Molecular diagnosis of occult bacteremia using Universal PCR method in 3 to 36 months children with fever without source and a negative blood culture

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(Received 26 Oct, 2013 Accepted 30 Apr, 2014)

ABSTRACT

Introduction: Early diagnosis of bacteremia and its complications is the most important part of the care and management of the children. The utility of polymerase chain reaction (PCR) techniques has been shown to identify pathogens in less and more optimal time. The aim of our study was to evaluate the importance and prevalence of occult bacteremia using universal PCR of blood in 3-36 month children with fever without source in Pediatric Medical Center comparing other routine methods like blood culture.

Methods: 100 febrile children in 3-36 month age suspected to Bacteremia with fever without source, who was admitted in children's hospital of Bandar Abbas, were evaluated. All of them had negative blood culture. Blood samples were taken from all patients for PCR and other clinical tests such as CRP, ESR and CBC.

Results: The mean temperature of the body was $>39^{o}$c at presenting time. 23 patients had positive PCR test, which none of them had positive blood cultures. Laboratory findings such as WBC, ESR, and CRP had no significant difference to PCR results.

Conclusion: Universal PCR technique is more sensitive and specific than conventional blood culture and other methods to diagnose organism cause bacteremia.

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Key words: Bacteremia, Fever, PCR

Introduction:

About 30% of febrile patients 3 months to 3 years have less than a week of fever and had no localized infection protests in examination that are known as fever without a source. In these cases, distinguishing serious bacterial infections and self-limiting viral diseases are difficult for a physician. (1) A big concern in cases of children with fever without source is the possibility of occult bacteremia. Occult bacteremia (bacteremia without specifying the infection source), due to Streptococcus pneumonia, Haemophilus influenza type b, Neisseria meningitides, and Salmonella species occur in 1.5% of children aged 3 up to 36 months with relatively good condition along with fever (rectal temperature ≥ 38 ° C) (2). Increasing incidence of bacteremia, among young children is due to safety defects or lack of maturity in the production of IgG opsonic antibodies against

polysaccharide antigens in encapsulated bacteria. Streptococcus pneumonia is the most common reason of reported occult bacteremia. No laboratory testing (except blood culture) or clinical evaluation can accurately reflect the occult bacteremia. (3)

White blood cell counting (CBC) is studied widely as a potential tool in the diagnosis of occult bacteremia. (4) Some studies indicate that the ability to predict bacteremia in febrile children by erythrocyte sedimentation rate (ESR) is not more useful than WBC counting. (5) But, in other studies, it indicated that the concentrations of c - reactive (CRP) serum might be (in the diagnosis of viral from bacterial infections) more accurate than complete blood count (6,7). In some studies, increasing levels of serum procalcitonin is more appropriate in diagnosing of bacteremia (8,9). With respect to the incidence of 3 percent bacteremia in children with fever without source, early treatment with antibiotics in several studies indicate a better prognosis than those who were not treated early (10). The findings of blood cultures are commonly used to detect infection. However, blood culture lacks the sensitivity for more samples that are infectious; especially after antibiotic treatment as well as slow growth pathogens (11). Nucleic acid amplification technology was necessary to respond to this need, which was useful even in the presence of bacteria growth inhibitors, including antibiotics. The ability of UPCR method is built based on the bacteria phylogenetic category that is gene arrangement base (rDNA). In this way, the primers are used that amplifies the number of 997 base pair from rDNA 16 S gene, which has almost all bacteria of the gene sequence (rRNA), and it is rDNA 16 S gene which is shown the existence of this common gene sequence in different types of bacteria with a direct replication of bacterial genes in clinical samples. In Anthony et al study, among 158 positive blood culture samples, 119 cases (75.3%) are correctly detected by PCR method. (17) In another study, using the technique of Universal PCR, among the 101 samples, 25 cases were positive, of which, 10 cases had negative blood culture and 15 had positive blood cultures. The sensitivity and specificity of PCR test are respectively calculated 65% and 87, at the same time the blood culture in these samples, was positive in 22 cases, of which, 7 people had negative PCR and 15 had positive PCR (19). The extent of fever without source, as a main complaint among children 3 to 36 months and the possibility of occult bacteremia in these children and the weakness of the classical diagnostic methods, as created the need for sensitive methods to infer the presence of bacteria in the blood, the technique can become a diagnostic tool for confirming the existence of occult bacteremia in children (12,13).

Although, the PCR is a method with the highest sensitivity to detect bacterial DNA, but it is more complex technically than standard tests and can be performed only in special laboratories, but the possibility of false positives is too much due to high sensitivity.

Methods:

In this cross sectional study, 100 febrile infants without lesions referred to Children's Hospital in Bandar Abbas on the date October 2010 to October 2011 were studied. In this study, people who had a fever equal to or greater than 39 degrees Celsius entered the research and in Laboratory evaluation, one case had white blood cell $\geq$15000 in milliliter cases, or neutrophils above 60% or Sediment over 30 or C-reactive protein over 20 or band cell over 500. First in the questionnaire, the patients' characteristics such as age and gender were collected. Then a blood sample and a urine sample were taken from the patients and were sent for culture to the microbiological laboratory. Eventually, 100 blood samples with blood culture result and negative urine were transferred for molecular analysis to Molecular Medicine Research Laboratory of Bandar Abbas and stored at - 80 °C until DNA was extracted. DNA extraction was done by using DNA extraction kit prepared from Fanavaran Gene Company according to the manufacturer's instructions. Extracted DNA from fresh biological material (blood, cellular environment, tissue, etc.) with high purity and OD = 1.6-2.0 at a wavelength of 260 nm, will be in the form of large molecules in the range

of 60-50 thousand base pairs. According to previous studies, it was shown that part of the 997-base pair sequence rRNA 16s as a primer can be used to amplify DNA of bacteria in UPCR method. The Primer used for UPCR includes (25):

TEHF: 5' CAG. CAG CCG CGG TAA 3'

TEHR:5' ACG GTT ACC TTG TTA CGA CTTC 3'

With the thermo cycler instrument, the proliferation responses are as follows: a denaturation at 94 ° C for 30 s, annealing at 55°C for 45 seconds and extension at 72°C for 30 seconds. These processes were repeated for 30 times. Before these stages, pre-denaturation was done for 5 minutes and after this stage, the next extension was done for 5 minutes. PCR product related to samples and negative control was studied by using electrophoresis in 1.5% agarose gel. At this stage, the size of the PCR product is 1000 base pairs. The easiest way to observe the DNA in agarose gel is using Ethidium bromide fluorescent dye. This dye will penetrate between two DNA chains and is observed under ultraviolet light to red - orange fluorescence and with the use of Gel Documentation device with UV light the DNA fragments were observed and photographed. Row A - I is positive samples, J is positive control, and K is Marker Size 100 base pairs, L is negative control (Figure 1).

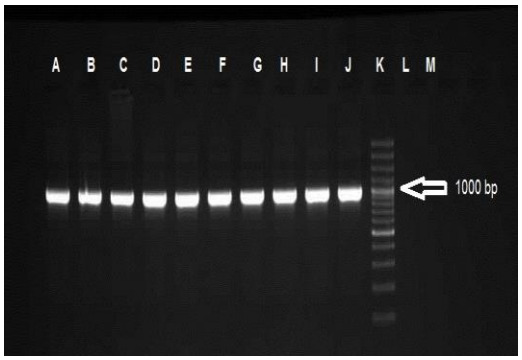

Figure 1. Bacterial sequences bands by UPCR

After commissioning UPCR, the test was done on 100 samples of extracting DNA of patients. To control the test, the negative control was used that the negative pipe was prepared in a similar way of tests, but DNA was removed and the distilled water was added. The product obtained from agar gel was taken and in the case of observing the considering band due to Electrophoretic movement and its weight beside DNA weighted index was considered positive in terms of Bacteria. Negative and positive control with patients' samples must be negative and positive, respectively; otherwise, the tests were repeated. Finally, through the completion of questionnaire and patients' medical records, clinical and demographic characteristics include: age, sex, rectal temperature degree, counting WBC, ESR, CRP … were recorded.

Statistical analysis was used by SPSS (IBM SPSS Statistics) software version 19, Chi-and Fisher's square statistical test and proportions (binomial) to investigate the relation (correlation) between factors risk factors with pathogens..

Results:

In this study, 100 children were studied with a fever diagnosis without a source. Children aged were 3 months to 3 years (36 months) with a mean age of 22 months and a median of 21.5 months. The frequency of male patients was 50% and female were 50%, respectively. The mean (range) of body temperature during the evaluation was > 39°C. Blood culture results were negative for all children and they had normal urine analysis and negative urine culture. UPCR was reported positive in 23 patients, including 9 males and 14 females. ESR mean in the case of patients was 21.24 (range 4 to 125) mm per hour and the average of white blood cells was 10677 (range 1,400 to 30,500. Available CRP in 50 percent of patients with CRP in their case was reported negative (Table 1).

Table 1. Demographic characteristics and laboratory findings

Risk factors	Groups of patients (N=10)
Age (months)	3-36
Sex (male)	50
Gender (female)	50
Temperature (0C)	≥39
WBC (Lμ)	1400-30500
ESR (mm per hour)	4-125
CRP	Neg-3^{+}

With respect to the results obtained by Fisher's exact test, no significant difference was observed statistically between positive and negative UPCR results with age, sex, erythrocyte sedimentation rate, C-reactive protein, and white cell of peripheral blood ($P > 0.05$).

Conclusion:

The aim of this study was to evaluate Para clinical outcomes, especially UPCR results in patients suspected with bacteremia that were hospitalized with a diagnosis of fever without source. Bacteremia is one of the most serious infectious diseases and may be caused by gram-positive and gram-negative microorganisms' wide range that in younger ages, it is not associated with infection (14). However, no specific clinical finding for bacteremia is a diagnostic purpose (15). At present, blood culture is a standard method for diagnosis of bacteremia and yet it has limitations such as low sensitivity and long duration of diagnosis (more than 2 days) (16). On the other hand, early detection of bacteremia and its complications is considered the most important part of care, in which appropriate treatment is crucial. Unnecessary antibiotic treatment has side effects and leads to the induction of resistance in the body (17). Blood culture has a positive impact on the treatment of antibiotic and should be provided before antimicrobial treatment. However, the relevant information with the pathogen causing the infection and its sensitivity will be provided at the start of treatment, which is of course in these cases, the use of UPCR is beneficial (15,18).

In a large number of studies, PCR test, for certain strains, such as pneumococcus, etc., with creating specific conditions to isolate specific microorganisms in culture, the number of positive culture will get higher and, therefore, with the number of cases that their PCR was indicated, much higher compliance is established. However, in this study, conventional blood culture method and PCR with universal primers in all laboratories have been compared with each other in unit samples. In all these cases such as our test, the PCR number of positive results was more than the positive culture that these results might be due to the PCR test accuracy compared with culture. Blood cultures will be negative because of different reasons such as antibiotics and error of the test …over there or in our center (17,18,20-22). PCR results could be ready within a few hours and provide information about the disease earlier than 70 percent of blood cultures. However, PCR can detect more cases of bacteremia compared with the cultivation and identify the non-viable microorganisms DNA. Therefore, we can calculate both positive and negative PCR result adaptation to cultivation. Most positive PCR patients with negative blood cultures are classified as probable bacteremia (23). PCR techniques have also been successful to detect organisms from the direct blood sample, Buffy coat serum, or negative culture samples. These results indicate that PCR-based methods may be more sensitive than conventional bacteriological methods that are shown in our study.

In our study, the severity of fever and laboratory findings, CBC, ESR, CRP, among patients with blood culture results and positive or negative UPCR were not significantly different. Thus, the importance of using more accurate diagnostic methods, such as UPCR is determined. In a study of Isaac man, by examining PCR evaluation to identify pneumococcal bacteremia in patients who had a positive blood culture, had a higher temperature, more WBC count and less neutrophil count than negative cultures, regardless of the PCR results. Of course, there was no significant difference between patients with positive PCR and negative culture with patients with PCR and negative cultures in terms of these clinical findings (24).

In the present study, UPCR results were statistically analyzed with age, sex, and lab results such as ESR, WBC, CRP that statistically, they had

no significant differences and this recommends the importance of using more rigorous methods of finding microbial. One limitation of this study was the low number of investigated patients that needs the similar studies necessity in larger and greater sample size.

In general, it seems that the PCR method to detect organisms responsible for bacteremia has more sensitivity and greater specificity compared to conventional laboratory methods. Of course, since the Gold Standard test does not exist to evaluate the false-positive or real PCR results in terms of identifying the organism, the usual approach of bacteremia diagnosis interpret based on PCR data or blood cultures will be done based on data the overall clinical background and course of the disease. It seems that an alternative method for making decisions based on the results of PCR and culture does not exist. However, the everyday use of it, requires further investigation.

Acknowledgment:

Thanks from Hormozgan University of Medical Sciences for funding the project, dr.Mousavi and his noble lab personnel, personnel of Hormozgan University of Medical Sciences, Molecular Research Center and noble families of patients that have helped us sincerely.

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تشخیص مولکولی باکتریمی مخفی با روش Universal PCR در کودکان ۳ تا ۳۶ ماه با تب بدون کانون دارای کشت خون منفی

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چکیده

مقدمه: *تشخیص زود هنگام باکتریمی و عوارض آن مهمترین قسمت مدیریت درمان کودکان به شمار می‌آید. استفاده از روش واکنش زنجیره‌ای پلیمراز (PCR) موجب شناسایی پاتوژن ها در زمان کوتاه‌تر و مناسب تر شده است. هدف از این مطالعه، بررسی باکتریمی مخفی در کودکان ۳ تا ۳۶ ماه با تشخیص تب بدون کانون با روش Universal PCR(UPCR) در بیماران تب دار با کشت خون منفی مراجعه‌کننده به بیمارستان کودکان می‌باشد.*

روش کار: *۱۰۰ کودک تب‌دار ۳ -۳۶ ماهه مشکوک به باکتریمی که با تشخیص تب بدون کانون به بیمارستان کودکان بندرعباس مراجعه نموده و کشت خون آنها منفی گزارش شده، مورد بررسی قرار گرفتند. از همه بیماران نمونه‌های خون برای UPCR پس از آزمایش‌های تشخیصی مثل CRP، ESR، WBC جمع‌آوری شد.*

نتایج: *درجه حرارت بدن در زمان مراجعه $\leq^{c}$ ۳۹ گزارش گردید. از این ۱۰۰ نمونه، ۲۳ مورد PCR مثبت گزارش شد که هیچ کدام کشت خون مثبت نداشته و در وضعیت آزمایشگاهی آنها مثل CRP، ESR، WBC با یافته‌های UPCR از دیدگاه آماری اختلاف معنی‌داری مشاهده نگردید.*

نتیجه‌گیری: *براساس نتایج آزمایشگاهی و آماری این تحقیق می‌توان گفت روش UPCR برای تشخیص ارگانیسم‌های مسئول باکتریمی نسبت به روش های مرسوم مثل کشت خون می‌تواند سریع تر و دقیق تر باشد.*

کلیدواژه‌ها: باکتریمی – تب – PCR

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دریافت مقاله: ۹۲/۸/۴ اصلاح نهایی: ۹۳/۱/۲۹ پذیرش مقاله: ۹۳/۲/۱۰