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Diagnostic accuracy of buffy coat culture compared to total blood culture in late-onset sepsis of the newborn☆

Víctor Javier Lara-Díaz [a,*], Jessica De La Vega-Méndez [a,b], Víctor Arízaga-Ballesteros [a], Beatriz R. Tinoco-Torres [b], Jorge Eugenio Moreno-Cuevas [a]

[a] Tecnológico de Monterrey, Escuela de Medicina y Ciencias de la Salud, Programa Multicéntrico de Neonatología, ITESM y Cátedra de Terapia Celular, Ave. Ignacio Morones Prieto 3000 poniente, Edificio CITES, tercer piso, oficina 307, Col. Doctores, CP 64710, Monterrey, Nuevo León, Mexico
[b] Servicios de Salud del Estado de Nuevo León, Hospital Metropolitano "Dr. Bernardo Sepúlveda", Servicio de Laboratorio Clínico, Monterrey, Nuevo León, Mexico

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SUMMARY

Objectives: To study the potential of buffy coat culture as a diagnostic tool for neonatal late-onset sepsis.
Methods: This was a study of diagnostic accuracy in newborn infants born at 28–41 weeks of gestation, weighing >800 g, with ≥8 points on the NOSEP-1 scale. Paired samples for total blood culture (TBC) and buffy coat culture were drawn. We established the positivity rate, sensitivity, specificity, predictive values, and likelihood ratios, and compared time to positivity and contamination rates.
Results: Fifty-two newborns were included in the study. Twenty-one TBC and 22 buffy coat cultures were positive. The positivity rate for TBC was 40.4% and for buffy coat culture was 42.3% (p = not significant). Three TBC were positive with negative buffy coat culture. Four buffy coat cultures were positive with negative TBC; Kappa agreement was 0.723, p <0.001. Buffy coat culture sensitivity was 86% (95% confidence interval (CI) 68.5–95.4%), specificity 87% (75.4–93.7%), positive predictive value 82% (65.4–91.1%), negative predictive value 90% (77.9–96.8%), positive likelihood ratio 6.64 (2.79–15.05), and negative likelihood ratio 0.16 (0.05–0.42). We found no difference in time to positivity in hours; Wilcoxon Z = 1224, p = 0.22. The contamination rate was 1.9% for both methods.
Conclusions: Buffy coat culture is as good as TBC for the microbiological diagnosis of late-onset sepsis of the newborn. Buffy coat culture allows the use of remaining plasma for further analysis.

1. Introduction

Neonatal late-onset sepsis (LOS), also known as nosocomial sepsis, occurring after 3 days of age (although some authors consider it after 7 days of age), is a serious problem in neonatal intensive care. The incidence varies between 5% and 32% and depends upon birth weight, gestational age, and the type of germ isolated. It also has a high mortality, with a range of 10–55%, especially in very low birth weight neonates and those infected by Gram-negative organisms or fungi. The most commonly isolated agents are Gram-positive bacteria, and among these, coagulase-negative staphylococci account for most episodes.[1–7] A presumptive diagnosis is made with the presence of signs and symptoms of infection,[8] and confirmation with a positive total blood culture (TBC), which is considered the reference test or gold standard. However, the TBC technique in not without faults, and its diagnostic performance varies, with a sensitivity range of 30–80% and a specificity range of 70–100%.[9–15] Performance is also influenced by the volume of blood used, the culture media, and the manufacturer.[16–21]

Separation and staining of the leukocyte-rich layer of a blood sample, also known as the 'buffy coat',[22,23] which contains mononuclear cells and granulocytes, has been used as an alternative tool for the identification of microorganisms, especially in the diagnosis of tropical diseases.[24] However confidence in its use as a diagnostic tool for sepsis has not yet been attained because of its low sensitivity and specificity.[25–31] We propose the use of buffy coat culture as an alternative to TBC as a new diagnostic tool for LOS in newborns. To our knowledge, there has been no prior attempt to evaluate the diagnostic performance of buffy coat culture for the detection of neonatal LOS compared to the current gold standard, TBC. We hypothesized that the buffy coat culture would perform as well as the reference method (TBC).

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* Corresponding author. Tel.: +52 81 8888 2147; fax: +52 81 8888 2052.
E-mail address: lara-diaz.vj@itesm.mx (V.J. Lara-Díaz).

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2. Materials and methods

This was a prospective study of the diagnostic accuracy of a new test (buffy coat culture) compared to the reference test (TBC) in neonatal LOS.

2.1. Patients

The study population consisted of all newborns admitted to the neonatal intensive care units (NICUs) of Hospital Materno Infantil de Alta Especialidad and Hospital Metropolitano "Dr. Bernardo Sepúlveda", in the metropolitan area of Monterrey, Nuevo León, Mexico, during the period July 2010 to March 2011. Both hospitals are part of the network of the Secretaría de Salud del Estado de Nuevo León. The study protocol was approved by the research ethics committees of both hospitals and the research ethics committee of Escuela de Medicina y Ciencias de la Salud, Tecnológico de Monterrey. Informed consent was obtained from the parents of all the subjects included.

The selection of patients was done sequentially during morning visits among hospitalized newborns who were suspected for LOS, based on a score of $\geq$8 points on the NOSEP-1 scale;[32] this scale has been validated in our population.[33] Attending physicians did not participate as researchers, but as patient recruiters. The medical researchers were not involved in clinical care decisions. Laboratory personnel remained blinded to the clinical diagnosis of the patients included in the study.

Neonates of both genders were included, born at any gestational ege, who were $\geq$4 days old, and who were being treated in the NICUs of the study hospitals. We excluded patients whose guardians refused to participate and infants weighing less than 800 g, as the extraction of blood for research purposes should not exceed 8% of the estimated blood volume, and at this particular weight the resulting volume would be insufficient for the study and would require an action to replace the blood drawn. Those patients from whom it was not possible to obtain adequate samples were also excluded, as well as those whose samples were damaged during the laboratory process.

2.2. Methods

Paired 2–3-ml blood samples were drawn at the same time and by the same route (peripheral puncture, central line, or percutaneously inserted central catheter). The first sample was used for the reference standard method, TBC, and was inoculated into pediatric blood culture BACTEC PEDS PLUS/F bottles, following the manufacturer's recommendations. The second sample was kept in a sterile tube with ethylenediaminetetraacetic acid (EDTA) (Vacutainer, Cat. No. 367-861) in order to proceed to the buffy coat preparation. This was carried out as follows: the sample was centrifuged at 3500 rpm for 10 min at room temperature in a VanGuard Hamilton Bell V6500 Centrifuge or Mx PowerSpin UNICO centrifuge. This separated the whole blood into an upper layer (plasma), an intermediate layer (rich in leukocytes or buffy coat), and a lower layer (erythrocyte concentrate). Under sterile conditions in a laminar flow hood, plasma was removed with a sterile syringe leaving roughly 1 mm of plasma just above the buffy coat. The buffy coat and residual plasma were slowly aspirated and re-suspended in 0.5 ml of ultrapure water (Gibco Ultrapure distilled water, Cat. No. 10977, Invitrogen) and transferred to a BACTEC PEDS PLUS/F blood culture flask. As expected, some mixing of the buffy coat with the bottom layer of erythrocytes occurred. All samples were processed by one of the authors (JDLV); incubation was done in the laboratory of the hospital at which the blood was drawn.

Cultures were considered contaminated when organisms such as *Propionibacterium acnes*, *Micrococcus* species, viridans group streptococci, *Corynebacterium* species, or *Bacillus* species, as well as when atypical organisms usually unrelated to neonatal infection, were isolated and the patient did not exhibit disease attributable to such serious pathogens. *Staphylococcus epidermidis* was considered a pathogen. The proportion of contaminated cultures in each group was recorded as previously recommended.[34] Each case with a positive TBC was considered as confirmed LOS.

All samples were analyzed in an automated BACTEC 9120 or 9050 (Becton Dickinson Cat. No. 445-800 and 445-570). Cultures were regarded as negative at 7 days if there was no evidence of bacterial growth. The culture media used were those recommended in the literature.[19,35–38] The plates were incubated as recommended: for blood agar, in an Accurate Thermo Scientific or Felisa incubator at 37 °C with 5.5% CO_2, and for MacConkey agar, Columbia agar, and potato dextrose agar, at 37 °C. We quantified the number of microorganisms on each culture plate in accordance with the Kass account, in colony-forming units (CFU) per milliliter. When the growth of two different strains was detected, both were isolated and reseeded on separate culture plates. Inoculums were then prepared to detect the specific strain and its antibiotic susceptibility. A suspension of bacterial colonies taken from the culture plates was used for a turbidity measurement and further analyzed using the VITEK 2 system (bioMérieux, Lombard, IL, USA), as published.[39] Laboratory personnel responsible for reporting the results of the cultures were unaware of the sample preparation technique.

2.3. Statistical analysis

Data were recorded on an Excel 2003 spreadsheet (Office 2003 for Windows, Microsoft Corp. Richmond, VA, USA) and then imported to SPSS v17 (SPSS Inc., Chicago, IL, USA) for analysis. The comparison of diagnostic tests was done with contingency tables, with confidence intervals (CI) at 95%. We compared the time to positivity, measured in hours, and the rate of contaminated cultures. We also measured data regarding diagnostic test performance. As data were not normally distributed, we used the Wilcoxon *Z* test or Kruskal–Wallis test. Cohen's Kappa test was used to evaluate agreement between the tests.

The sample size was calculated as 52 episodes of suspected LOS, with a test power of 80%, contrast ratios of 20%, and a one-sided hypothesis; blood samples had to be taken simultaneously to show that the new method (buffy coat) would be as good as the reference method (TBC). Detecting no difference under these conditions, we could assume that buffy coat culture was as good as TBC in terms of positivity rate, time to positivity, and contamination rates.

3. Results

During the study period, July 2010 to March 2011, there were 13 429 live births at the study hospitals. Of these, 1179 (8.8%) were admitted to the NICUs, and it was from this group that we selected candidates for the study; 54 patients were eligible. Two patients were excluded because the parents refused to participate in the study. The demographic characteristics of the 52 included patients are shown in Table 1.

As can be observed in Figure 1, a Standard for Reporting of Diagnostic Accuracy flow chart,[40] there was no significant difference in the positivity rates of the two methods ($p = 0.48$). Twenty-one of 52 TBC were positive (40.4%), while 22 of 52 buffy coat cultures were positive (42.3%) (p = not significant).

Buffy coat culture sensitivity was 86% (95% confidence interval (CI) 68.5–95.4%), specificity 87% (75.4–93.7%), positive predictive value 82% (65.4–91.1%), negative predictive value 90% (77.9–96.8%),

Table 1
Demographic characteristics of the study population ($n = 52$ neonates)

Feature	
Gestational age (weeks), mean (SD)	33 (3.9)
Birth weight (kg), mean (SD)	1.97 (1.2)
Gender, *n*	
Female	26
Male	26
Delivery, *n*	
Vaginal	14
Abdominal	38
Apgar, median (min–max)	
1 min	7 (0–9)
5 min	8 (1–10)
10 min	9 (7–10)
NOSEP-1, mean (SD)	11.8 (3.7)
Hospital	
Hospital Metropolitano	12
Hospital Materno Infantil	40

SD, standard deviation.

positive likelihood ratio 6.64 (2.79–15.05), and negative likelihood ratio 0.16 (0.05–0.42); the distribution of data is shown in Figure 1. The post-test probability was 90%.

In the analysis with Cohen's Kappa, a good agreement was shown between the two culture methods (Kappa 0.723, standard deviation (SD) 0.097 (95% CI 0.436–0.884), $p < 0.001$). Three events of negative buffy coat culture had a positive result for the reference test; coagulase-negative staphylococci were isolated from all of them, with a time to positivity around 24 h. Furthermore, four positive buffy coat cultures had a negative reference test. Organisms isolated in those buffy coat cultures were *S. epidermidis* in two, *Staphylococcus aureus* in one, and *Klebsiella pneumoniae* in one. In this set, the time to positivity for the first three was less than 3 days, and for the latter was more than 4 days. No differences in the rates of discordant results were found with Fisher's exact test.

The time to positivity, the time required for a positive culture with the methods under study, was measured in hours. We found a mean 23.14 (SD 21.8) for TBC and 31.2 (SD 29.8) for buffy coat culture. No difference in the time required for a positive result was observed: Wilcoxon's $Z = 1224$, $p = 0.22$. The rate of contaminated cultures was 1.9% in both procedures.

Blood samples were obtained at an average postnatal age of 25.6 days, with a range of 4–127 days (SD 26.4 days).

4. Discussion

The buffy coat culture technique as performed in this study has not been previously reported. There was no significant difference between the percentage of buffy coat culture positivity (42.3%) and TBC positivity (40.4%). This is the most objective estimate of the prevalence of confirmed LOS in our population. The prevalence of a positive rate in our series is similar to that reported by other researchers.[3,15,17,41] We recognize that the success rate is variable, as it depends mainly on the volume of sample used; currently the positivity rate of TBC in infants is not more than 50% at the best centers.[10,13–17,20,41] Our rate of positivity is significantly higher than that found by Lee et al., who reported 9.89% positivity in 192 samples obtained from infants with nosocomial sepsis, and that reported by Guerti et al., 15% of 2916 cultures from 1828 neonates, and is lower than the 63% rate reported by Macharashvili et al.[19,42,43]

The sensitivity and specificity of buffy coat culture (86% and 87%, respectively) are similar to those reported for TBC, which has been reported to have a sensitivity of 30–80% and specificity of 70–100%. These values are modified when more than one blood culture sample is obtained, and also depends on several factors, such as the inoculated volume, the amount of bacteria present in the sample, the incubation time, the sensitivity of the automated system, and the type of organism isolated, as some do not grow on conventional culture media and there is no single culture medium that is suitable to enhance the recovery of all microorganisms.[10,13–15,17,35,41]

Comparing the buffy coat culture to the reference test or gold standard – the traditional TBC – in order to determine sensitivity and specificity, is perhaps not the most orthodox choice, as the TBC shares the same positivity rate as the buffy coat; however, TBC is the most widely used diagnostic test available.

The test of concordance between the two techniques showed good agreement and we can affirm that the tests are similar for the isolation of microorganisms in neonatal LOS. Discordant results were distributed evenly among the two tests.

It is clear that the processing time prior to the incubation of the sample for buffy coat culture is greater than that needed for the TBC samples, so this factor was not included in the analysis. As far as we can make out, this factor seems to have no impact on the time to positivity.

As regards the time for positivity, the literature tells us that this varies depending on the organism isolated. Up to 5 days is

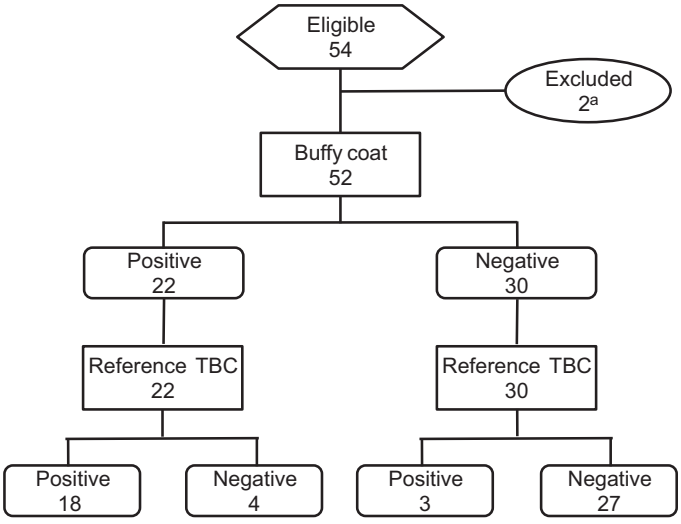

Figure 1. Flow chart of the study. The positivity rate for total blood culture (TBC) was 40.4% and for buffy coat culture was 42.3% (p = not significant); Kappa agreement 0.723, $p < 0.001$. [a]Legal guardians did not grant permission for inclusion in the study.

generally allowed for a culture to become positive, and most laboratories wait until 7 days to declare a culture as negative. However, after 5 days the cultures may be false-positives (contamination). Some bacteria and fungi may require up to 7 days, as is the case for fastidious organisms such as Legionella, Brucella, and Mycobacterium, which are rare in the neonatal period.[35] Continuous surveillance automated systems have reduced the time to positivity, but there is little information with regard to these systems in the culture of newborn infant samples. Janjindamai and Phetpisal evaluated the time to positivity in 75 infants with suspected sepsis using the BacT/Alert system. The positivity rate was 70.2% at 24 h, 91.8% at 36 h, and 95.9% at 48 h. At 36 h of incubation, the sensitivity, specificity, and negative predictive value were 70.3%, 100%, and 93.3%, respectively.[44] These results differ from those reported by Guerti et al, who noted a time to positivity of 21.33 h, interquartile range of 13.17–32.46 h. They also reported an overall positivity rate of 21.8%, 56.1%, 88.6%, and 96.6% at 12, 24, 48, and 72 h, respectively.[42]

The time to positivity could be reduced by modifying the buffy coat culture technique, perhaps by adding a step to ensure leukocyte lysis and the 'setting free' of microorganisms contained in their cytosol and vacuoles. This opens the field for future research.

Regarding the rate of contamination found, it was slightly less than that reported in the literature (a contamination rate of between 2% and 3% is generally acknowledged). This contamination is mostly related to the sampling technique, since the possibility of contamination occurring in continuous automated systems is remote, although not nonexistent.[18,45,46] Rohner and Auckenthaler report a higher contamination rate of up to 4%,[36] while Bekeris et al. reported a contamination rate of 2.89% for all cultures in a study conducted in 356 clinical laboratories. Of these, the rate of contamination in samples from neonates was 0.75% to 4.27%, with a median of 2.08%.[34] A concern of our team when we started this protocol was related to the possibility that the sample processing for buffy coat culture could increase the rate of contamination. Although the sample size in our study was not calculated to detect differences in this parameter, the finding of the same rate of 1.9% with both methods is encouraging. An in-depth review of blood culture contamination has been published recently.[46]

In sick newborn patients, the blood loss by phlebotomy is higher in relation to the total blood volume than in older patients, especially in preterm infants and during the first 2 weeks of life. Besides cultures, many other studies in blood are ordered, and therefore the optimal use of blood drawn for laboratory analysis becomes especially important in these patients.[47,48] The advantages of buffy coat culture are evident when one considers its ability to isolate the germ with the same rate as TBC; other diagnostic test rates are equal to or better than the TBC, and this modification allows the remaining plasma to be used for further analysis.

Buffy coat culture, as proposed in this study, also has the advantage of being a simple process that can be performed in any hospital with a standard microbiology laboratory, since the materials required are readily available. In contrast, highly refined techniques based on sophisticated methods or molecular biology for the detection and sequencing of bacterial ribosomal DNA, are expensive and not readily available to all microbiology laboratories.[49]

Our study is novel as there is no prior proposal of buffy coat culture in closed bottles in the case of suspected neonatal LOS or nosocomial neonatal sepsis. In subsequent studies we propose to use the plasma concomitantly for the determination of other biomarkers of sepsis. A disadvantage that should be noted is that sample acquisition should be performed by trained personnel and the whole process is more time-consuming than direct inoculation of the total blood sample into the blood culture vial. To our knowledge, this is the first study reporting the diagnostic accuracy of buffy coat culture for the microbiological diagnosis of LOS in the newborn.

In conclusion, the positivity rate and time to positivity were not significantly different between buffy coat culture and TBC, therefore buffy coat culture appears as good as TBC for the microbiological diagnosis of neonatal LOS in our study population (28–41 weeks of gestation). Additional studies are needed and should include patients with less than 28 weeks of gestation (and weighing less than 800 g) who are at a considerably higher risk for LOS. It should be noted that the buffy coat culture technique allows the use of remaining plasma for further analysis.

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This entire study was approved by the ethics and research commissions of Escuela de Medicina y Ciencias de la Salud, Tecnológico de Monterrey, and the ethics and research committee at Hospital Metropolitano "Dr. Bernardo Sepúlveda", Servicios de Salud del Estado de Nuevo León.

Conflict of interest: The authors hereby declare no conflict of interest exists.

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