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Association of *Escherichia coli* with Fever following Mitral Valve Replacement in Patients Suffering Rhumatic Mitral Valve Disease

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Abstract: Two patients (adult females) were admitted to Ahmed Gasim Medical Hospital, Khartoum North, Sudan, with history of severe combined mitral regurgitation and stenosis. The patients were referred to the thoracic surgery unit for mitral valvectomy and valve replacement. Both patients received early empirical prophylactic antibiotic therapy half an hour before as well as post operatively. However, they developed fever shortly after the operation. Blood samples were collected and submitted to the diagnostic laboratory for conventional and molecular microbiological examinations. Conventional bacteriological examination revealed that blood cultures were negative for any bacterial growth. However, the Polymerase Chain Reaction (PCR) detected a 486 bp PCR product specific for *E. coli*. The identity of the nucleic acid sequence was confirmed by nested amplification of a 186 bp PCR product from the primary PCR product. The scientific data presented in this study indicated that PCR provides a rapid method for detection of *E. coli* during bacteraemia, irrespective of their viability. However, conventional bacterial isolation methods failed to diagnose *E. coli* infection in patients receiving high doses of antibiotics.

Key words: Mitral valve, *E.coli*, bacteremia, diagnostics, PCR, sudan

INTRODUCTION

Episodes of pyrexia and sepsis are caused by microbial infections. However bacteraemia is diagnosed in only small number of blood cultures [1]. *Escherichia coli* is one of the most common microorganisms isolated during hospital-acquired bacteraemia in intensive care patients[2]. Several factors could contribute to isolate microbes from blood. Of these factors, some species of bacteria are difficult to culture[3]. In addition, certain species of bacteria are slow growers, such as Mycobacterium sp.[4,5]. Moreover, bacteraemia is often transient, thus the number of viable microorganisms circulating in the blood decreases rapidly after the onset of bacteraemia due to phagocytic and other host defense mechanisms. Further more, the majority of intensive care patients with sepsis are already under antimicrobial treatment, thus compromising the results of microbiological culture techniques[1,6,8]. It is, therefore, becoming increasingly obvious that the amplification of bacterial DNA by Polymerase Chain Reaction (PCR) would be advantageous for a variety of circumstances including the problems associated with conventional microbiological techniques[9]. The potential of PCR technology to detect intracellular or non growing microorganisms may be useful for diagnosing bacteraemia in hospitalized patients. PCR has already been successfully applied in the detection of fastidious microorganisms [9,10] and, in particular, for detecting bacteraemia [11,13]. In the present study, blood samples were collected from two critically ill patients, who developed fever shortly after miteral valvectomy and valve replacement, for detection of bacteraemia using bacterial culture methods and PCR-based detection assay.

MATERIALS AND METHODS

Conventional isolation and identification: Blood samples were grown on Mac Conkey's medium as selective and differential medium to exclude non-lactose fermentor of the family Enterobacteriaceae. Lactose is included as a fermentable carbohydrate with a pH indicator, usually neutral red. Strong acid producers like *Escherichia*, *Klebsiella* and *Enterobacter* produced red colonies. Loopful of suspension from each medium was streaked on Eosin Methylene Blue (EMB) agar, plates were incubated 18 24hrs at 37°C and examined for typical *E. coli* colonies, dark centered with or without metallic

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sheen. Two typical colonies from each (EMB) plate were picked and transferred to nutrient agar slants for morphological and biochemical tests. Slants were incubated at 37°C for 18-24 hrs. Gram stain on each culture was performed, for all cultures appearing as Gram-negative, short rods or cocci, were identified by Triple Sugar Iron (TSI) biochemical scheme, motility test, indole test, urea's test, lysine decomposition test and DNAse hydrolysis test [14]. The rest of the samples were isolated using multiple tube technique and identified as described previously [15].

Extraction of bacterial nucleic acids from blood samples: Extraction of bacterial nucleic acids from blood samples of the patients was made possible using a commercially available QIAamp tissue kit (QIAGEN Inc. Chatsworth,CA) according to the anufacturer's instructions. Briefly, (200 mL of blood, 30 µLof proteinase K stock solution and 200 mL of lysing buffer were pipetted into 1.5 mL epindorf tube and the mixture was incubated at 70° C for 10 minutes at room temperature. 200 µLof absolute ethanol was then added to the sample and mixed by vortexing. The mixture was then transferred to the QIAamp spin column and placed in a clean 2mL-collection tube and centrifuged at 8000 RPM for 1 minute at room temperature. The QIAspin column was washed twice using 500 mL of washing buffers W1 and W2, respectively by spinning for 1 minute. The QIAamp spin column was placed in a clean 1.5 mL epindorf tube and the DNA was eluted with 200 µLof double distilled water preheated at 70° C at room temperature. Maximum DNA yield was obtained by spinning at 12,000 RPM for 1 min at room temperature. The bacterial DNA concentration was determined by spectrophotometer at 260-wave length. Five microliters of the suspended nucleic acid was used in the PCR amplification.

Primers selection: A pair of oligonucleotide primers (P1 and P2) was derived from a highly conserved region of nucleotide sequences of the uidA gene of *E. coli*, encoding-glucuronidase specific for *E. coli* [16]. Primers (20mer each) Primers 1 and 2 (P1 and P2) were selected for the synthesis of specific PCR product. P1 (5'-ATC ACC GTG GTG ACG CAT GTC GC-3') included 23 bases of the positive sense strand. P2 (5'-CAC CAC GAT GCC ATG TTC ATC TGC-3') included 24 bases of the complementary strand. The PCR using primer P1 and P2 would result in a 486 bp PCR product. For nested amplification, two pairs of internal primers were designed (P3 and P4). Oligonucleotides P3 (5'-TAT GAA CTG TGC GTC ACA GCC-3') and P4 (5'-CAT CAG CAC GTT ATC GAA TCC-3') were used in the nested PCR for the amplified products from the primary PCR to amplify a 186-bp fragment. All primers were synthesized on a DNA synthesizer (Milligen/Biosearch, a division of MilliporeBurlington, MA, USA) and purified using oligo-pak oligonucleotide purification columns (Glen Research Corporation, Sterling, Virginia, USA).

Polymerase Chain Reaction (PCR): A stock buffered solution containing (250 micro liter (µL) 10X PCR buffer, 100 µLof 125 mM Mg cl_2, 12.5 µLof each dNTPs (ATP, TTP, GTP and CTP) at a concentration of 10mM) was prepared in 1.5 mL tube. The primers were used at a concentration of 20 picogram. Double distilled water was added to bring the volume of the stock buffer solution to 1.5 mL. For each PCR amplification, 2.0 µLof the target DNA and 2 µLof primers was added to 45 µLof the stock solution in PCR tubes and mixed by vortexing. 1.0 µLof Taq DNA polymerase (Perkin Elmer) was used at a concentration of 5.0 units. All PCR amplification reactions were carried out in a final volume of 50 µL. The thermal cycling profiles were as follows: a 2-min incubation at 94°C, followed by 30 cycles of 94°C for 1 min, 57°C for 30 sec and 72°C for 45 sec. A final incubation at 72°C for 10 min was carried out to ensure complete synthesis of the expected PCR products.

Thermal profiles were performed on a Techne PHC-2 thermal cycler (Techne, Princeton, NJ.). Following amplification, 10 µLfrom each PCR reaction containing amplified product were loaded onto gels of 1.0% SeaKem agarose (FMC Bioproduct, Rockland ME) and electrophoresed. The gels were stained with ethidium bromide and the specific 486 bp PCR products were identified following visualization under UV light.

Nested polymerase chain reaction: For nested PCR, 5 µLof the amplified product were added to 45 µLof the second master mixture. The master mixture for the nested PCR contained the following:

2 µL of primers P3 and P4, 42 µLof the PCR stock solution, 1.0 µL of Taq DNA polymerase (Perkin Elmer) was used at a concentration of 5.0 units. All PCR amplification reactions were carried out in a final volume of 50 µL. The nested PCR amplification was performed with an initial denaturation step at 95°C for 2 min; 35 cycles at 95° C for I min, 57° C for 30 sec and 72°C for 45 sec; and a final elongation phase at 72°C for 10 min.

The product of the nested PCR was electrophoresed with 3 µL of gel loading buffer (0.25 g of bromphenol blue and 40 g of saccharose dissolved in 100 mL of distilled water) through a 2% agarose gel at 80 V for 45min. Molecular size markers (Promega, Madison, Wis.) were run concurrently. The gel, stained with ethidium bromide

(0.5 μg mL^{-1}) was examined under UV light for the presence of a 186 bp band and photographed for documentation.

RESULTS

Conventional bacteriological techniques showed that no bacterial growth was evident from blood culture under aerobic or anaerobic condition. However, the PCR-based detection assay, using the outer pair of primers (P1 and P2), afforded specific detection of a 486 bp PCR product of *E. coli* from a blood sample of one patient (Fig. 1). The nested amplification, using primers P3 and P4, confirmed the identity of the primary PCR product and increased the sensitivity of the PCR assay. The nested 186 bp PCR products were visualized onto an ethidium-bromide stained agarose gel from blood samples of the two patients (Fig. 2). The specificity studies indicated that, the primary or nested PCR product was not amplified from blood of clinically normal individuals as shown in lane 3 of Fig. 1 and 3.

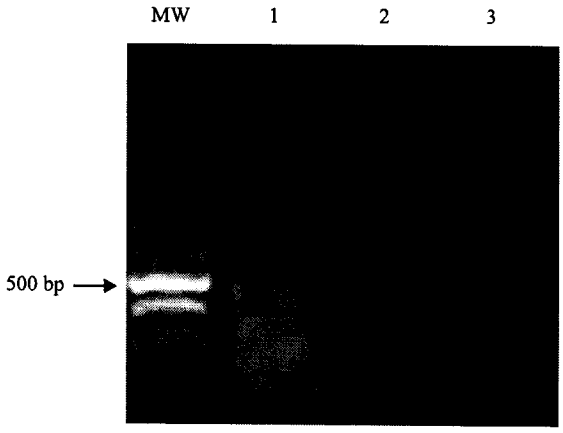

Fig. 1: Visualization of the primary *E. coli* specific 486 bp PCR Product from DNA extracted from blood of the patients. Lane MW: 100 bp Molecular weight marker, Lane 1-2: blood sample from the vazectomized patients, Lane 3: blood sample from clinically normal individual

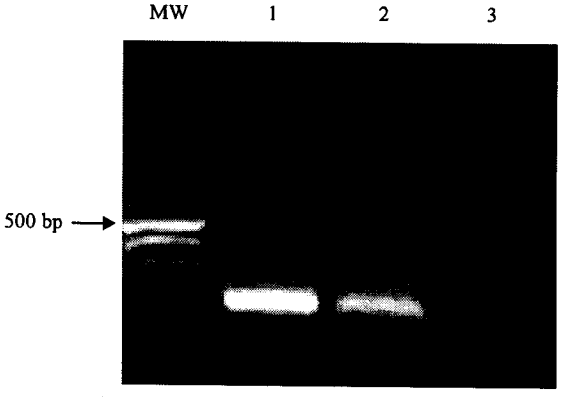

Fig. 2: Visualization of the Nested 186 bp specific *E.coli* PCR Product from nucleic acids extracted from blood of the two patients. Lane MW: 100 bp Molecular weight marker, Lane 1-2: blood sample from the vazectomized patients, Lane 3: blood sample from clinically normal individual

DISCUSSION

The sensitivity of the described PCR assay was comparable to that of bacterial isolation methods used for detection of bacteremia [12,17,19]. It is well documented that, in bacteremia due to *E. coli*, the number of pathogens is usually less than 1 microorganism per mL of blood in most adult patients [7]. Therefore, large blood volumes of 20 to 30 mL are required to be used in order to obtain a successful bacterial isolation attempts and to avoid false-negative results [20]. This problem could probably be solved in the near future using PCR technology as only viable or intact pathogens are necessary for conventional isolation methods[5,21]. Consequently, a variety of different DNA isolation kits have now been developed and made commercially available.

Present result that conventional isolation is much less efficient than PCR in detecting bacteremia during antimicrobial treatment is most probably due to the killing effect of antibiotics on *E. coli*. It is worth mentioning that PCR positive but bacterial isolation negative result, from the same clinical specimen, is not surprising. This because PCR detect bacterial nucleic acids, killed organism as well as infectious (intact) bacteria [9,22]. Therefore, the biological significance of a PCR result has to be interpreted with caution in light of the presence of bacteria nucleic acids and absence of infectious bacteria. Killed bacteria are not detectable by conventional methods, while PCR detects bacterial DNA independently of viability [23]. From clinical point of view, it is vital to develop improved techniques for diagnosing bacteremia, because the emergence of bacteremia is of substantial prognostic and therapeutic importance[24,25]. The occurrence of secondary bacteremia Garner *et al.*, [26] is a signal that the host's defenses have failed either to contain an infection at its primary site or that the physician has failed to control the infectious process [27,28]. Thus, the detection of bacteria in the patient's blood, regardless of whether they are still viable or have been killed by antibiotics, implies that the treatment regimen

might be insufficient and has to be augmented accordingly. Strikingly, it was shown recently that bacterial DNA has substantial immunostimulatory properties comparable to those of endotoxin and that its presence can cause sepsis-like symptoms in mice [29]. The presence E.coli nucleic acid sequences in the blood would probably interfere with cytokines release particularly IL2 thus leading to the onset of pyrexia in these valvectomized patients.

To advance beyond the current knowledge of diagnosis of bacteraemia, we have evaluated PCR assay for detection of *E. coli* in blood samples from patient admitted to Hospital for valvectomy due to severe mitral valve regurgitation and valve stenosis. The result of this study indicated that the nested PCR could be used as an alternative to conventional bacterial isolation procedure to diagnose bacteraemia. The rapidity, sensitivity and specificity of the PCR assay would greatly facilitate rapid detection of *E. coli* in blood samples from feverish patients following mitral valvectomy or infectious endocarditis. Because the nested PCR amplification assay is an extremely sensitive procedure, care must be taken to avoid cross-contamination between tubes during pipetting of reagents. Negative and positive controls should be included in each PCR reaction to estimate the lower limit of specificity and the higher limit of sensitivity.

In conclusion, The PCR-based detection assay provides a rapid method for diagnosis of bacteraemia, caused by different species of bacteria, including *E. coli* irrespective of their viability. However, conventional bacterial isolation methods failed to diagnose *E. coli* from blood cultures of infected patients receiving high doses of antibiotics.

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