ISSN: 0975 -8542

Journal of Global Pharma Technology

Available Online at: www.jgpt.co.in

RESEARCH ARTICLE

Molecular Identification of Bacterial Infections in Children with Intermittent Peritoneal Dialysis

Rafeef Yousif Rasheed*, **Azhar A.f. Al Attraqchi**, **Shatha Hussin Ali**

College of Medicine, Alnahrain University, Baghdad, Iraq.

***Corresponding Author Email:** *rafoof_2006 @yahoo.com*

Abstract

Background: Bacteria is a major cause of peritonitis infections and it is very important to have a reliable test to detect these bacteria in peritoneal fluid by molecular methods. Aims of the study: Detection of the most common bacteria in peritoneal fluid from pediatric patients with acute and chronic renal failure who undergo peritoneal dialysis, through molecular diagnostic methods. Methods: One hundred clinical peritoneal fluid isolates were submitted to molecular methods (Polymerase chain reaction (PCR) and DNA sequencing) for detection. Results: By molecular methods according to PCR technique, the following bacteria were identified: *S. aureus* which was seen in 17 (17.0%) of samples, *S. epidermids* which was identified10 (10.0%) and Pseudomonas spp. which was identified 10 (10.0%) and according to DNA sequencing technique, the following bacteria were identified: *S. aureus*10(10.0%) of samples, *S. epidermids* 16 (16.0%), *E.coli* 6 (6.0%),Bacillus spp. 8 (8.0%). *Streptoccous* and *Micrococcus*1 (1.0 %) for each one. Conclusion: The most common bacterial were the best. pathogene responsible for peritonitis were *S. aureus*, *Psudomonas*spp., *S. epidermids,* and *E. coli*.

Keywords: *Peritonitis, Molecular detection of bacterial peritonitis, Peritoneal dialysis infections.*

Introduction

Kidney failure is a medical condition in which the kidneys fail to adequately filter waste products from the blood. The two main forms are acute kidney injury, which is often reversible with adequate treatment, or chronic kidney disease, which is often not reversible. Peritonitis represents one of the most important complications of peritoneal dialysis (PD) [1].

Patients in established renal failure are at high risk of developing infection due to decreased immune defenses and because that dialysis techniques increase the potential of microbial contamination. The incidence of peritonitis has markedly decreased since the lates 1980s, but the infection remains a significant complication of chronic PD [2].

Gram-positive cocci such as *Staphylococcus epidermidis* (*S. epidermidis*) and *Staphylococcus aureus* (*S. aureus*)are the most frequent causative agents of PD-associated peritonitis [3]. Gram-negative bacilli are the major cause of spontaneous bacterial peritonitis (SBP) [4].

The most incidence bacteria in ascetic fluid cultures, included *E. coli* (46%), Streptococcus (30%) and Klebsiella (9%) [5]. Gram-positive cocci have generally accounted for less than 25% of spontaneous bacterial peritonitis (SBP) [6].*Staphylococcus aureus* accounts for a smaller proportion of peritonitis episodes (12%-20%) but is overrepresented in the more severe forms of peritonitis leading to hospitalization and catheter removal [7].

Culture method, remains the gold standard for diagnosis of bacterial typically becomes positive 24-72 hours, after sampling [8], but the development of rapid diagnostic methods has been identified as an important medical need to supplement conventional culture diagnostics and molecular techniques have potential to fulfill this need. Nucleic acid based diagnostic systems, including polymerase chain reaction (PCR) and gene Sequencing methods as well as the application of DNA is well known sensitive techniques for a more rapid detection and the specific identification of pathogens [9].

Materials and Methods

Preparation of samples

One hundred pediatric patients suffering from acute and chronic kidney disease were enrolled in this study; the samples used in this study were peritoneal fluid. Patients were admitted to Children Welfare Teaching Hospital and Central Teaching Hospital for Children. Samples were collected during the period from May 2016 to February 2017. Patients' age range was between 8 days and 14 years.

The preparation of samples and DNA extraction and PCR done in medical microbiology laboratories of College of Medicine, Al-nahrain University. Ten ml of each peritoneal fluid sample was collected at third day of dialysis.

Molecular Method for the Diagnosis of Bacteria

DNA Extraction

DNA was extracted from each peritoneal fluid sample using a freezing-thawing technique [10] for cell lysis, and the QIA amp® DNA Mini Kit protocol for Gram-positive bacteria beginning with the RNase treatment step.

Oligonucleotide Primers

The sequence of oligonucleotide primers (16S rRNA gene(universal gen) and Gap gene)that were used in conventional PCR to detect the presence of *Staphylococcus aureus* [11] and Exo A gene for *Pseudomonas aeruginosa* according to [12] The primers were Synthesized in Bioneer® (South Korea).Table(1).

Table 1: Primers sequences with their relevant product size for bacteria

Gen	Primer ID	Sequence 5'–3'	Annealing temperature	Product length
16S rRNA	F R	GGAATTCAAAGG AATTGACG GGGGC CGGGATC CCAGGCCCGGGAACGTATTCAC	50°C	479 bp
Gap	F R	ATG GTTTTGGTAGAATTGGTCGTTTA GACATTTCGTTATCA TACCAAGCTG	50°C	933 bp
Exo A	F R	GACAACGCCCTCAGCATCACCAGC CGCTGGCCCATTCGCTCCAGCGCT	68°C	396 bp

Polymerase Chain Reaction PCR

Procedure of PCR had done according to [13] Tables (2, 3).

Table 2: PCR reaction mixture composition used for amplification of 16S rRNA, Gap genes for Staphylococcus aureus and Pseudomonas aeruginosa(Conventional PCR)

Reagents	(Volume /µl)
Forwad Primer	1
Reverse Primer	1
DNA template	1
PCR master mix	5
(DNAse free) water	12
Total volume	20

Table 3: The PCR thermo cycler program for bacterial genes

Steps	Temperature	Time	Cycles
Initial denaturation	94°C	5 min	
Denaturation Annealing Elongation	94°c 60°c 72°c	60 sec 60 sec 70 sec	30
Final extension Hold	72°c 4°c	10min	

Electrophoresis

DNA samples were electrophoresed by horizontal agarose gel electrophoresis according to [14].

Gene Sequencing

PCR product for 16S rRNA gene of bacteria was sent for Sanger sequencing using ABI3730XL, automated DNA sequence, by Macrogen Corporation-Korea. The results were received by email then analyzed using genius software.

Results

DNA Extraction

The final concentration of extracted DNA was ranged from 4.9 to 167.8ng/µl and purity ranged from 1.17 to 1.9.

PCR and DNA Sequencing Technique for Bacterial Identification

According to PCR technique, the following bacteria were identified: *S. aureus* which was seen in 17 (17.0%) of samples, *S. epidermids* which was identified10 (10.0%) and Psudomonas spp. which was identified10 (10.0%), as shown in Table (3), Figures (1, 2). According to DNA sequencing technique, the following bacteria were identified: *S. aureus* 10(10.0%) of samples, *S. epidermids* 16(16.0%), *E.coli* 6 (6.0%), Bacillus spp. 8 (8.0%), *Streptoccous* and *Micrococcus*1 (1.0%) for each one, as shown in Table (3).

Table 4: Bacterial isolated by PCR and Sequencing.

Technique	Bacteria	No.	%
PCR	*S.aureus*	17	17.0
	S.epidermids	10	10.0
	Psudomonas spp.	10	10.0
SEQ	*S.aureus*	10	10.0
	S.epidermids	16	16.0
	E.coli	6	6.0
	Bacillus	8	8.0
	Streptoccous	1	1.0
	Micrococcus	1	1.0

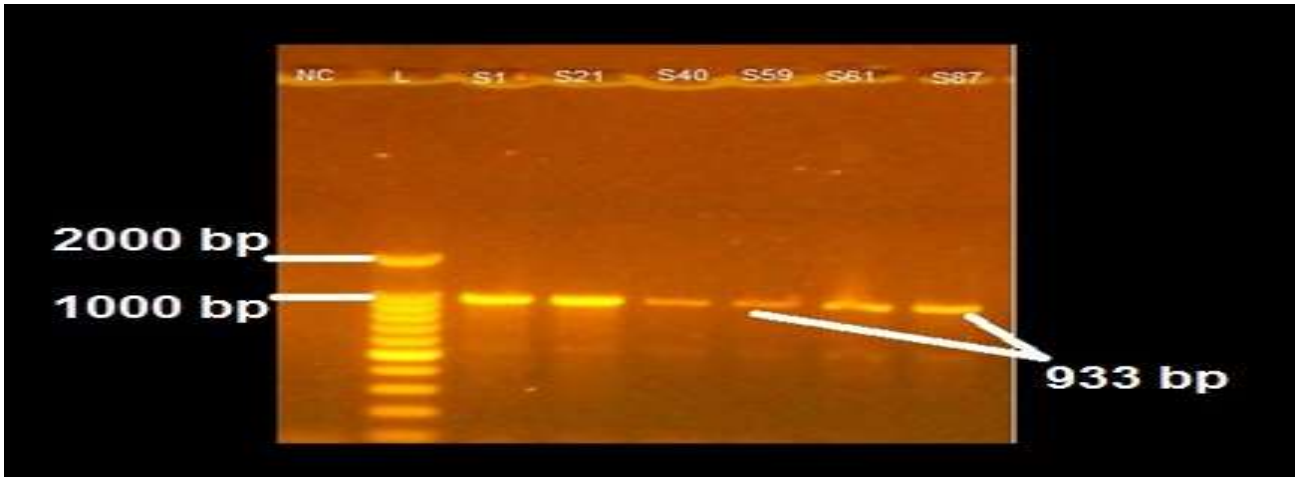

Figure 1: Gel electrophoresis of conventional PCR products of Gap gene for *S.aureus* (933 bp); negative control; MW, 2000bp ladder; (2% agarose, 5v/cm (70)1hr)

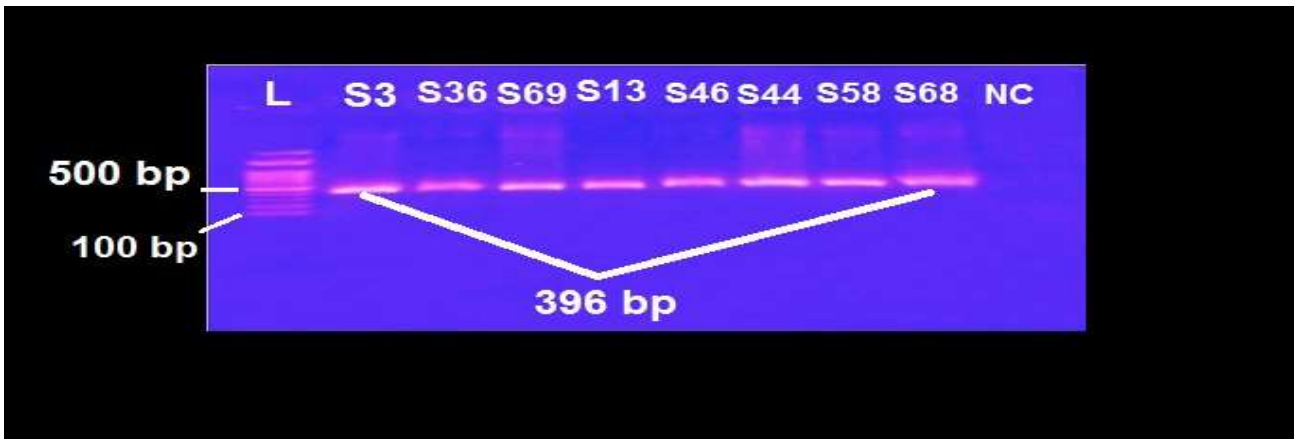

Figure 2: Gel electrophoresis of conventional PCR products of Exo A gene for Psudomonas spp.(396 bp); negative control; MW,1000bp ladder; (2% agarose,5v/cm (70)1hr

Discussion

Isolated Bacteria by PCR and DNA Sequencing Technique

PCR Technique

In the present study, and according to PCR technique, the following bacteria were diagnosed: *S. aureus* which was seen in 17(17.0 %) of samples, *S. epidermids* which was isolated from 10 (10.0%) of samples and *Psudomonas* spp. which represent 10(10.0%) of samples. In a study carried out by Chang *et al*., (2014)[15],PCR coupled with electro spray ionization mass spectrometry (PCR/ESI-MS) was compared with culture for pathogen detection in peritoneal dialysis

(PD)-related peritonitis, of 21 samples of PD effluent, PCR/ESI-MS identified microorganisms in 18 (86%) samples, including *Mycobacterium tuberculosis* in 1 culture-negative sample. Of 15 double-positive samples, PCR/ESI-MS and culture reached levels of agreement of 100% (15/15) and 87.5% (7/8) at the genus and species levels, respectively.

This means that the results of coupled (PCR/ESI-MS) incompatible to these obtained by culture method in sensitivity, but PCR alone is not sensitive enough in comparison with the gold standard method (culture). In another study, broad-range PCR (16S PCR) coupled with high-resolution melt analysis (HRMA) was compared with standard culture techniques for diagnosis of Spontaneous bacterial peritonitis (SBP) in 106 peritoneal fluid samples from patients with suspected Spontaneous bacterial peritonitis (SBP). The sensitivity and specificity for (16SrRNA) PCR for detecting eubacterial DNA compared with those of standard culture techniques were 100% (17/17) and 91.5% (85/89), respectively [16].

Some PCR-based methods for detecting bacterial DNA have also been applied to the microbiological diagnosis of spontaneous bacterial peritonitis (SBP) (Soriano *et al*., 2011 [17]. However, these methods have received several major criticisms regarding the detection of bacterial DNA. First, most previous studies enrolled a limited number of patients, and a recent report including a large number of patients showed poor results for diagnosis. Furthermore, previous studies have revealed serious concerns regarding contamination of bacterial DNA in the PCR system [18].

Commercially available Taq-polymerases may be contaminated with bacteria DNA (18). Moreover, the reagents used for DNA extraction procedures carry a risk of exposing the clinical samples to exogenous bacterial DNA [19]. Although PCR is a very sensitive method for detecting DNA, PCR-based methods display discrepant and controversial findings with respect to diagnostic performance in detecting the causative pathogen(s) in bacterial peritonitis patients [17], perhaps, or at least in part, due to the problems described above. Therefore, no definitive PCR-based method for providing an accurate diagnosis of bacterial peritonitis has been established. In addition, antibiotic therapy may reduce the Bacterial load and may also alter the bacterial target by damaging the bacterial cell wall/membrane, releasing the DNA and providing a free-circulating rather than a cell-associated DNA source [20].

If free DNA is targeted, then uncomplicated commercial nucleic acid purification systems can be used, whereas if the DNA source is cell-associated, steps to lyse the bacterial cell must be applied (White & Barnes, 2009) [20].

DNA Sequencing Technique

In the present study, according to DNA sequencing technique, the following bacteria were diagnosed: *S. aureus* as 10 (10.0 %) of samples, *S. epidermids*as 16 (16.0 %) of samples, *E.coli* as 6 (6.0%) of samples, *Bacillus* spp.8 (8.0%), *Streptoccous* spp. as 1 (1.0%) of samples and *Microccous* spp. as 1 (1.0%) of samples. These results also solidify the fact that the most common organisms associated with bacterial peritonitis are *S.aureus* and *S. epidermids* and this is similar to what is stated in most published articles (Renaud *et al*., 2011 [21]; Akoh, 2012 [22].

To identify the bacterial species, 16S rRNA gene sequencing was used. Based on that method, the most common colonizing bacterium was *S. epidermidis,* found on 5 of 11 culture-positive catheters [23]. Method, while molecular method identified the etiological agents in 40 (88.9 %) samples [24].

This higher sensitivity of DNA sequencing is controversial to current findings. 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 [24].

References

1. Johnson DW, Chang S, Excell L, Livingston B, Bannister K, McDonald SP (2007) Peritoneal dialysis. In: McDonald SP, Excell L, eds. ANZ DATA Registry Report 2006. Adelaide, South Australia: Australian and New Zealand Dialysis and Transplant Registry, 87-103.
2. Bender FH, Bernardini J, Piraino B (2006) Prevention of infectious complications in peritoneal dialysis: best demonstrated practices. (2006) Kidney Int. Suppl., 70: S44-S54.
3. Gupta S, Muralidharan S, Gokulnath H (2011) Epidemiology of culture isolates from peritoneal dialysis peritonitis patients in southern India using an automated blood culture system to culture peritoneal dialysate. Nephrology (Carlton) 16: 63-67.
4. Runyon BA, Squier S, Borzio M (1994) Translocation of gut bacteria in rats with cirrhosis to mesenteric lymph nodes partially explains the pathogenesis of spontaneous bacterial peritonitis. J. Hepatol., 21: 792-6.
5. Bhuva M, Ganger D, Jensen D (1994) Spontaneous bacterial peritonitis: an update on evaluation, management, and prevention. Am. J. Med., 97: 169-75.
6. Yakar T, Guclu M, Serin E, et al (2010) A recent evaluation of empirical cephalopsporin treatment and antibiotic resistance of changing bacterial profiles in spontaneous bacterial peritonitis. Dig. Dis. Sci., 55: 1149-54.
7. de Lourdes Ribeiro, de Souza, de Cunha M, Montelli AC, Fioravante AM, et al (2005) Predictive factors of outcome following staphylococcal peritonitis in continuous ambulatory peritoneal dialysis. Clin. Nephrol., 64:378-82.
8. Heimo W, Andreas H (2008) A multiplex real-time PCR assay for rapid detection ad differentiation of 25 bacterial and fungal pathogens from whole blood samples.Article in Medical Microbiology and Immunology. Impact Factor: 3.04 ·
9. Struelens MJ, de Mendonca R (2001) The emerging power of molecular diagnostics: towards improved management of life threatening infection. Intensive Care Med., 27(11):1696-1698.
10. Reischl U, Pulz M, Ehret W, Wolf H (1994) PCR-based detection of Mycobacteria in sputum samples using a simple and reliable DNA extraction protocol. Biotechniques, (5):844-5.
11. Moussa IM, Shibl AM (2009) Molecular characterization of methicillin-resistant Staphylococcus aureus recovered from outpatient clinics in Riyadh, Saudi Arabia. Saudi Med. J., 30 (5): 611-617.
12. GL Gray, DH Smith, JS Baldridge, RN Haskins, ML Vasil, EY Chen, HL Heyneker (1984) "Cloning, nucleotide sequence, and expression in Escherichia coli of the exotoxin A structural gene of Pseudomonas aeruginosa". Proc. Natl. Acad. Sci. USA, 81: 2645-2649.
13. Brown DJF, Edwads DI, Hawkey PM et al (2005) Guidelines for the laboratory diagnosis and susceptibilitytesting of methicillin-resistant Staphylococcus aureus(MRSA). J. Antimicrob. Chemother., 56:1000-1018.
14. Sambrook J, Russell DW (2001) Molecular Cloning: A Laboratory Manual Cold Spring Harbor. New York, USA, Cold Spring Harbor Laboratory Press, N.Y., .
15. Chang Y-T, Wang H-C, Wang M-C, et al (2014) Rapid Identification of Bacteria and Candida Pathogens in Peritoneal Dialysis Effluent from Patients with Peritoneal Dialysis-Related Peritonitis by Use of Multilocus PCR Coupled with Electrospray Ionization Mass Spectrometry. Onderdonk AB, ed. Journal of Clinical Microbiology, 52(4):1217-1219.
16. HardickJ, Won H, Jeng K, et al (2012) Identification of Bacterial Pathogens in Ascitic Fluids from Patients with Suspected Spontaneous Bacterial Peritonitis by Use of Broad-Range PCR (16S PCR) Coupled with High-Resolution Melt Analysis. Journal of Clinical Microbiology, 50(7):2428-2432.
17. Soriano G, Esparcia Ó, Montemayor M, et al (2011) Bacterial DNA in the diagnosis of spontaneous bacterial peritonitis. Alimentary Pharmacology and Therapeutics, 33(2):275-284.
18. Philipp S, Huemer HP, Irschick EU, Gassner C (2010) Obstacles of multiplex real-time PCR for bacterial 16S rDNA:

primer specifity and DNA decontamination of Taq polymerase. Transfusion Medicine and Hemotherapy, 37(1):21-28.

19. Tilburg JJHC, Nabuurs-Franssen MH, van Hannen EJ, Horrevorts AM, Melchers WJG, et al (2010) Contamination of commercial PCR master mix with DNA from Coxiellaburnetii. Journal of Clinical Microbiology, 48(12):4634-4635.
20. Barnes RA, White PL, Bygrave C, Evans N, Healy B, Kell J (2009) Clinical impact of enhanced diagnosis of invasive fungal disease in high-risk hematology and stem cell transplant patients. J. Clin. Pathol., 62: 64-69.
21. Renaud CJ, Subramanian S, Tambyah PA, Lee EJ (2011) The clinical course of rapidly growing nontuberculous mycobacterial peritoneal dialysis infections in Asians: A case series and literature review. Nephrology (Carlton), 16:174-179.
22. Akoh JA (2012) Peritoneal dialysis associated infections: An update on diagnosis and management. World Journal of Nephrology, 1(4):106-122.
23. Pihl M, Davies JR, Johansson A-C, Svensäter G (2013) Bacteria on Catheters in Patients Undergoing Peritoneal Dialysis. Peritoneal Dialysis International : Journal of the International Society for Peritoneal Dialysis, 33(1):51-59.
24. Ahmadi SH, Neela V, Hamat RA et al (2013) Rapid detection and identification of pathogens in patients with continuous ambulatory peritoneal dialysis (CAPD) associated peritonitis by 16s rRNA gene sequencing. Trop. Biomed., 30(4):602-7.

