RESEARCH ARTICLE

Serum PCR in diagnosis of neonatal sepsis caused by bacteria and enteroviruses.

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Abstract
Although blood culture is considered the gold standard for detection of neonatal infections of bloodstream, its result can take from 48-72 h and has low sensitivity. In this study, we determined the usefulness of the panbacterial polymerase chain reaction (PCR) and the panviral RT-PCR in serum, as tests for the early diagnosis of neonatal sepsis. We studied 195 patients with the clinical diagnosis of neonatal sepsis, up to the age of 28 days, hospitalized in the Neonatal Intensive Care Unit (NICU) of the *Hospital Infantil de México "Federico Gómez"*, and 195 umbilical cord samples obtained from healthy newborns. Blood cultures, and DNA / RNA extraction were done with a system of primers of the rRNA 16s region, which is highly conserved in a large number of bacteria and a highly conserved non-coding enteroviral 5´ (5'-NCR) region, respectively. The results showed that among the 90 newborns with sepsis and negative PCR, 19 were randomly selected to perform RT-PCR; 26% of them had a positive result. Statistical analysis included the PCR test's sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV) using the clinical diagnosis as the gold standard. PCR sensitivity was 77%, specificity was 83%, PPV was 92% and the NPV was 59%. In summary, panbacterial PCR is useful, quick and effective in the diagnosis of sepsis and should be used as a complement to the microbiological and clinical diagnoses. Although the frequency of enteroviral sepsis was low, in newborns with a negative panbacterial PCR, a panviral RT-PCR is recommended. Our results indicate that molecular diagnosis provides the balance of cost-benefit.

Keywords: Early diagnosis; Neonatal sepsis; Polymerase chain reaction; Reverse transcriptase polymerase chain reaction.

1. Introduction

Sepsis was defined as the presence of infection along with other listed general systemic signs and symptoms[1]. It is still one of the main infectious problems in the neonatal intensive care unit (NICU), with high associated mortality rates ranging between 10% -30%. It is the most common cause of neonatal death, so early diagnosis and treatment are paramount[2, 3]. Newborns are at increased risk of infection as a result of, among others, immunological immaturity[4]. In Mexico, the causes of death during the 1st week of life are bacterial sepsis or congenital pneumonia. After the 1st week, bacterial sepsis predominates in terms of frequency. An incidence of 4 to 15.4 cases per 1000 liveborns has been reported. It is estimated to develop in 2.3% of all births[5].

Blood culture is the definitive tool used in the diagnosis of neonatal sepsis[6, 7].

However, this gold standard method is time-consuming and may yield false positive as well as false negative results. Blood culture limitations have spurred the search of biomarkers in the diagnosis of neonatal sepsis. The combination of several biomarkers such as the total neutrophil count, immature to total (I/T) neutrophil ratio, and C-reactive protein (CRP) promises a quick and exact diagnosis. The sequential detection and follow-up of CRP allows the exclusion of microbial infections, thus precluding the use of unwarranted antibiotic therapy[8].

Recently, molecular methods have surged as promising diagnostic tools in neonatal sepsis. The polymerase chain reaction (PCR), is a technology based on microbial DNA extraction from blood samples and it has been proven to recognize panbacterial sequences such as those associated to the rRNA 16S gene[9, 10].

Further, enterovirus infection is the most frequently diagnosed viral infection in the neonatal period and it is associated with a broad spectrum of signs and symptoms, ranging from a non-specific febrile illness to potentially deadly multisystem involvement. The most common symptoms include fever, irritability, lethargy, anorexia and skin rash. Severe disease may include sepsis and / or meningoencephalitis that can develop during the first 2 weeks of life and is deadly[11, 12]. The diagnosis can be established with the reverse transcriptase polymerase chain reaction (RT-PCR) in blood and cerebrospinal fluid.

The aim of this study was to determine the usefulness of the panbacterial PCR and panviral RT-PCR as diagnostic tests of neonatal sepsis.

2. Materials and methods

We studied 195 neonatal patients up to the age of 28 days, hospitalized in the Neonatal Intensive Care Unit (NICU) of the *Hospital Infantil de México Federico Gómez* (HIMFG), with the clinical diagnosis of neonatal sepsis. As controls, we obtained 195 blood samples in which the absence of microorganisms was confirmed by blood culture; these were provided by the umbilical cord hematopoietic stem cell Bank, of the Central Blood Bank at the *Centro Médico Nacional La Raza, IMSS*, in Mexico City, in 2015–2016. The protocol was accepted by the local Research and Ethics Committee of the Hospital, and informed consent was obtained from all parents or guardians of participants included in the study.

2.1 Clinical diagnosis of neonatal sepsis

There is no validated scale for the diagnosis of neonatal sepsis based on risk factors and non-specific clinical and laboratory parameters. Signs and symptoms of sepsis refer to the presence of at least two of the following criteria, one of which should be fever or an abnormal total leukocyte count[1, 13, 14]: Temperature > 38.5° C or < 36° C**,** tachycardia, **a**pnea, intercostal retractions,

cyanosis, increased or decreased leukocyte count, neutrophilia, bands > 10%, immature to total neutrophil ratio (I/T) > 0.2, hematocrit < 40, platelets < 150 000.

2.2 Inclusion criteria

Newborn patients with changes in breathing, heart rhythm, ventilation and heart rate, changes in body temperature, abnormalities in skin perfusion, cold extremities and a general sickly appearance of the baby. They may also present food intolerance, vomiting and / or diarrhea.

2.3 Exclusion criteria

Samples of patients who do not have blood culture. Samples of patients in whom blood collection is not conducted with the appropriate antisepsis. Samples of patients in whom the amount of sample obtained is insufficient.

The following maneuvers were performed on patients under aseptic conditions: venipuncture or catheter aspiration, obtaining approximately 500 μL of blood for PCR and RT-PCR. Among the 90 newborns with a negative PCR, 19 were chosen at random to conduct RT-PCR. Also, the entire laboratory panel and complete blood count were obtained as well as blood cultures (BacT / Alert^{TM}), before initiating antibiotic therapy; the same tests were conducted in the control cord blood samples. All procedures were performed in the Hospital's laboratories by authorized personnel and following conventional techniques. The control samples were also tested by PCR and 19 samples were tested by RT-PCR. Due to the low sensitivity of blood cultures, the gold standard was the clinical diagnosis including clinical and laboratory parameters as well as blood culture.

2.4 DNA/RNA extraction. We were particularly careful during and after obtaining the samples to avoid environmental contamination. For example, the skin surface was cleaned with 70% isopropyl alcohol + 2% chlorhexidine pad before obtaining the samples and these were immediately placed in sterile microcentrifuge tubes. The DNA/RNA was isolated with guanidine isothiocyanate and phenol using 500 μL of Trizol reactant (Gibco BRL) in accordance with the procedure described by Chomczynski[15]. This method is based on the use of a reactant containing phenol and guanidine isothiocyanate. The biological sample (serum) is homogenized in the reactant and simultaneous RNA and DNA isolation is accomplished in one step through liquid-phase separation. RNA isolation can be completed in approximately 1 hour and that of DNA, in approximately 3 hours. DNA was resuspended in 50 lμ of distilled water after precipitation with ethanol 75%. This solution was heated to 55º C for 20 min. Absorbance was detected at 260/280 nm.

2.5 Panbacterial PCR. The broad range PCR primers have been previously used to detect DNA[16, 17]. Briefly, this is a non-annealing assay system using two primers: 5'-GCGTTAATCGGAATTACTGGGCGTAAG-3 'and 5'-GGTTGCGCTCGTTGCGGGACTTAACC-3'. The cycle parameters were 4 min at 95º C, after 35 cycles of 1 min at 95º C, 1 min at 52º C, 1 min at 72° C and finally, 10 minutes at 72° C. This primer system is known as panbacterial because it amplifies sequences of 16S ribosomal RNA gene (rRNA) from a number of bacterial species. As designed, the system amplifies a ~ 577 pb DNA fragment (depending on the microorganism)[17] that encompasses the region with the 501-1,077 nucleotides in the standard 16S rRNA gene (*Escherichia coli*). Design of consensus primers based on this highly conserved DNA sequenced regions

allows amplification of rDNA coding sequences from many, although not all, bacterial rRNA 16S genes, determined by a homology search of primer sequences in GenBank as follows: Bacillus; Borrelia; Campylobacter; Escherichia; Listeria; Neisseria; Proteus; Rickettsia; Salmonella; Staphylococcus; Yersinia; Bordetella; Chalmydia; Klebsiella; Mycoplasma; Pseudomonas; Shigella; Serratia; Streptococcus, and others. Its sensitivity allowed the detection of bacterial cells in the 10-50 cell range.

In every run, water controls were inserted (i.e., without adding the template) between the analyzed samples. Pure *E. coli* DNA was used as a positive control in the panbacterial screening system. Amplification products were analyzed on agarose gels stained with ethidium bromide. All assays were performed in duplicate.

2.6 Panenteroviral RT-PCR. Primer sequences were selected from highly conserved regions of the non-coding 5' (5'-NCR) region[18, 19]. Primers consisted of 20 to 21 bases with 100% homology with the known enteroviral RNA sequences.

Briefly[20], a 50 μL reaction mixture was prepared. The reaction mixture consisted of 50 mM KCl, 10 mM Tris-HCl (pH 8.9), 3.6 mM $MgCl_2$, 10 mM of each deoxynucleoside triphosphate, (Access Quick™ RT-PCR system), 5 U of reverse transcriptase of the avian myeloblastosis virus (Promega ™), 5 U of *Tfl* DNA polymerase, 20 pmol of each 1 (5´-CAAGCACTTCTGTTTCCCCGG-3´) and 3 (5´-ATTGTCACCATAAGCAGCCA-3´) primer and RNA isolated from the sediment of our clinical samples. After incubation at 37º C for 60 min, the sample was denatured at 94º C for 5 min. Amplification was performed in 40 cycles in which samples were denatured for 1 min at 94º C, primers were aligned for 1 min at 42º C and extension lasted 2 min at 72º C. The obtained product consisted of 440 bp. In all runs, a positive and a negative control were used. Reactions were analyzed by electrophoresis in 2% agarose gels.

2.7 Semi-nested PCR. A second set of 2 (5´-TCCTCCGGCCCCTGAATGCG-3´) and 3 primers were used to conduct the PCR with the previously obtained product. A 155 bp PCR product was generated.

The test had been previously validated[19]. Analytic sensitivity was 0.1 fg of the Coxsackie B3 clone that was used, the approximate equivalent to 10 genomes.

3. Results

In the study patient group, 37% were male and 63% were female. In the control group, genders were equally represented.

The most frequently detected risk factors in the patient group with a diagnosis of sepsis were the following: use of a catheter (47%); mechanical ventilation (42%); low birth weight (20%) and surgical procedures (15%). There were no risk factors in the control group. The following changes were observed in serum and hematologic tests (Table 1).

Table 1. Neonatal data, main clinical signs and laboratory parameters.

Data	Media (rank)
Gestational age (weeks)	35.6 (28-41)
Age at onset of sepsis (days)	25 (1-28)
· Main clinical signs	
Dysthermia	82%
Respiratory alterations	68%
·Laboratory parameters	
Neutrophilia	59%
Immature to total neutrophil ratio >0.2	24%
Hematocrit <40	72%
Platelets < 150000	54%

Blood cultures were positive in 54 (28%) cases. The most frequently isolated bacterial species was *Staphylococcus epidermidis* (30 samples) as a single microorganism, and it was associated to *Staphylococcus auricularis* in one sample. *Enterococcus faecalis* (8 samples); *Escherichia coli* (5 samples); *Staphylococcus haemolyticus* and *Staphylococcus hominis* (3 samples); *Enterobacter cloacae* (2 samples); *Listeria monocytogenes*, *Staphylococcus saprophyticus* and *Staphylococcus auricularis* (1 sample of each).

Considering the number of patients with a clinical diagnosis of sepsis established by the clinician and detected by panbacterial PCR, in 195 problem cases the 577 bp band was detected in 105 cases (54 %). Each problem sample was processed paired with a control sample with a negative blood culture (Figure 1).

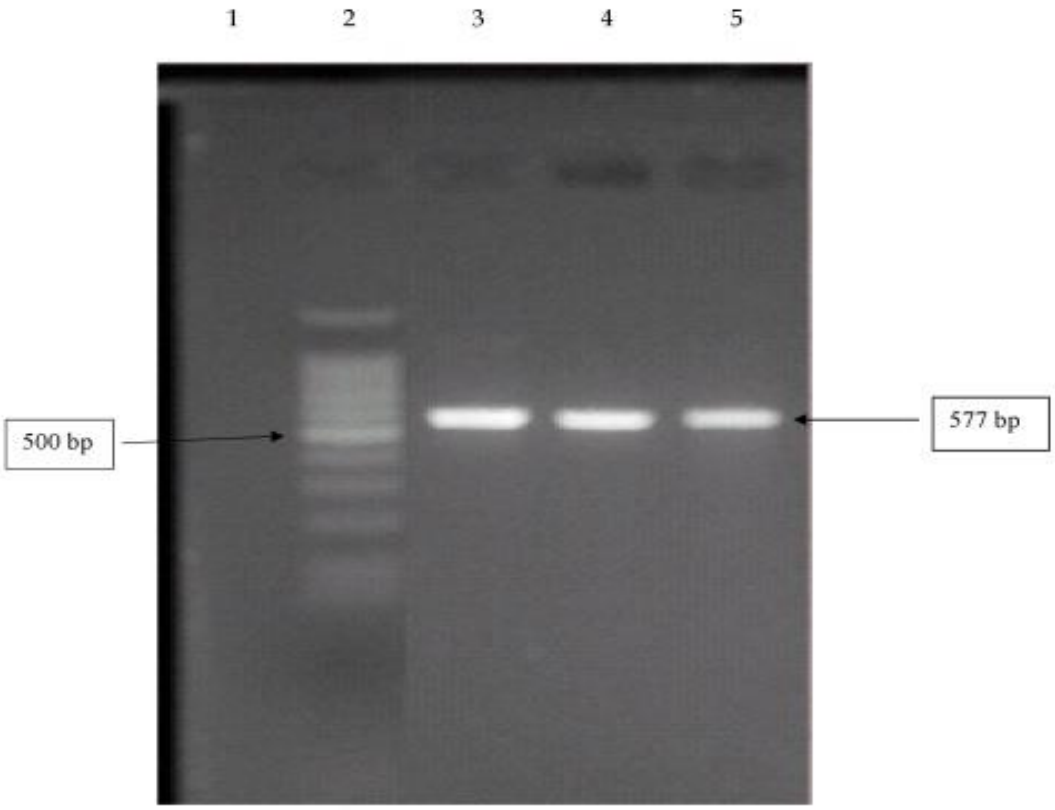

Figure 1.

Fig. 1 Gel electrophoresis 1.5% agarose. Lane1, water (negative control); lane 2, 100 bp (Promega) marker; lane 3, positive control (*E coli*); lane 4 and 5, serum samples positive patients

Table 2. Comparison between the results of the final clinical diagnosis (clinical sepsis) vs blood culture BACTEC™ PED PLUS BD vs panbacterial PCR.

PCR	Blood culture	Final clinical diagnosis	n=195
Negative	Negative	Negative	45
Positive	Positive	Positive	49
Positive	Negative	Positive	60
Negative	Negative	Positive	17
Negative	Positive	Positive	14
Positive	Negative	Negative	10
Positive	Positive	Negative	0
Negative	Positive	Negative	0

Table 2 shows the comparison of results with both methods and the final diagnosis. The molecular diagnosis (PCR) further supports the clinical diagnosis of sepsis by a factor of four compared to blood culture and using the same gold standard.

With these results, we calculated the test´s sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV) for PCR and blood culture, with traditional 2 x 2 contingency tables. We considered the clinical diagnosis as the gold standard and determined the usefulness of PCR in the confirmation of the clinical diagnosis, as a diagnostic support, and in treatment reestablishment in cases with a positive test and compatibility with other laboratory tests and the medical examinations (Table 3).

Table 3. Statistical values obtained from panbacterial polymerase chain reaction (PCR) and blood culture BACTEC™ PED PLUS BD compared with the clinical diagnosis.

N=195	Panbacterial PCR	Blood culture BACTEC™ PED PLUS BD
Sensitivity	77%	44%
Specificity	83%	100%
Efficacy (Youden index)	0.60	0.44
Positive predictive value	92%	100%
Negative predictive value	59%	41%
Accuracy	79%	60%

With these results, we proved the advantages of PCR over blood culture, such as sensitivity, efficacy and accuracy – among others -; it also has limitations such as bacterial identification but PCR appears to be an ideal complement to blood culture.

Subsequently, among the 90 newborns with sepsis and negative PCR, 19 were randomly selected to perform a panenteroviral RT-PCR; 26% has a positive RT-PCR. In positive controls, the initially expected 440 bp band was amplified, while in the negative control and in the serum sample from patient 71 suspected of harboring enteroviral sepsis, this band was not observed (Figure 2). In order to increase the test´s sensitivity, a second semi-nested PCR was performed with the initial amplification product; a 155 bp product was obtained in the serum of patients 71-73, 75 and 78, thus confirming its positivity (Figure 3).

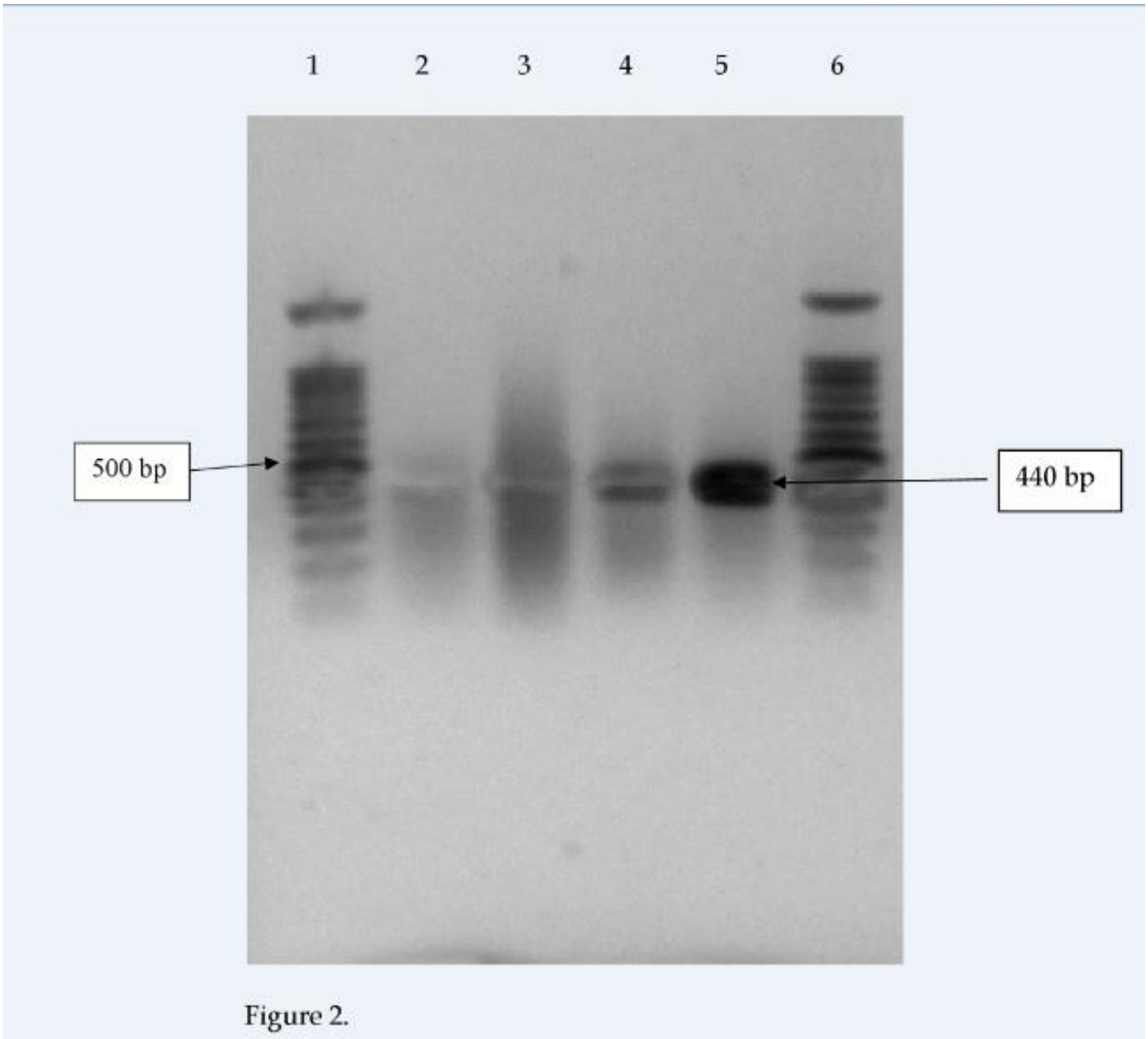

Fig. 2 Gel electrophoresis 2% agarose stained with ethidium bromide. Lanes 1 and 6, 100 bp (Promega) marker; lane 5, positive control (Echovirus 30); lane 4, positive control (Echovirus 30) diluted 1: 100; lane 3, serum sample patient 71; lane 2, water (negative control)

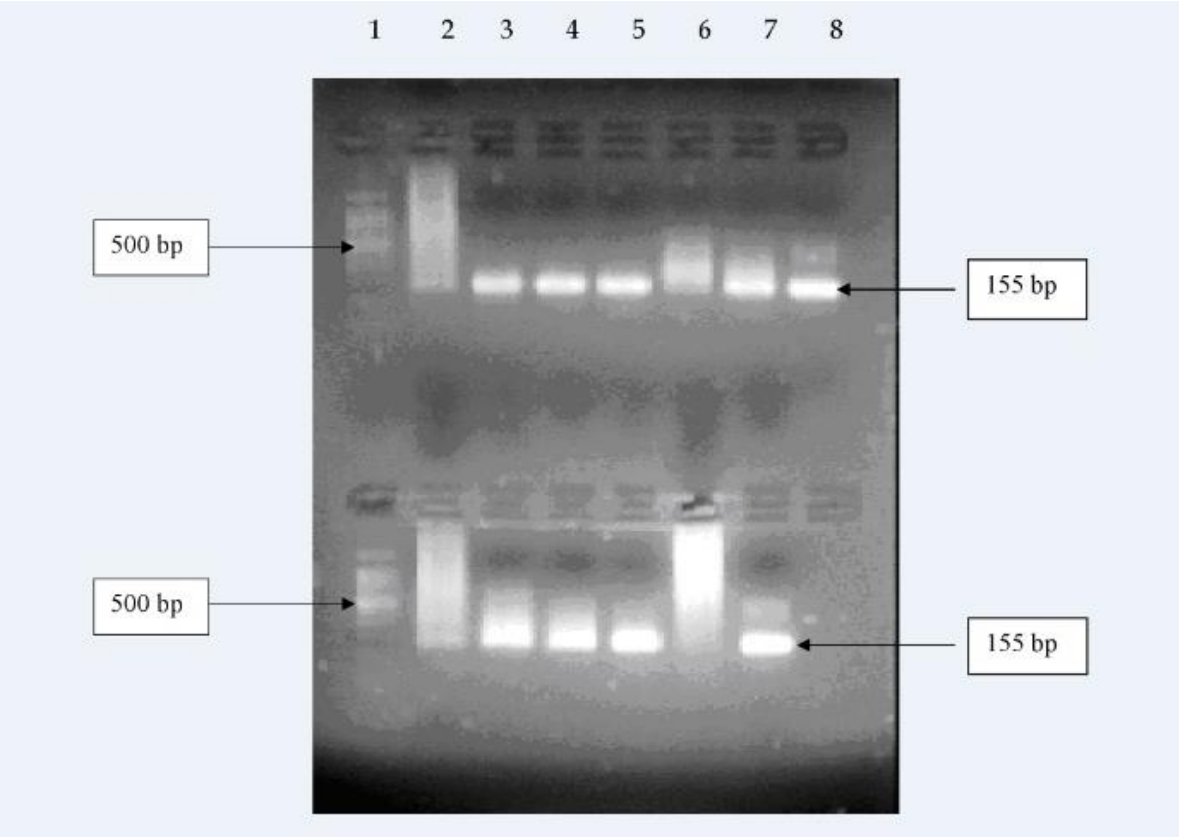

Fig. 3 Semi-nested PCR electrophoresis. Top: lane 1, 100 bp marker; lanes 3-5 and 7, serum samples positive patients 71-73 and 75; lane 8, positive control (Echovirus 30). Lower: lane 1, 100 bp marker; lane 5, serum sample positive patient 78; lane 7, positive control (Echovirus 30); lane 8, negative control

4. Discussion

Female patients were more frequent than males in our study group. Our results diverge from those reported in the literature[13, 21]. As in most reported cases, clinical signs were characterized by their variety and non-specificity. The main clinical manifestations were dysthermia, as reported by other authors[22, 23] and respiratory abnormalities[13, 24].

Significant laboratory abnormalities included a hematocrit <40 and neutrophilia, in agreement with other authors[25]. Microorganisms were obtained in 28% of blood cultures, as reported by others[3].This low rate of microorganism recovery was concerning but could result from the small blood volumes that were cultured. The recommended blood sample to culture medium ratio is 1:10, and a minimum of 2 blood cultures should be obtained in order to increase microorganism detection. This was difficult in most of our patients due to their weight, associated pathologies, etc. Other possible causes could be the transient nature of bacteremia or low microorganism density.

The most frequently isolated microorganism was coagulase-negative *Staphylococcus* (CoNS) in 70% of positive blood cultures. This finding coincides with results previously published by our group[16] in Mexico[5] and in other countries[26-29], where it is isolated in approximately 47- 78% of

positive blood cultures. CoNS have emerged as predominant pathogens in late and nosocomial sepsis in industrialized countries and in some developing regions. In terms of toxin production, CoNS are not as virulent as Gram negative bacteria and fungi, which partially explains the low proportion of short-term infectious complications and deaths.

Among CoNS, 8% were *S hominis*, and responsible for 48% of late appearing sepsis in low birth weight newborns[30].However, there are few reports of nosocomial dissemination and may thus be ignored; since this is the second report in our national literature on the subject, it is pivotal to recognize this microorganism as an important pathogen in the NICU setting. Our study cases with *S hominis* were all low weight newborns.

The second most frequently isolated microorganism was *Enterococcus faecalis* (15%), unlike reports by other authors. Enterococcus is the main infection-causing species and a cause of nosocomial infection[31]. Its habitat is the digestive tract and it is present in adult feces in >90%; by one week of age, newborns are colonized and the microorganism may lead to septicemia outbreaks in the NICU. Our study cases infected by *Enterococcus faecalis*, had fever and prolonged hospitalizations.

In third place and in accordance with other authors[5, 26] we isolated Gram negative bacilli (13%).

Our group previously reported that in neonatal sepsis, a Gram-stained buffy coat smear yielded good specificity when considering blood culture as the gold standard, as well as when the gold standard was the clinical diagnosis[16].

Panbacterial PCR is an ideal complementary test to blood culture[32]. 16S rRNA PCR increased the sensitivity of bacterial DNA detection in neonates with signs of sepsis and coincided with the sensitivity, specificity and accuracy of previous report [33, 34].

Panenteroviral RT-PCR was positive in 26% of newborns with a negative panbacterial PCR. Enteroviruses are the main cause of viral infection in children. Although most infections are mild and self-limited, severe disease may develop, such as viral sepsis syndrome, myocarditis and meningoencephalitis [35-37]. Enteroviruses are also a frequent cause of fever in the newborn. The diagnosis can be established by RT-PCR in blood and yields more sensitive and timely results[33]. Our study cases with neonatal enteroviral infection were all patients with fever.

Broad-range PCR assays have significant advantages when performed in conjunction with microbial cultures as a complementary test. The high specificity of PCR assay has the potential of decreasing antibiotic exposure by aiding physicians to make earlier decisions about discontinuation of antibiotics. Costs, availability of equipment and technical skills in the microbiologic laboratory are important considerations that will impact applicability[38].

5 Conclusions

From a molecular diagnostics viewpoint, it was interesting to compare the low recovery rate of microorganisms in blood cultures when the PCR yielded positive results. We thus confirmed that panbacterial PCR possesses numerous advantages over blood culture, including its sensitivity, efficacy and accuracy; it can also detect lower bacterial concentrations, microorganisms need not be viable and it shortens treatment duration. However, it has limitations such as bacterial identification, it is costly and blood culture is still necessary to determine antimicrobial sensitivity. When the laboratory has the appropiate infrastructure (safety cabinet, electrophoresis chamber, power source, UV transilluminator and image analyzer), the PCR test becomes

cheaper and provides the optimal balance of cost-benefit and effectiveness.

Enteroviral infections in newborns are frequently associated to non-specific findings and may be confused with bacterial infections. Due to the severe complications caused by enteroviral infections, physicians should diligently search for signs of sepsis, myocarditis and meningoencephalitis, particularly in cases of sterile sepsis.

DECLARATION

Conflict of interests

The authors declare that they have no conflict of interest.

The submission has not been previously published, nor presented before another journal for consideration.

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Authorship

All authors declare that they have made contributions of the idea, study design, data collection, data analysis and interpretation, critical review of the intellectual content and final approval of the manuscript that we are sending.

Ethical responsibilities

Protection of people

The authors declare that the procedures followed were in accordance with the ethical standards of the responsible human experimentation committee and in accordance with the World Medical Association and the Declaration of Helsinki.

Rigth to privacy and informed consent.

The authors have obtained the informed consent of the parents or guardians of the patients referred to in the article.

References

1. Bone RC, Grodzin CJ, Balk RA. Sepsis: a new hipótesis for patogénesis of the disease process. Chest 1997; 112:235-43. doi: 10.1378/chest.112.1.235
2. Chu SM, Hsu JF, Lee CW, Lien R, Huang HR, Chiang MC, Fu RH, Tsai MH. Neurological complications after neonatal bacteremia: the clinical characteristics, risk factors, and outcomes. PLoS ONE 2014; 9: e105294. doi: 10.1371/journal.pone.0105294
3. Rohit A, Maiti B, Shenoy S, Karunasagar I. Polymerase chain reaction- restriction fragment length polymorphism (PCR-RFLP) for rapid diagnosis of neonatal sepsis. Indian J Med Res 2016; 143: 72-78. doi: 10.4103/0971-5916.178613
4. Pérez RO, Lona JC, Quiles M, Verdugo MA, Ascencio EP, Benítez EA.
   Early neonatal sepsis, incidence and associated risk factors in a public hospital in western Mexico. Rev Chilena Infectol 2015; 32: 387-392. doi: 10.4067/S0716-10182015000500003
5. Leal YA, Álvarez-Nemegyei J, Velázquez JR, Rosado-Quiab U, Diego-Rodríguez N, Paz-Baeza E et al. Risk factors and prognosis for neonatal sepsis in southeastern Mexico: analysis of a four-year historic cohort follow-up. BMC Pregnancy Childbirth 2012; 12:48. doi: 10.1186/1471-2393-12-48
6. Korber F, Iris Zeller I, Grünstäudl M, Willinger B, Apfalter P, Hirschl AM et al. SeptiFast versus blood culture in clinical routine – A report on 3 years experience. Wien Klin Wochenschr 2017; 129:427-434. doi: 10.1007/s00508-017-1181-3
7. StraubJ, Paula H, Mayr M, Kasper D, Assadian O, Berger A et al. Diagnostic accuracy of the ROCHE Septifast PCR system for the rapid detection of blood pathogens in neonatal sepsis – A prospective clinical trial. PLoS ONE 2017; 12: e0187688. https://doi.org/10.1371/journal.pone.0187688.
8. Dong Y, Speer CP. The role of Staphylococcus epidermidis in neonatal sepsis: guarding angel or pathogenic devil? Int J Med Microbiol 2014; 304:513-20. doi: 10.1016/j.ijmm.2014.04.013
9. Dutta S, Narang A, Chakraborty A, Pallab Ray P. Diagnosis of Neonatal Sepsis Using Universal Primer Polymerase Chain Reaction Before and After Starting Antibiotic Drug Therapy. Arch Pediatr Adolesc Med 2009; 163:6-11. doi: 10.1001/archpediatrics.2008.513
10. Liu CL, Ai HW, Wang WP, Chen L, Hu HB, Ye T et al. Comparison of 16S rRNA gene PCR and blood culture for diagnosis of neonatal sepsis. Arch Pediatr 2014; 21:162-9. doi: 10.1016/j.arcped.2013.11.015
11. Lindamood KE, Fleck P, Narla A, Vergilio JA, Degar BA, Baldwin M et al. Neonatal enteroviral sepsis/meningoencephalitis and hemophagocytic lymphohistiocytosis: Diagnostic challenges. Am J Perinatol 2010; 28:337-46. doi: 10.1055/s-0030-1268710
12. Schlapbach LJ, Ersch J, Balmer C, Prêtre R, Tomaske M, Caduff R et al. Enteroviral myocarditis in neonatos. J Pediatr Child Health 2013; 49: E451–E454. doi: 10.1111/jpc.12248
13. Bhat AA, Shamim A, Gul S, Akther R, Bhat I. Neonatal sepsis- Early detection comparing Procalcitonin and CRP as markers and newer tools.

IJMDS 2016; 5:1048-1055. doi:10.19056/ijmdsjssmes/2016/v5i1/83574

14. Chandna A, Rao MN, Srinivas M, Shyamala S. Rapid diagnostic tests in neonatal septicemia. Indian J Pediatr 1988; 55:947-53. doi: 10.1007/BF02727835
15. Chomczynski P. A reagent for the single-step simultaneous isolation of RNA, DNA and proteins from cell and tissue samples. Biotechniques 1993; 15: 532-7.
16. García-Elorriaga G, Cortés-Torres N, Ballesteros-del Olmo JC, del Rey-Pineda G, González-Bonilla C. The usefulness of the buffy coat smear and panbacterial polymerase chain reaction in early diagnosis of neonatal sepsis. Rev Invest Clin 2012; 64: 275-283.
17. Gérard HC, Wang Z, Wang GF, El-Gabalawy H, Goldbach-Mansky R, Li Y et al. Chromosomal DNA from a variety of bacterial species is present in synovial tissue from patients with various forms of arthritis. Arthritis Rheum 2001; 44: 1689-97. doi: 10.1002/1529-0131(200107)44:7<1689::AID-ART293>3.0.CO;2-K
18. Iizuka N, Kuge S, Nomoto A. Complete nucleotide sequence of the genome of coxsackie B1. Virology 1987; 156: 64-73. doi: 10.1016/0042-6822(87)90436-3
19. Zoll GJ, Melchers WJF, Kopecka H, Jambroes G, Van der Piel HJA, Galama JMD. General primer-mediated polymerase chain reaction for detection of enterovirus: application for diagnostic routine and persistent infections. J Clin Microbiol 1992; 30: 160-165. doi: 10.1128/JCM.30.1.160-165.1992
20. García-Elorriaga G, Esparza-García A, Méndez-Rojas C, del Rey-Pineda G, González-Bonilla C. CSF RT-PCR standardization in pediatric patients with enteroviral central nervous system infection. Rev Invest Clin 2012; 64: 59-66.
21. Tsai MH, Chu SM, Lee CW, Hsu JF, Huang HR, Chiang MC et al. Recurrent late-onset sepsis in the neonatal intensive care unit: incidence, clinical characteristics and risk factors. Clin Microbiol Infect 2014; 20: O928–O935. doi: 10.1111/1469-0691.12661
22. Chiabi A, Takou V, Mah E, Nguefack S, Siyou H, Takou V et al. Risk Factors for Neonatal Mortality at the Yaounde Gynaeco-Obstetric and Pediatric Hospital, Cameroon. Iran J Pediatr 2014; 24: 393-400.
23. Tsai MH, Chu SM, Hsu JF, Lien R, Huang HR, Chiang MC et al. Polymicrobial bloodstream infection in neonates: Microbiology, clinical characteristics, and risk factors. PLoS ONE 2014; 9: e83082. doi: 10.1371/journal.pone.0083082
24. Tsai MH, Chu SM, Hsu JF, Lien R, Huang HR, Chiang MC et al. Risk factors and outcomes for multidrug-resistant Gram-Negative bacteremia in the NICU. Pediatrics 2014; 133: e322-e329. doi: 10.1542/peds.2013-1248
25. Bhandari V, Wang C, Rinder C, Rinder H. Hematologic profile of sepsis in neonates: neutrophil CD64 as a diagnostic marker. Pediatrics 2008; 65:19-25. doi: 10.1542/peds.2007-1308
26. Lahra MM, Beeby PJ, Jeffery HE. Intrauterine inflammation, neonatal sepsis, and chronic lung disease: a 13-year hospital cohort study. Pediatrics 2009; 123:1314–19. doi: 10.1542/peds.2008-0656
27. van den Hoogen A, Gerards LJ, Verboon-Maciolek MA, Fleer A, Krediet TG. Long-term trends in the epidemiology of neonatal sepsis and

antibiotic susceptibility of causative agents. Neonatology 2010; 97:22–8. doi: 10.1159/000226604

28. Borges Peixoto P, Henrique Massinhani F, Netto Dos Santos KR, Cardoso Chamon R, Beatriz Silva R, Lopes Correa FE et al. Methicillin-resistant *Staphylococcus epidermidis* isolates with reduced vancomycin susceptibility from bloodstream infections in a neonatal intensive care unit. J Med Microbiol.2020; 9:41-45.doi: 10.1099/jmm.0.001117
29. Huggard D, Powell J, Kirkham C, Power L, O'Connell NH, Philip RK. Time to positivity (TTP) of neonatal blood cultures: a trend analysis over a decade from Ireland. J Matern Fetal Neonatal Med. 2021; 34:780-786.doi: 10.1080/14767058.2019.1617687
30. Chaves F, García- Alvarez M, Sanz F, Alba C, Otero JR. Nosocomial spread of a *Staphylococcus hominis* subsp. *novobiosepticus s*train causing sepsis in a neonatal intensive care unit. J Clin Microbiol 2005; 43:4877–4879. doi: 10.1128/JCM.43.9.4877-4879.2005
31. Furtado I, Niz Xavier PC, Martinelli Tavares LV, Alves F, Fonseca Martins S, de Sousa Martins A et al. Enterococcus faecium and Enterococcus faecalis in Blood of newborns with suspected nosocomial infection. Rev Inst Med Trop. Sao Paulo 2014; 56:77-80. doi: 10.1590/S0036-46652014000100012
32. Reier-Nilsen T, Farstad T, Nakstad B, Lauvrak V, Steinbakk M. Comparison of broad range 16S rDNA PCR and conventional blood culture for diagnosis of sepsis in the newborn: a case control study. BMC Pediatrics 2009; 9:5. doi: 10.1186/1471-2431-9-5
33. El Gawhary S, El-Anany M, Hassan R, Ali D, El Gameel el Q. The Role of 16S rRNA Gene Sequencing in Confirmation of Suspected Neonatal Sepsis. J Trop Pediatr 2016; 62:75-80. doi: 10.1093/tropej/fmv066
34. El-Amir MI, El-Feky MA, Abo Elwafa DA, Abd-Elmawgood EA. Rapid diagnosis of neonatal sepsis by PCR for detection of 16S rRNA gene, while blood culture and PCR results were similar in *E.coli*-predominant EOS cases. Infect Drug Resist. 2019; 12:2703-2710. doi: 10.2147/IDR.S213958
35. Morriss FH Jr, Lindower JB, Bartlett HL, Atkins DL, Kim JO, Klein JM et al. Neonatal Enterovirus Infection: Case Series of Clinical Sepsis and Positive Cerebrospinal Fluid Polymerase Chain Reaction Test with Myocarditis and Cerebral White Matter Injury Complications. Am J Perinatol Rep 2016; 6:e344-e351. doi: 10.1055/s-0036-1593406
36. Schröder D, Siauw C, Wirbelauer J. Rare Differential Diagnosis of a Neonatal Bacterial Sepsis: The Neonatal Viral Myocarditis. Z Geburtshilfe Neonatol. 2018; 222:82-85. doi: 10.1055/s-0043-121222
37. Chuang YY, Huang YC. Enteroviral infection in neonates. J Microbiol Immunol Infect. 2019; 52:851-857. doi: 10.1016/j.jmii.2019.08.018
38. Pammi M, Flores A, Versalovic J, Leeflang MM. Molecular assays for the diagnosis of sepsis in neonates. Cochrane Database Syst Rev. 2017; 2:CD011926. doi: 10.1002/14651858.CD011926