2006) and Drancourt *et al.* (2000) showed that 16S rRNA gene sequencing provided genus identification >90% in most cases but less in species identification (65 to 83%) and between 1 to 14% unidentified isolates, which was almost similar to the results obtained in this study (Drancourt *et al.*, 2000; Mignard & Flandrois, 2006). However a distinctive result was observed in three samples by 16S rRNA gene sequencing (culture-negative but PCR-positive). Among 3 new samples identified by 16S rRNA gene sequencing, 2 showed >99% similarity for *Escherichia fergusonii* and *E. coli* while the third sample showed *Citrobacter freundii*. Clinical information from these three CAPD patients showed, they had antibiotic therapy prior to hospital admission. It was clear that 16S rRNA typing is a useful and rapid method to identify bacterial pathogen in culture negative samples and also in CAPD patients especially who have recently received antibiotic therapy. The greatest fallback of the 16S rRNA gene sequencing was it did not clearly distinguish the number of the genetically closely related organisms in some cases such as: *Streptococcus salivarius* or *Streptococcus vestibularis*, *E. fergusonii/ E. coli* or *Shigella flexneri*, *Burkholderia cepacia* or *Burkholderia gladioli* and *Enterococcus faecium* or *Enterococcus ratti* (Table 2). As seen in the present study, similar results were also observed by Kim *et al.* (2012) where, by culture method the organism was identified as *E. coli* but by 16S rDNA it showed homology toward *E. fergusomii*, *E. coli* and *S. flexneri*.

In conclusion, despite some limitations of 16S rRNA gene sequencing for bacterial identification such as high genetic similarity in some strains and inadequate database in Gene Bank, 16S rRNA gene sequencing could be used for direct detection of bacterial pathogen from peritoneal fluid as a supplementary test to the routine culture methods for the diagnosis of bacterial peritonitis in CAPD patients.

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