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Original Article

The application of PCT and CRP combined with 16s rRNA in the early diagnosis of neonatal septicemia

Na Wen*, Jin Shi*, Jiang Wu, Shixiong Yang

*Department of Pediatrics, Edong Healthcare Group, Huangshi Maternity and Children's Health Hospital, Huangshi 435000, Hubei, China. *Equal contributors.*

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Abstract: Neonatal septicemia (NS) is characterized by high morbidity and high mortality. Pathogens in newborns can grow rapidly in the blood and cause an inflammatory reaction, leading to multiple organ damage or even death. However, the early stages of NS often exhibit no obvious symptoms. Therefore, the early diagnosis of NS is of great significance for its treatment. It was found that procalcitonin (PCT), C-reactive protein (CRP), and bacterial 16s rRNA have a good diagnostic value for early NS. However, their combined application has not been reported for the early diagnosis of NS. 92 patients admitted to the neonatology department from January to February 2018 were enrolled in our study, including 50 NS patients and 42 non-infected neonates. The PCT and CRP content in the patients' serum was determined by immunofluorescence (IFAT). The 16s rRNA content in their blood was measured by fluorescence quantitative PCR (FQ-PCR). The sensitivity and specificity of the combined diagnosis were analyzed. The serum PCT and CRP levels were significantly higher in the sepsis group compared with the control group ($P<0.05$). In the sepsis group, the positive rate of 16s rRNA was 98% (49/50), and the positive rate of the blood culture was 24% (12/50) ($P<0.05$). In the control group, the blood samples were negative for 16s rRNA and for the bacterial culture. The sensitivity and the specificity of the combined diagnosis were 100% and 98.2%, respectively. The combination of PCT, CRP, and 16s rRNA can improve the diagnostic efficiency of NS. Their combination is simple and rapid and can provide early and sensitive diagnostic methods for NS, which can help to evaluate the therapeutic effect of the disease.

Keywords: Neonatal septicemia, procalcitonin, C-reactive protein, 16s rRNA, FQ-PCR, immunofluorescence

Introduction

Neonatal septicemia (NS) is a serious infectious disease that occurs in the neonatal period with the characteristics of high morbidity and high mortality [1, 2]. Because of the immature immune system, the immune function is poor and prone to infection. After the pathogen enters the body, the infection range is difficult to limit and leads to an extensive, systemic inflammatory response [3]. Pathogenic bacteria enter the blood circulation through various ways and proliferate rapidly in the blood. The disease progresses rapidly, which can cause damage to multiple organs and even neonatal death. It is a critical illness for newborns and highly concerning for pediatricians [4, 5]. The causes of neonatal sepsis are complex and include a variety of bacteria, including *Staphylococcus aureus*, *Escherichia coli*, *Streptococcus*, etc., which can be caused by intrauterine infection or intrapartum and postpartum infections [6, 7]. The early clinical manifestations of NS are often atypical, especially in premature infants. Clinical symptoms often only show a decrease or refusal of milk intake, galactorrhea, lethargy or irritability, low crying, fever or hypothermia. It can also appear as nonspecific symptoms, such as normal body temperature, low response, pale or dull, wilting, and weight loss [8, 9]. Therefore, finding a diagnostic marker for the early diagnosis of NS has important clinical significance and can provide timely help for clinical treatment.

It was found that PCT is a type of protein and is an important parameter for determining bacterial inflammatory infections [10]. The PCT level is significantly increased during sepsis and multiple organ failure, or it is accompanied by

severe bacterial, fungal, and parasitic infections [11, 12]. The expression of PCT in neonatal sepsis has also been reported, but the diagnosis of NS by PCT alone still has the problem of poor specificity and sensitivity [13, 14]. As an acute phase protein, C-reactive protein (CRP) increases rapidly within a few hours after the onset of various diseases such as acute inflammation, tissue injury, myocardial infarction, surgical trauma, and radiation injury and then decreases to a normal value if the diseases are in remission. The changes in CRP are positively correlated with the severity of infection. The detection of the bacterial 16s ribosomal RNA (16s rRNA) gene can also be used as an important way to evaluate NS [15, 16]. However, the diagnostic value of the PCT, CRP, and 16s rRNA combination in early diagnosis of NS has not been reported.

Materials and methods

General information

The clinical data of 92 patients admitted to the neonatology department from January to February 2018 were included, including 50 NS patients and 42 non-infected neonates. All newborns were aged 1-28 days (d), including 48 males and 44 females, 52 premature infants and 40 full-term infants. The diagnostic criteria for NS were established by the Chinese Medical Association Pediatrics Branch [3]. The exclusion criteria included simultaneous infection with other blood disorders, autoimmune diseases, malignant tumor complications, and patients who were unwilling or unable to cooperate with the study and follow-up [3]. This study was approved by the medical ethics committee. The parents or guardians of all the selected subjects signed an informed consent.

Main reagents and instruments

A LUMI test kit was purchased from the German BRAHMS diagnostic company. A CRP ELISA kit was purchased from Shanghai Chuangyang Biotechnology Company. The PCR reagent was purchased from TransGen BioTech. The whole blood DNA extraction kit and the bacterial DNA extraction kit were purchased from Omega Bio-tek. The primers were synthesized by Genepharma. The BRAHMS™ PCT detector was purchased from the German BRAHMS diagnostic company. The Labsystem Version 1.3.1 microplate reader was purchased from the Bio-rad Corporation. The ABI 7700 Fast Quantitative PCR Reactor was purchased from ABI. The HITACHI 27060 automatic biochemical analyzer was purchased from Hitachi. The BACT/ALERT 3D blood culture instrument was purchased from Mérieux. The ABI 9700 PCR instrument was purchased from ABI.

Methods

General information collection: The general information such as age, sex, weight, and the infection site of each group were recorded.

Blood collection: 3 ml of upper extremity venous blood was collected in the early morning after admission. Next, the blood was centrifuged at 2000 rpm for 5 min, and the serum was stored at -70°C. 1 ml serum was used for the blood culture, 1 ml serum was used for the fluorescent quantitative PCR to detect bacterial 16s rRNA, and 1 ml serum was used to detect the PCT content.

IFAT detection of PCT content: According to the kit's instructions, the serum and acridinium ester-conjugated PCT monoclonal antibody in a test tube coated with an anti-catacalcin monoclonal antibody. The excess tracer solution was washed away at room temperature in a dark room. The measuring tube was directly placed on a microplate reader to measure the PCT content. The positive standard was PCT≥2 ng/mL.

Detection of CRP by ELISA: The CRP level was measured using an ELISA kit according to the manufacturer's instructions. Briefly, in the precoated 96-well plate, the samples, standards and then the HRP-conjugated antibodies were added, followed by incubation and washing. After that, the TMB substrate was added for color development followed by measuring the absorption value at a wavelength of 450 nm. Positive was defined as CRP≥10 mg/L.

Bacterial blood culture: 1 ml blood was added to a 20 ml culture flask containing a blood enrichment solution. The sample was cultured in a blood culture apparatus, and the negative result was determined after 5 days of culture.

FQ-PCR: Anticoagulated blood was added to PBS and centrifuged at 2000 rpm. The sediment was resuspended in a TE buffer prepared

Table 1. Primer sequences

Gene	Forward 5'-3'	Reverse 5'-3'
16s rRNA	AGAGTTTGATCMTGGCTCAG	GGTTACCTTGTTACGACTT

Table 2. General information and comparisons

Index	Control	NS	*P* value
Age (day)	18.0 ± 3.0	19.0 ± 2.0	0.069
Gender (male/female)	26/24	22/20	0.975
Weight (kg)	2.3 ± 1.2	2.1 ± 1.3	0.445
Prenatal (n, %)	30 (60.0%)	24 (57.1%)	0.781
Morbidity (n, %)	14 (28.0%)	10 (23.8%)	0.648

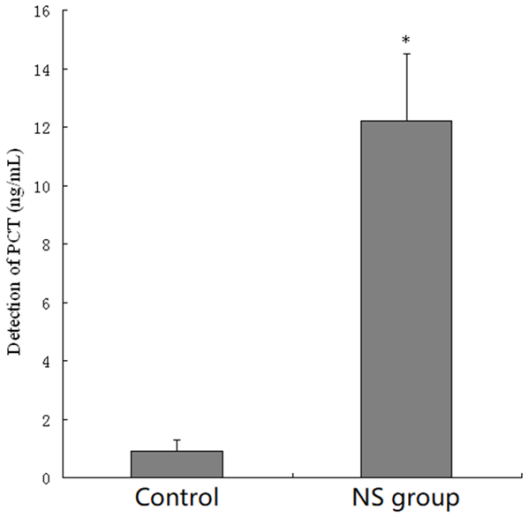

Figure 1. PCT content analysis. *P=0.013, compared with the control.

with EDTA and a Tris hydrochloric acid buffer, proteinase K solution, and 10% SDS solution. Next, it was water bathed at 56°C for 5 h and cooled down at 4°C. After an equal volume of pH 8.0 Tris-HCl phenol was added, the sample was centrifuged at 10,000 rpm for 15 min. The upper aqueous phase was mixed with an equal volume of chloroform and isoamyl alcohol, and centrifuged at 10,000 rpm and 4°C for 10 min. 10% volume upper aqueous phase was added with 3 mol/L sodium acetate with pH 5.2 and mixed with 100% ethanol after mixing, and then it was incubated at -20°C overnight. After being centrifuged at 10,000 rpm at 4°C for 20 min, the supernatant was mixed with 1 ml of 70% ethanol and centrifuged at 500 rpm and at 4°C for 10 minutes. After we discarded the supernatant, the solution was dried at room temperature for 10 minutes, dissolved in 20 μl TE buffer, and stored in a refrigerator at -20°C. The primers for the bacterial 16s rRNA gene were designed around highly conserved regions (**Table 1**). The FQ-PCR reaction system contained 5 μl 10×PCR buffer, 4 μl four types of dNTP mixtures, 0.5 μl upstream primer, 0.5 μl downstream primer, 0.5 μl Taq DNA polymerase, 2 μl template, and 37.5 μl DEPC water. The reaction conditions were offset at 95°C for 10 min, followed by 35 cycles of 94°C for 45 s, 60°C for 35 s, and 72°C for 30 s. GAPDH was used for a reference. The initial cycle number (CT) was calculated according to the fluorescence quantification. The standard curve was drawn based on the standard CT value. The CT value of the specimen was set at 40.0 for negative, ≤35 for positive, and 35-40 for re-doing, such as >38 for positive.

Statistical analysis

SPSS 18.0 software was applied for the data analysis. The measurement data is presented as the mean ± standard deviation ($\overline{x}$ ± S) and was compared using ANOVA. The enumeration data were compared using a χ^2 test. P<0.05 was considered a significant difference.

Results

General information

The general data of the two groups were analyzed. There were no statistical differences in age, sex, or weight between the two groups (**Table 2**).

PCT content analysis

The PCT levels in the NS group and the control group were analyzed separately. The PCT content in the serum of the NS group was (12.2 ± 2.3) ng/mL, which was significantly higher (P<0.05) when compared with the control group (0.9 ± 0.4) ng/mL (**Figure 1**).

CRP level analysis

ELISA was performed to measure the CRP level in the NS group and the control group and showed a significantly higher CRP level in the NS group (16.1 ± 2.3 mg/L) than the level in the control group (1.6 ± 0.6 mg/L) (P<0.05) (**Figure 2**).

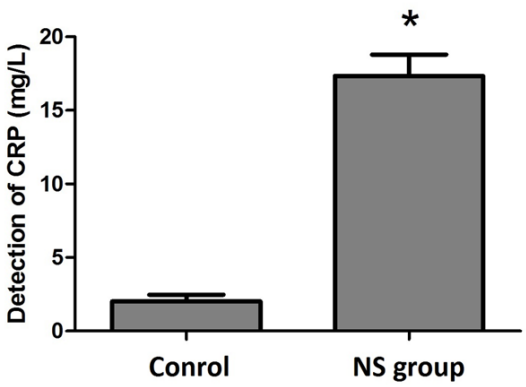

Figure 2. CRP level. *P=0.016, compared with the control.

Table 3. FQ-PCR detection of 16s rRNA and the blood culture positive rate

Index	Control	NS
16s rRNA (n, %)	0 (0)	49 (98%)
Blood culture (n, %)	0 (0)	36 (72%)

FQ-PCR detection of 16s rRNA and the blood culture positive rate

The 16s rRNA and the blood culture positive rate in the NS group and the control group were determined by FQ-PCR. It was shown that the 16r RNA positive rate in the NS group was 98% (49/50), which was clearly higher than that of the blood culture positive rate (24%, 12/50) ($P<0.05$). In the control group, both the 16s rRNA detection and the blood culture of the blood samples were negative (**Table 3**).

Diagnostic analysis of PCT and 16s rRNA in the early diagnosis of NS

With PCT≥2 ng/mL as the positive standard, the sensitivity and specificity of PCT in diagnosing NS were 81.2% and 67.8%, respectively. Using PCT≥5 mg/L as the positive standard, the sensitivity and specificity of PCT in the diagnosis of NS were 83.5% and 90.8%, respectively. The sensitivity and specificity of 16s rRNA in the diagnosis of NS were 98.0% and 94.1%, respectively. The sensitivity and specificity of the combined diagnosis were 100% (**Figure 3A**) and 98.2% (**Table 4**; **Figure 3B**).

Discussion

The onset of NS is concealed with unobvious early symptoms. However, untimely diagnosis and treatment may cause the mortality rate to be high [17, 18]. Therefore, it is urgent to find reliable biomarkers for the early diagnosis of NS with high sensitivity and specificity, thereby helping pediatricians to diagnose and treat the disease in a timely manner. Early diagnosis will be helpful in reducing the economic burden and in relieving the contradiction between doctors and patients. Previously, the initial diagnosis was mainly based on the clinical symptoms and signs, the high risk factors in a patient's medical history, C-reactive protein (CRP), and peripheral blood changes. However, the diagnosis was confirmed depending on the detection of pathogens or pathogen antigens. Therefore, a positive blood culture is still the "gold standard" for the diagnosis of NS [19, 20]. However, the blood culture takes a long time and is easily disturbed. The culture time of a positive result needs at least 36 hours, and it is not easy to completely separate the bacteria even if there is an infection. High blood culture negative rates have been reported in confirmed NS [21]. This study found that the blood culture negative rate in children diagnosed with NS was 76%, which affects the early diagnosis rate. The 16s rRNA gene primers were designed using highly conserved regions of bacteria to establish FQ-PCR detection. It was revealed that 16s rRNA has high specificity and sensitivity to the detection of bacteria, so it can effectively reduce the rate of misdiagnosis or missed diagnosis. Thus, it lowered the rate of the missed diagnosis of NS caused by using the "gold standard" blood culture [22].

PCT can reflect the activity of the systemic inflammatory response, but it does not increase in diseases such as autoimmune diseases, allergic diseases, and viral infections. Bacterial endotoxin can effectively induce PCT elevation, so PCT can be used to identify bacterial and non-bacterial infections and inflammation [23]. PCT detection takes a short amount of time, is simple to do, and is reliable, so it can dynamically monitor the treatment status of NS [14]. In order to further improve the diagnostic specificity and sensitivity of neonatal sepsis, finding effective early diagnosis markers is beneficial in improving the diagnostic value and can effectively reduce the mortality of the disease. As an acute phase protein, the CRP level is lower in healthy individuals and rapidly increases in patients with invasive bacterial infections, inflammation, or damaged tissues [24]. The

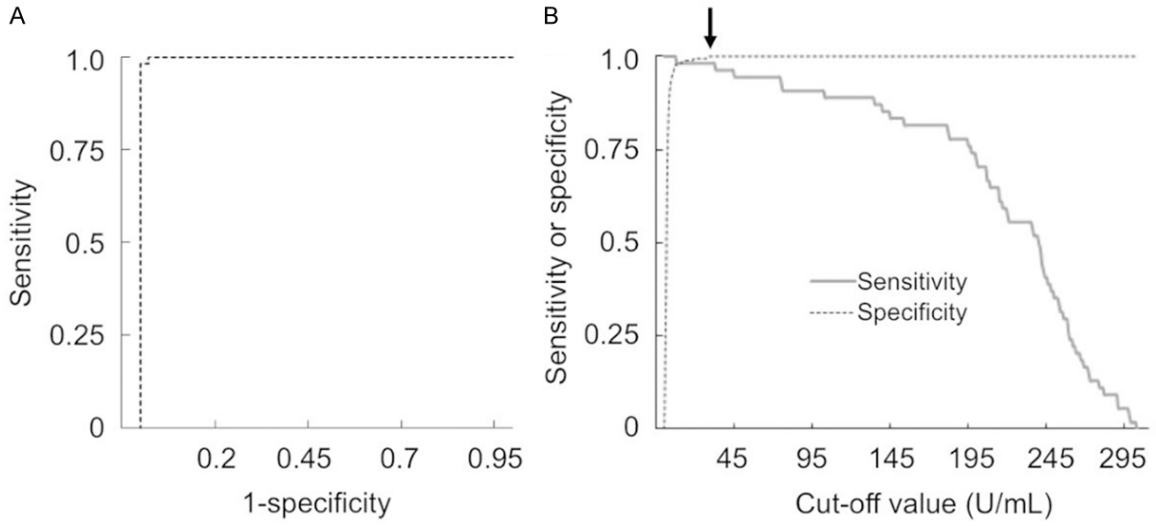

Figure 3. ROC curve analysis.

Table 4. Diagnostic analysis of PCT and 16s rRNA in the early diagnosis of NS

Index	Sensitivity	Specificity
PCT	81.2%	67.8%
CPP	83.5%	90.8%
16s rRNA	98.0%	94.1%
PCT+16s rRNA	100%	98.2%

increase of CRP is very quick and can be detected within 6-12 hours after the inflammation occurs [25], suggesting it might be used as an early indication for the evaluation and diagnosis of diseases [26]. In the present study, we found that, based on the blood culture as the gold standard of diagnosis, the combination of the two markers showed a higher sensitivity and specificity than the single marker. However, there was still were missed diagnoses. 16s rRNA has a higher sensitivity and specificity in the detection of bacteria, so this study combined PCT and CRP with 16s rRNA to analyze their diagnostic significances in NS. It was confirmed that their combination obviously improved the sensitivity and specificity, thus providing a basis for the early diagnosis of NS. However, due to the limited number of patients enrolled in the present study, a large cohort clinical study is required to confirm their diagnostic value in the future.

Conclusion

The combination of PCT, CRP and 16s rRNA can improve the diagnostic efficiency of NS. Their combination is simple and rapid and can provide early sensitive diagnostic methods for NS, which can help to evaluate the therapeutic effect of the disease.

Disclosure of conflict of interest

None.

Address correspondence to: Dr. Jin Shi, Department of Pediatrics, Edong Healthcare Group, Huangshi Maternity and Children's Health Hospital, No. 80, Guilin South Road, Huangshi 435000, Hubei, China. Tel: +86-0714-6353505; Fax: +86-0714-6353505; E-mail: doulian108999@126.com

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