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BACTEREMIA SCREENING OF CHILDREN AND THE FIRST DETECTION OF COLIFORMS IN BLOOD USING PCR TARGETING A PARTIAL SEQUENCE OF THE LacZ GENE

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ABSTRACT
Blood samples were collected from 135 children under 11years of age suspected with fever and sepsis in 2013, obtained from Welfare Teaching Hospital/Medical City/Baghdad. The blood samples were tested for bacteremia using conventional blood culture and polymerase chain reaction (PCR) targeting the 16s rRNA and the *Lac Z* gene. The results indicated that 69 (51.1% of the 135 screened) blood samples show positive cultures consisting of 55 (79.7% out of 69) gram-positive bacterial isolates and 14 (20.3% out of 69) gram negative isolates. *Enterobacter spp* was detected in 8 patients (11.6% out of 69, *E. coli* detected in 5 (7.2% out of 69 patients), *Klebsiella pneumoniae* detected in 1 (1.5% out of 69). All blood samples tested, 74 (54.8% out of 135) showed positive signals by PCR using the broad range primer targeting the 16s rRNA. Gram positive bacteria was detected in 60 samples (81% out of 74), whereas Gram negative bacteria was detected in 14 samples (19%out of 74). This study shows the potential approach of the PCR targeting the 16S rRNA gene and *LacZ* gene amplification for rapid detection of coliforms in blood in children.

KEYWORDS: PCR, Bacteria, Blood, bacteremia, coliform, gene.

INTRODUCTION

Bacteremia is associated with high morbidity and mortality worldwide. Despite the availability of effective antibiotics and improved treatment of circulatory failure and organ dysfunctions, mortality may be ≥ 20% in older patients with coexisting chronic diseases. Prompt detection and treatment is therefore an important goal for improving patient prognosis (Dellinger *et al*., 2008). Bacteremia is one of the most common causes of hospitalization and mortality in children and neonates under 5 years old of age. Septicemia represents the most important cause of neonatal morbidity and mortality (Stoll, 2004) with incidence ranges from 1 to 8 cases for every 1000 infants, but much higher values are found in preterm, low birth weight newborn infants (Puopolo *et al*., 2005). The majority of the bacteremia cases are caused by a number of pathogens including *Staphylococcus spp., Enterobacter spp., Streptococcus spp., K. pneumonia and Pseudomonas spp* (Weinstein *et al.*, 1995). Early onset neonatal sepsis (EOS), occurring in the first 72 hours of life, remains an important cause of illness and death among very low birth weight (VLBW) preterm infants. Gram-negative organisms continue to be the predominant pathogens associated with EOS (Stoll *et al*., 2005). Late onset sepsis, occurring after 3 days of age, is an important frequent problem in infants (Stoll *et al*.,1996). Molecular biology techniques, such as polymerase chain reactions (PCR) have been used as a specific and sensitive method for diagnosis of different bacterial, viral and protozoan infections (Stoker, 1990; Abbas *et al*., 2011 and 2013). Portions of DNA encoding the 16s ribosomal RNA (rRNA) have been used by PCR to define organisms as bacteria, allowing detection of even small amounts of bacteria and diagnosis of sepsis (McCabe *et al*.,1995; Andrade *et al*.,2008). The purpose of this study was to evaluate the potential of a broad diagnostic approach based on the 16S rRNA gene and LacZ gene amplification for detection of neonatal bacteremia. This approach led to the rapid detection of bacteremia in children.

MATERIALS & METHODS

Blood culture

Blood samples were drawn from children below 11 years who were referred to Welfare Teaching Hospital in city of medicine and suffered from fever (axillary > 38.3), blood samples (1-3ml venous) were collected in screw cupped tubes containing supportive media Brain Heart Infusion broth (BHIB).

Collection of samples: The blood samples were collected from children according to Fischbach (2000) under aseptic conditions. The blood samples were inoculated into culture bottle contained BHIB and transferred immediately to the laboratory to incubate it at 37 C for 2-7 days.

Laboratory tests: After the incubation period, signs of growth appeared in blood culture bottles, were turbidity, gas and flocculation. An amount of 0.1 ml of blood culture was taken from this culture to precede the routine diagnostic tests for *Enterobacteriaceae*, gram staining for full cellular morphology, MaCconkey agar, Kligler iron agar, Semisolid mannitol, Pepton water, Simon citrate agar and Urea agar.

Polymerase Chain Reaction (PCR)

Samples: One hundred thirty five blood samples have been collected from children below 11years who were presented to welfare Teaching Hospitals in medical city in Baghdad. Children suffered mainly from fever (about 38.5 °C).

Sample preparation: Blood samples (0.5-3 ml) were placed in a sterile vacutainer containing EDTA (Ethylenediamine-tetraaceticacid) and stored at -70ºC till processed.

Bacterial DNA extraction: Bacterial genomic DNA was extracted from blood using DNA blood mini kit (Qiagen, Germany) following the manufacturer's protocol. The resultant DNA extract with a final elution volume of 100 µl was frozen at -20^{o}C until use.

PCR and agarose gel electrophoresis

Each sample was subjected to consensus primer mediated PCR method. The primers supplied by (bioneer, korean), were complementary to the 16S rRNA gene sequences. PCR was carried out in a 25 µl reaction mixtures, with the universal broad-range primers; DG74 (5'-AGGAGGTGA TCCAACCGCA-3') and 65ab (5'AACTGGAGGAAG GTGGGGAY-3'). PCR was carried out with the Gram-positive-specific primers; DG74 and 143 (5'-GAYGA CGTCAARTCMTCATGC-3'). PCR was carried out using the Gram-negative-specific primers; DG74 and 68d (5'-AYGACGTCAAGTCMTCATGG-3') (Greisen *et al*., 1994; Klausegger *et al*., 1999). The initial denaturation was done at 95^{o}C for 5 min and was followed by 30 cycles of denaturation at 95^{o}C for 30 sec, annealing at 59^{o}C for 30 sec and extension at 72^{o}C for 1min. The final extension step was done at 72^{o}C for 5 min. The expected amplicons were 400 bp. Specimens with positive results by universal broad range PCR primers were further subjected to Gram speciation, using Gram-type specific primers to identify Gram-positive and -negative bacteria. Both sets of primers yielded amplicons of 400 bp. LacZ gene PCR have been used for the detection of coliform was carried out with the primers LacZ forward (5'-ATGAAAGCTGGCTACAGG AAGGCC-3') and LacZ reverse (5'-GGTTTATGCAGC AACGAGACGTCA-3') (Bej *et al*., 1991).The initial denaturation was done at 95^{o}C for 3 min and was followed by 30 cycles of denaturation at 94^{o}C for 1 min, annealing at 55^{o}C for 1 min and extension at 72^{o}C for 1min. The final extension step was done at 72^{o}C for 10 min. The expected amplicons were 264 bp.

RESULTS

Molecular diagnostics targeting the 16s rRNA revealed positive amplicons (400 bp) from 74 samples (54.8% of the 135 screened) (Table 1). In this study, gram-positive bacteria constitute 81% (60 out of 74) using gram negative specific primers (400 bp products). gram-negative bacteria constitute 19% (14 out of 74) using gram negative specific primers (400 bp products).

TABLE 1: Bacterial isolates detected by both PCR and Conventional blood culture

Culture result	Number of samples tested positive for each bacteria by culture	Gram type specific PCR	Number of samples tested positive for each bacteria by PCR
CoNS	55	Gram positive	60
Enterobacter sp.	8	Gram negative	8
Escherichia coli	5	Gram negative	5
Klebsiella pneumonia	1	Gram negative	1
Totatal of bacteria by blood culture	69	Total of bacteria by PCR	74

Abbreviations CoNS: coagulase-negative *Staphylococci*

TABLE 2: Diagnostic samples tested (Positive samples by gram negative bacteria) in the present study indicated with the case history, sex, age, PCR and culture

Iden. No	Case history	PCR results				Culture results	WBC counts/L
		PCR using the universal broad range primers[#]	PCR using gram positive specific primer[Ω]	PCR using gram negative specific primer[π]	PCR using the LacZ primer for detecting Coliform[¥]		
8	Male, 3day,fever	+ve	-ve	+ve	+ve	*E.coli*	14×10^{9}
10	Male, 6month,fever,	+ve	-ve	+ve	+ve	*E.coli*	3.5×10^{9}
13	Male,1year, fever	+ve	-ve	+ve	+ve	*Enterobacter spp.*	14×10^{9}
18	Male, 6month,fever	+ve	-ve	+ve	+ve	*Enterobacter spp.*	13×10^{9}
37	Male, 3day,fever	+ve	-ve	+ve	+ve	*Enterobacter spp*	13×10^{9}
49	Male, 18day, fever	+ve	-ve	+ve	+ve	*E.coli*	14.8×10^{9}
65	Male,2month, fever	+ve	-ve	+ve	+ve	*Enterobacter spp*	15×10^{9}
85	Female,4month, fever	+ve	-ve	+ve	+ve	*E.coli*	12.6×10^{9}
87	Male,5year, fever	+ve	-ve	+ve	+ve	*Enterobacter spp*	13×10^{9}
119	Male,2year, fever	+ve	-ve	+ve	+ve	*E.coli*	5.6×10^{9}
124	Male,25day, fever	+ve	-ve	+ve	+ve	*Enterobacter spp*	13.4×10^{9}
125	Female,3month, fever	+ve	-ve	+ve	+ve	*K. pneumoniae*	12.6×10^{9}
126	Female,2day,fever	+ve	-ve	+ve	+ve	*Enterobacter spp*	3.6×10^{9}
127	Female,3month,fever	+ve	-ve	+ve	+ve	*Enterobacter spp*	14×10^{9}

Abbreviations: +ve: positive, -ve:negative

TABLE 3: Rate of sepsis prevalence for gram negative bacteria from neonate

Gram negative bacteria	EOS (n=14)	LOS (n=60)
Enterobacter	2(14.3%)	6(10%)
E.coli	1(7.1%)	4(6.7%)
Klebsiella pneumonia	0	1(1.7%)

Abbreviations: EOS: Early onset sepsis, LOS, late onset sepsis

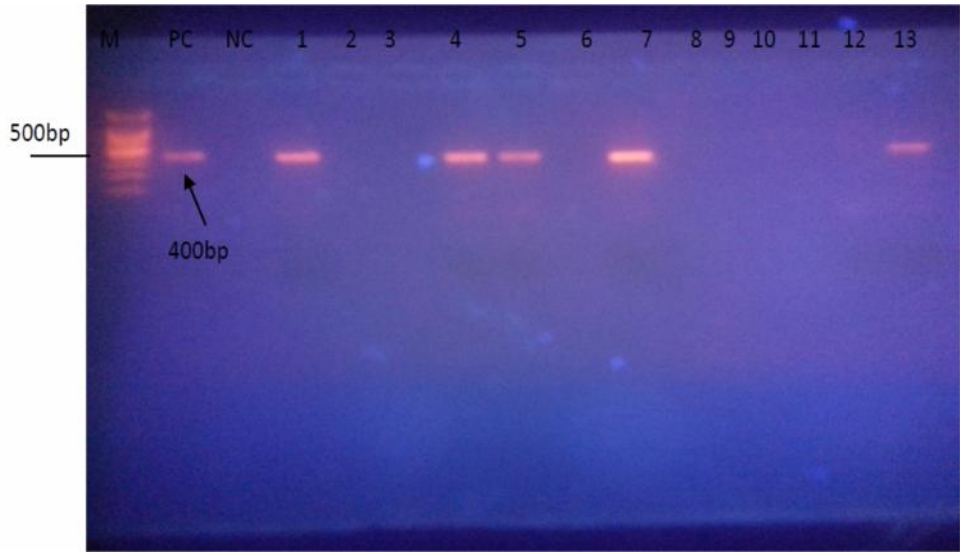

FIGURE 1: PCR amplicons from whole blood of bacteremic and non-bacteremic patients. With broad range 16s rRNA primers (400 bp): Abbreviations: PC: positive control, NC: negative control, M: marker,3: Sample ID No1 showed positive result in PCR and culture; 2,3 negative result in PCR and culture from patient ID No: 4, 7; 6, 7: positive result in PCR and culture from patient ID No 14,20; 8: negative result in PCR and culture from patient135; 9: positive result in PCR and culture from patient ID No 38;10,11,12,13: negative result in PCR and culture from patient ID No: 44,46,52,56; 14: positive result in PCR and culture from patient 50.

Two sets of primers (gram specific positive and gram specific negative PCR primers) were used for each sample tested positive for bacterimia after using the universal primers as shown in figure 2 Positive amplicons (400bp) were detected.

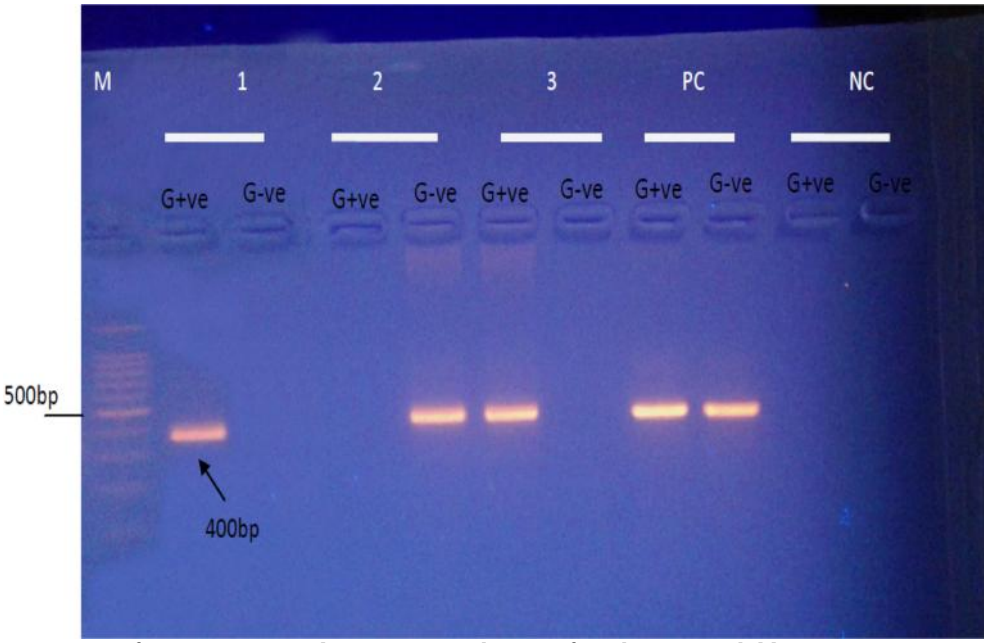

FIGURE 2: Detection of gram positive and gram negative bacteria from bactermic children using gram positive specific primers (400 bp) and gram-negative specific primers (400 bp) by PCR respectively.

Samples tested positive using gram negative specific PCR primers were tested further by PCR targeting LacZ gene of enterobacteriaceae as indicated in figure 3

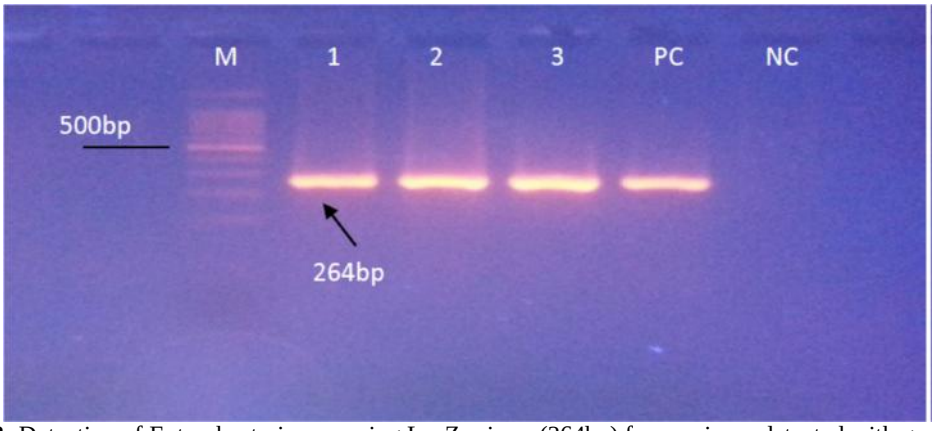

FIGURE 3: Detection of Enterobacteriaceae using LacZ primer (264bp) for specimen detected with gram negative bacteremia

DISCUSSION

Blood culture is considered to be the golden standard for diagnosis of neonatal bacterial sepsis (Washington and Hstrup, 1986)**.** In the present study 51.1% neonates (69 out of 135) suspected to have sepsis were positive by blood culture. These results were in agreement with Shaw *et al*. (2007) who reported comparable results (44.9) %. However, other studies (Buttery, 2002) demonstrated higher infection rates (80%) of suspected cases of neonatal sepsis. These differences in positivity of blood culture may be attributed to differences in blood volume withdrawn (Brown *et al.*,1995), blood culture techniques and exposure to antimicrobials in mother or the possibility of infection with viruses, fungi or anaerobes (Agnihotri *et al*., 2004). Another factor that may highly influence the sensitivity of the blood culture is starting antibiotic treatments at birth in all neonates with risk factors for early sepsis prior to diagnosis (Ishibashi *et al*., 2002; Manucha *et al.,* 2005). Therefore a large proportion of neonates not really infected are treated with broad-spectrum antibiotics. The possibility of having a 100% sensitive and specific method for the identification of bacteria in blood, with results available in a short period of time, could allow the onset of treatment only in neonates with infection, thus reducing the use of broad spectrum antibiotics, the need of close observation of suspected cases and medical costs. In comparison, PCR targeting the 16s rRNA in this study has been more sensitive and gave higher results (54% positive out of 135 tested children) than those obtained by the conventional blood culture method (51%). These results are in agreement with Fujimori *et al*. (2010), Reier-Nilsen *et al*. (2009), Ruppenthal *et al*. (2005) and El-Hawary *et al*. (2011). In this study, fever was the most common presenting fetal symptom. These results were in agreement with Ng *et al*. (1999) and Chiabi *et al. (*2011). However, Masood *et al*. (2011) reported that refusal to feed was the most common presenting complaint. In the study of Shitaye *et al*. (2010), hypothermia was the most prevalent clinical feature of sepsis. Gram positive bacteria in this study have been detected more frequently in children (81%) than gram negative bacteria (19%). A previous report from Berner and colleagues (1998) indicated that the gram-positive and gram-negative bacteria isolated from bacteremic children constitute 71% and 29% of the infections respectively. This result is in agreement with the previous studies (Kohli-Kochhar *et al*., 2011; Shah *et al*., 2012). However, the results are in contrast with a local study (Shams AL-Deen, 2001) who found that gram- negative bacteria isolated from bacteremic children are more common than gram-positive bacteria. Variable results are expected in such studies and the variation can be attributed to various risk factors represented by children's age, gestational age, body weight, education and hygienic conditions. *E. coli* is one of the most important pathogens causing sepsis in newborns and young infants (Huang *et al*., 2002). In the present study 6.75% (5 out of 74) isolates have been identified as *E. coli* by blood culture, PCR. Of these patients 6.7 % (4 out of 60) ranged from 2 weeks to 2 years and identified as late onset of sepsis. While, 7.1% (1 out of 14) has been one day old and identified as early onset of sepsis. This result contrasts a recent study from Badrawi *et al*. (2005) in Kasr El-Aini Hospital who found that *E. coli* represent 1% of the total isolates from neonates with EOS**.** In the present study *Klebsiella pneumonia* constitutes 1.35% (1 out of 74). These results are contrast with the result obtained by other study from El- Badawya *et al*. (2003) documented 26 %. The identification of Enterobacteriaceae is usually performed using *Lac Z* gene that encodes the β-galactosidase enzyme as target DNA (Bej *et al*., 1990). Results of I the present study, the *LacZ* primer described by Bej *et al*. (1990) was successful to detect enterobacteriaceae in blood with sensitivity of 100%. The WBC count has been the most widely studied laboratory parameter in occult bacteraemia and has been observed to consistently increase in patients with bacterial sepsis. With regard to the correlation of the leukocyte count with sepsis, there are no definite records as to how high the leukocyte count should be to obtain a positive blood culture or PCR. Patients can have leukopenia, yet show bacteraemia and a patient can have leukocytosis without bacteraemia. These results agree with the previous findings from Valle *et al*. (2010). The findings of our

study showed the direct correlation of WBC counts of > 12,000/mm3 to the occurrence of bacteraemia. All of the methods utilized demonstrated that the majority of specimens with a WBC count of < 12,000/mm3 were negative for microbial growth whereas those with a WBC count of > 12,000/mm3 were all positive. In conclusion, there is direct correlation of WBC counts of > 12,000/mm3 to the occurrence of bacteraemia.

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