Molecular Detecting of fungi and Bacteria in the Blood of Patients With Genital System Inflammatory Infection

Mohammad Ibrahim Khalil
Environmental college
Mosul University
mohbioecology@gmail.com

Shifaa Tayyar Jaffar
Education College of Girls
Mosul University
shifatayyar@yahoo.com

Butheina Jasim Yousif
Education College of Girls
Tikrit University
buthena600@gmail.com

Abstract

A *PCR* technique was used to detect fungi and bacteria in the blood of patients with inflammatory infection of genital system, three primer sets were used to detect *E. Coli* , *Candida* spp. and existence of other fungi The results showed infection by both microorganisms. All patients had bacteria in the blood stream while 30 % of them had a *Candida* spp. and the same percentage of other fungi species in blood.

Keywords: genital system inflammatory , Fungi and bacteria infection, *PCR* technique.

الخلاصة

أستخدمت تقنية الـ *PCR* للتحري عن الفطريات والبكتريا في دم المصابين المصاحب لالتهابات الجهاز التناسلي ، ثلاث انواع من البادئات استخدمت لتشخيص بكتريا *E.coli* والفطر *Candida* spp. وتواجد مجاميع اخرى من الفطريات واظهرت النتائج الاصابة بكلا النوعين حيث ان جميع المرضى كانو مصابين ببكتريا *E.coli* و30% منهم مصابين بالفطر *Candida* spp. و بنفس النسبة لفطريات اخرى .

الكلمات المفتاحية : التهابات الجهاز التناسلي ، الاصابات الفطرية والبكتيرية ، تقنية تفاعل سلسلة متعدد البلمرة (*PCR*).

Introduction

Conventional diagnosis of a microbial infection mainly relies on culture-based testing. These cultivations usually yield diagnostic results by consuming a long time after sampling. Furthermore, cultivation of microbial is not always successful under laboratory conditions. Such failures may occur due to unsuitable culturing conditions and methods for the microbial species investigation. Alternatively, the particular patient under investigation may have received antimicrobial therapy before sampling which can give misleading results as well as the detection of fungi still difficult even with improved automated blood cultures methods and materials (Martin *et al*., 2000). The sensitivity and specificity of diagnostic test have focused on culture independed methods, in particular nucleic acid-based methods. such as a *PCR* assays.

Numerous studies as mentioned by Lua and others (2007), have highlighted the advantages of using *PCR* technology to detect viable and nonviable fungal pathogens in a variety of clinical specimens. The majority of assays target multicopy genes in particular the ribosomal DNA(rDNA) genes(18S, 28S and 5.8S) .Recent efforts to improve molecular methods based on nucleic acid amplification and hybridization aim to circumvent these problems and hasten diagnostic procedures. In such methods, the pathogen is simultaneously detected and identified, which results in more rapid diagnoses than those obtained by conventional culturing methods and obviates the need for additional culture tests, rapid diagnostics can also reduce these of antimicrobial agents in addition to allowing a faster switch to the most optimum treatment, thus reducing both side-effects and costs (Alexander Pfaller and, 2006).

In the United states, *Candida* spp. are the fourth most common cause of blood stream infection (Pfaller and Diekema, 2007), also the *Candida* spp. can produce infection in mouth , vagina, respiratory and urinary tract (Edwards, 1994). To date most assays have been designed to detect *Candida* or *Aspergillus* species only where as there are more than 200 fungal species have been reported to cause disease in humans and companion animals, a *PCR* assay for the detection of fungal nucleic acid may be the optimal diagnostic approach because it offers the potential of being more sensitive than current culture based methods and encompassing multiple fungal genera and being applied to a variety of specimen types,also the blood culture may cause some confusion when the blood culture gives positive on 2 days when the *PCR* signal is negative but this happens in the end of infection, after the patients had received significant amount of antifungal infection therapy. Conversely, some patients who had a fungemia infection had a negative *PCR* signal on the last day that the blood culture was positive but positive *PCR* signal on the following day, (Burick *et al*.,1998)

Management of febrile neutropenia in hematological patient undergoing intensive chemotherapy is important, because the bacteria or fungi infections during prolonged neutropenia are major causes of morbidity and mortality in these patients. These infections can rapidly become life- threatening appropriately and promptly. Therapeutic decisions should ideally be made based on microbial isolation. However the sensitivity of microbial culture test remains low. Despite clinical best efforts it has been shown that specific pathogens were identified in only about 20% to 30% of febrile neutropenia cases, (Smith *et al.,* 2000).

polymerase chain reaction (*PCR*) based molecular detection of fungal DNA from blood may be promising tool for the early diagnosis of invasive fungal disease and *PCR* analysis of bacteria in blood is reported to be more sensitive than blood culture. The aim of this study is to find out the type of microbes which causes the inflammation which are not included in routine test and to test some primers in detection fungal and bacterial infection.

Materials and Methods

Blood collection:

Whole blood specimens were collected from 30 female patients at Al-Jemhoria hospital in Mosul city who suffer from inflammation in genital system, placed in vacationers contained 1.5 ml of acid citrate dextrose (ACD) anticoagulant and stored at 4 ˚C, (Barenfanger *et al.,* 1999).

DNA extraction for detection Microbial elements from whole blood:

DNA Extraction protocol:

Amount of 500 µl of blood Poured into a 1.5 ml eppendorf tube and adding 1000 µl of red cell lysis buffer,shacked gently (up to homogenizing), then spin for 2 minutes at 7000 rpm the supernatant discarded and steps repeated two or three more times to remove hemoglobin. It is important to breakdown the pellet by vortexing and rinsing it well in red blood cell lysis buffer in order to clean the white blood cells from residual of hemoglobin,the tube Placed on tissue paper for few seconds downward then 400 µl of nucleic lysis buffer,100 µl of saturated NaCl (5M) was added and mixed then 600 µl of chloroform was added to eppendorf tube and mix on a rotating blood mixer at room temperature then spin it for 2 minutes at 7000 rpm then 400 µl of supernatant was transferred to a new 1.5 ml tube. 800 µl of cold (-20°C) absolute Ethanol was added and shacked it gently then vortex . DNA should appear as

a mucus-like strand in the solution phase,the microfuge tube Spin for one minute at 12000 rpm to precipitate, then discard supernatant carefully and let tube be completely dried in room temperature (Eppendorf tube Placed downward on the tissue paper) then 50µl of TE was added to it then vortex; eppendorf tube of DNA was kept in 4°C or -20°C for later uses. One µl per *PCR* reaction routinely used without adverse effects (Iranpur and Esmailizadeh, 2010).

***PCR* primers**

Three primers sets were used in order to detect blood pathogens;

Sensitivity of 16S rRNA detection:

The universal bacterial specific primer pair P11P 5`GAG GAA GGT GGG GAT GAC GT`3and P13P 5`AGG CCC GGG AAC GTA TTC AC`3were used to amplify a portion of the 16S rRNA gene corresponding to the V6 region of the gene position 1175 to 1390 of *Escherichia coli* 16S rRNA gene generating a *PCR* product of ~216 bp (Miller *et al*., 2000).

Sensitivity of 18S rRNA detection:

The universal specific primer pair 18S (r) 5`ATT GGA GGG CAA GTC TGG TG`3and 18S (f) 5`CCG ATC CCT AGT CGG CAT AG`3 which bind with variable area of the 18S rRNA gene V7 to V9 were used to amplify a portion of gene corresponding to position 544 to 1033 in the case of *Candida albicans* gene reaction *PCR* products of ~ 500 bp(Miller *et al*., 2000).Besides, universal fungal detection primer pair were used in blood *PCR* reaction FF2 (F): 5` GGTTCTATTTTG TTG GTT TCT A `3 and FR1 (r): 5`CTC TCA ATC TGT CAA TCC TTA TT `3 (Zhou et al.,2000).

***PCR* reaction**

Amplification reactions were performed in total volume 25 µl, which included 2.5 µl of each primer (20 pmol), 2.5 µl of genomic DNA (5 µg/ml), and 12.5 µl of Master mix (Amersham Pharmacia, Piscataway, NJ, USA), and the volume completed to 25 µl by adding 5 µl of distilled water. *PCR* was performed by initial denaturation at 94°C for 4 min, followed by 35 cycles at 94°C for 2 min, 55°C for 2 min, and 72°C for 2 min, with a final extension at 72°C for 10 min (Kumar and Shukla, 2005).

Detection of amplicons

Following amplification, aliquots (15 µl) were removed from each reaction mixture and examined by electrophoresis (80 V, 45 min) in gels composed of 2% (w/v) agarose (Gibco, UK) in TAE buffer (40 mM Tris, 20 mM acetic acid, 1 mM EDTA, pH 8.3), pre-stained by ethidium bromide (5 mg/100 ml). gel were visualized under UV illumination using a gel image analysis system (UVP Products, UK) and all images archived as digital graphic files.

Results

The using of Iranpur and Esmailzadeh`s modification method gave a high purity and high concentration of DNA as well as the simplicity and cheapness.All 30 patients included in this study had a bacterial infection by showing positive (band) signal to primer pair of P11P which amplify a portion of 16S rRNA gene of *E.coli*as shown in figure (1)

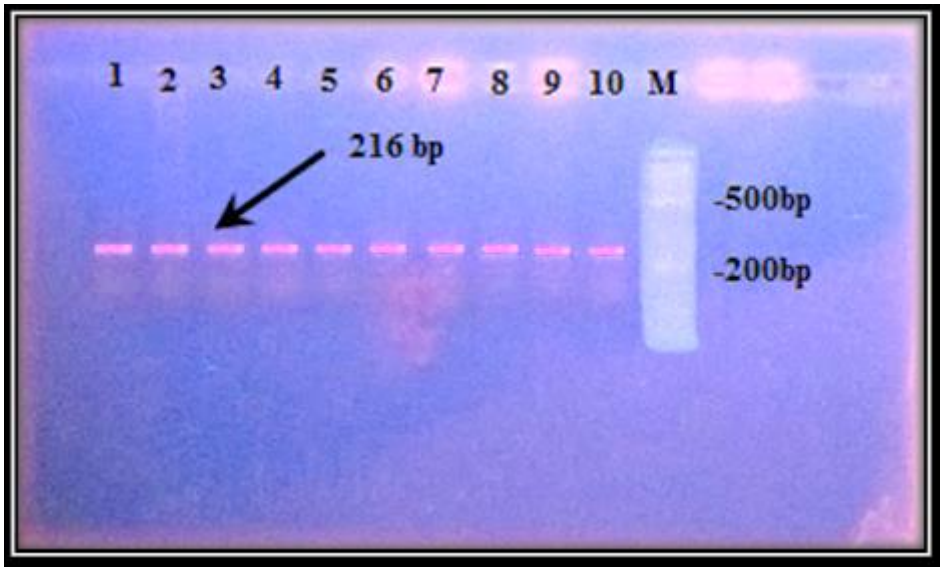

Figure (1): PCR products amplified by primers P11P/P13P from blood DNA (1-10) ,M: Molecular weight marker. the amplified PCR products were separated by 2% agaros gel electrophoresis (80 V,45

The blood DNA bands were in size 216 bp which indicated to infection with *E. coli* bacteria .

Sensitivity of 18S rRNA detection:

About 30 % of blood samples showed positive signal (bands) to 18S primer pair , which amplified a gene belonging to *Candida albicans*in size 505 bp as shown in figure (2).

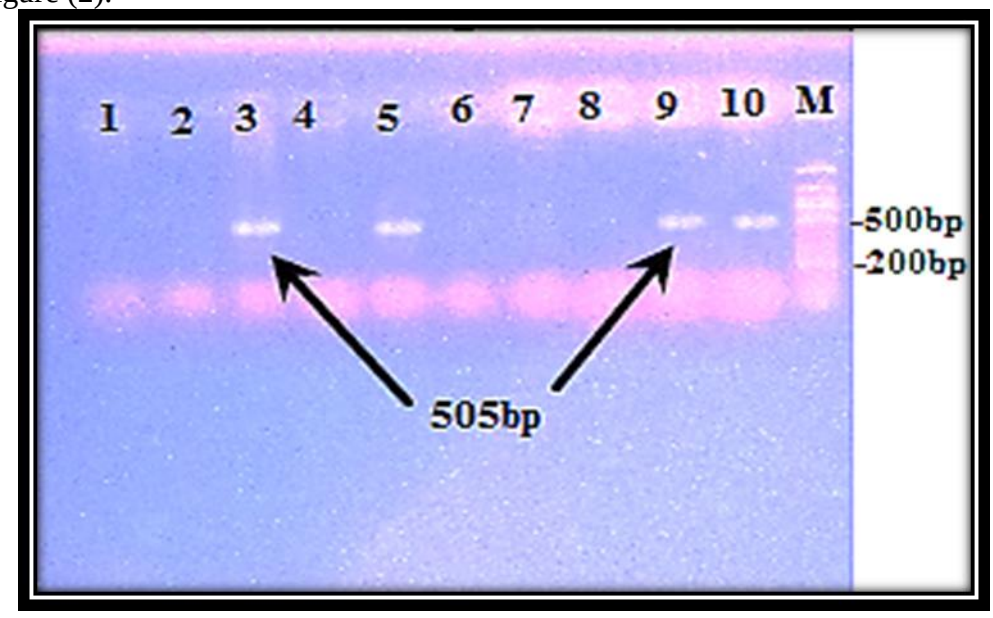

Figure (2): PCR products amplified by primers 18S from blood DNA (1-10) , M: Molecular weight marker. the amplified PCR products were separated by 2% agaros gel electrophoresis (80 V, 45

While 30 % of patients showed *PCR* products with universal fungal primer FF2 as shown in figure (3) in size 425 bp.

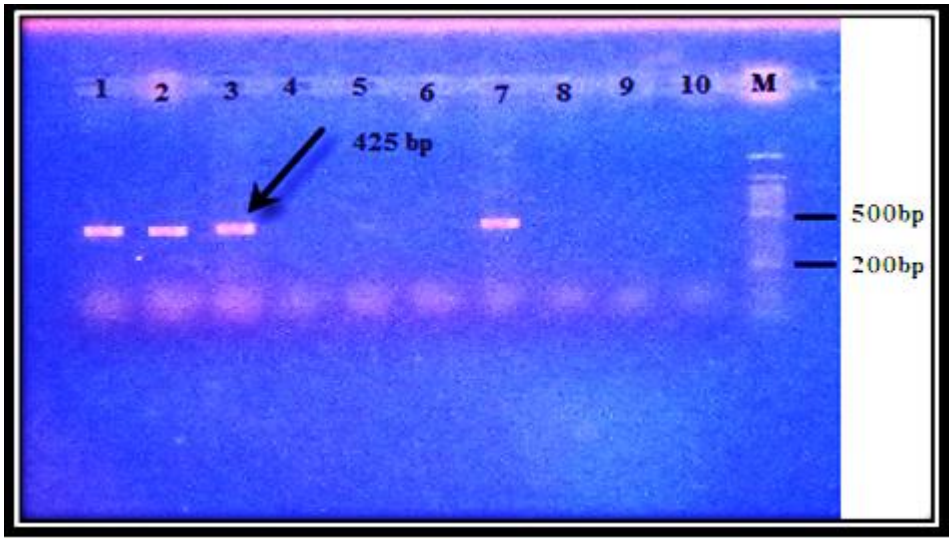

Figure (3): PCR products amplified by primers FF2 from blood DNA (1-10) , M: Molecular weight marker the amplified PCR products were separated by 2% agaros gel electrophoresis (80 V ,45

Discussion

Rapid and precise identification of pathogens(fungal and bacteria) to species level is critical to improving the fast diagnosis of diseases. The results from this study indicate that the application of pathogens *PCR* to amplify the ITS1 region of the rDNA gene cluster is a high sensitive and useful tool for the detection and identification of wide range of pathogens , while the using the Classic techniques to detection pathogens in many cases failed to diagnose these pathogens in patients who received medicine , the using PCR technique can offer complete diagnosis for the organism which cause the infection in fast way to treat this infection with suitable medicine.

The detection of *Candiad* spp. is very important , candidemia is defined by presence of *Candida* spp. isolate in blood culture occurred in 30 % of our patients. Several variables were associated with development of candidemia by multivariate analysis such as fever , dwelling in urinary catheter , parenteral nutrition, previous use of antifungal drugs and many reports either risk factors associated with death due to candidemia . systematic candidiasis is the complication affecting individuals with reduced immune function or any other type of weakening of their defense, almost any organ of the body may be involved, after beginning as an episode of candidemia, during which candida can be isolated from blood (Edwards, 1994).

Also the results show the relation between fungi and bacteria infection; chronic sinus infection is the most common caused by fungi in blood, these are usually caused by a bacteria but if antibiotics are taken to kill bacteria the fungi can take over very quickly.

These results were identical with the results which optain by Pechorsky and Others (2009) ; they made a comparison between conventional and *PCR* methods and they found in 12.5% of the cases , *PCR* afforded identification of bacteria but conventional methods showed no bacteria in the sample. Also , the use of *PCR*

methods increased the sensitivityin detecting bacterial DNA in newborns with signs of species as mentioned by Tonje and Others (2009).

Blood culture remains the corner stone in the detection of microbiological agent responsible of action , however blood culture fails to yield positive results in many instances, there is a number of factors which may be responsible for culture negative results finding in febrile patients; the causative organisms may be fastidious in nature such as *Brucella* spp., *Neiseria* spp. and cell wall deficient organisms , some organisms are cell dependent such as *Coxiella burnetii* and *Chlamydia* spp. and detection of fungi is still difficult even with improved automated blood culture method and materials (Millsretal *et al*., 2000).

The molecular detection of microbiological agents of infection is now widely used, in particular , in cases where the agent is fastidious in nature, e.g. *Chlamydia* spp. and *Mycobacteria* spp. The molecular approach would be advantageous in particular when the infectious agent is believed to be fastidious or fungal in nature or when blood culture fails to identify the causative agent or where a quick diagnosis needs to be made. The identification of the infection organisms may thus be made earlier than conventional approach and through amplification of antibiotic – resistance gene *Loci* , the most appropriate antimicrobial therapy may be initiated sooner (Moore *et al*., 1999).

The use of *PCR* techniques in patients would help support the empirical indicator for antifungal therapy , while some studies found that the rate of previous antifungal therapy was significantly higher in patients with true candidemia than in patients with *PCR* candidemia suggesting that physicians are not alert to the risk of candidemia in high risk patients (Nakamura *et al*., 2010). *PCR* analysis proved more sensitive than blood cultures for the detection of bacteria in both prospective and sporadic studies , also the pilot and sporadic study showed that bacterial *PCR* analysis was more sensitive than blood culture results. The sensitivity of blood culture might be low because the volume of blood used for the culture . Bacterial *PCR* analysis would allow the early diagnosis of the empirical therapy resistant bacteria. A *PCR* assay for detection of fungal nucleic acids may be the optimal diagnosis approach because it offers the potential of being more sensitive than current culture based methods, encompassing multiple fungal genera and being applied to a variety of specimen types (Burik *et al*., 1998). Identification of the fungal species is essential for appropriate clinical decision making concerning both the significance of a particular isolate and the dosage and duration of antifungl therapy. Culture and microbiological determination of the species of fungi from clinical material usually require up to several days. By using the oligonucleotide probe hybridization assay; DNA extraction and amplification of the *PCR* products and determination of the fungal species can be performed within 12 hours (Einsele *et al*., 1997). As showed by Sabeeh(2013) who suggested the molecular analysis of candidmia is more sensitive and less time consuming that culture and other conventional methods did, also the *PCR* results showed 100% sensitivity and 96.6% specificity and it`s rapid, easy, reliable and also applicable in clinical laboratory for identification.

In the near future, *PCR* and other nucleic acid amplification technologies will be used to screen individual whole blood donors for infectious agents, such as HIV1/2, HCV, and human T cell lymphotrophic viruses I and II. Screening pools of plasma donors with *PCR* has already become a standard practice in some European countries (Innis *et al*., 1999).

Reference

Alexander, B. and Pfaller, M.(2006).Contemporary tools for the diagnosis and management of invasive mycoses. Clin. Infect. Dis. 43: 515-527

Barenfanger J, Drake C and Kacich G. (1999). Clinical and financial benefits of rapid bacterial identification and antimicrobial susceptibility testing. J. Clin. Microbiol, 37:1415-1418.

Buric ,V.; Myerson, D.; Schreckhise, R. and Bowden, R.(1998). Panfungal *PCR* Assay for detection of fungal infection in human blood specimens, J. Clin.Microbiol., 36(5): 1169-1175.

Edwards, M. C., and Gibbs, R. A. (1994) Multiplex *PCR*: advantages, development, and applications. *PCR* Methods Appl. 3, 65–75.

Einsele, H.; Holger, H.; Gottfried, R., Jurgen, L.; Ines, R.; Fer, C.; Raleigh, A.; Joanne,V.; Dan, E.; Lothar, K.; and Ulrike, S.(1997). Detection and Identification of Fungal Pathogens in Blood by Using Molecular Probes, J. Clin. Microbiol., 35(6): 1353–1360.

Innis, Michael A.; Gelfand, David H.; Sinisky, John J.(1999). *PCR* application, Academic press, USA.

Iranpur, V. and Esmailizadeh, A. (2010) Rapid Extraction of High Quality DNA from Whole Blood Stored at 4ºC for Long Period. Department of Animal Science, Faculty of Agriculture, Shahrekord University, Shahrekord, Iran. www. Protocol-online.org.

Lau, A.; Sharon, C.; Sorrell, T.; Dee, C.; Malik, R.; Patricia, M. and Hilliday, C. (2007). Development and clinical application of a panfungal *PCR* assay to detect and identify fungal DNA in tissue speciments, , J. Clin.Microbiol., 45(2):380-385.

Martin, C., Roberts, D. and Van der Weide, M., (2000) Development of a *PCR*-based line probe assay for identification of fungal pathogens. J. Clin. Microbiol. 38: 3735–3742.

Millar, Beverley C.; Xu Jirua ; Moorea, John E.; Earleb, John A. (2000) A simple and sensitive method to extract bacterial, yeast and fungal DNA from blood culture material, J. Microbiol. Metho., 42: 139–147.

Moore, J.E., Millar, B.C., Yongmin, X., Crowe, M., (1999). A rapid molecular assay for the detection of antibiotic resistant organisms in infective endocarditis. Eur. Heart J. 20, 559.

Nakamura, A.; Sugimoto, Y.; Ohishi, K.; Sugawara, Y.; Fujieda, A.; Monma, F.; Suzuki, K.; Masuya, M.; Nakase, K.; Matsushima, Y.; Wada, H.; Katayama, N. and Nobori,T. (2010). Diagnostic value of *PCR* analysis of bacteria and fungi from blood in empiric therapy resistance febrile neutropenia, J. Clin. Microbiol., 48(6): 2030-2036.

Pechorsky, A., Nitzan, Y. and Lazarovitch, T. (2009). Identification of pathogenic bacteria in blood cultures: comparison between conventional and PCR methods, J. Microbiol. Metho., 78(3): 25-30.

Pfaller, M. and Diekema, D. (2007). Epidomiolog of invasive Candidiasis : apersistent puplic health problem. Clin. Microbiol. Rev. j. Med. 324: 1060-1062.

Sabeeh, S.; Al-attraqchi,A. and Alaswad,E. (2013). Journal Diyala of Medicin. 5(2): 29-35.

Smith,T.,O'Connor, L., Glennon,M.,and Maher ,M.(2000) . Molecular diagnostics in food safety: rapid detection of food-borne pathogens. Irish J. Agr. Res. 39: 309 – 319.

Tonje, R., Farstad, T., Nakstad, B. , lauvark, V. and Steinbakk, M. (2009). Comparison of beoad rang 16S rDNA PCR and conventional blood culture for diagnosis of species in newborn:a case control study, BMC Pediatrics, 9(5):1-8.

Zheng, X., Kolbert, C.P.,Varga-Delmore, P., Arruda, J., Lewis, M.,Kolberg, J. et al., 1999. Direct mecA detection from blood culture bottles by branched-DNA signal amplification. J. Clin.Microbiol. 37: 4192–4193.

Zhou, G. ; Whong, W.; Ong, T. and Chen,B.(2000). Development of fungus specific *PCR* assay for detecting low level fungi in an indoor environment. Molecuar and cellular Probes, 14:339-348.