

DIAGNOSIS OF NEONATAL GROUP B *STREPTOCOCCUS* SEPSIS BY NESTED-PCR OF RESIDUAL URINE SAMPLES

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ABSTRACT

Group B *streptococcus* (GBS) remains the most common cause of early-onset sepsis in newborns. Laboratory gold-standard, broth culture methods are highly specific, but lack sensitivity. The aim of this study was to validate a nested-PCR and to determine whether residue volumes of urine samples obtained by non invasive, non sterile methods could be used to confirm neonatal GBS sepsis. The nested-PCR was performed with primers of the major GBS surface antigen. Unavailability of biological samples to perform life supporting exams, as well as others to elucidate the etiology of infections is a frequent problem concerning newborn patients. Nevertheless, we decided to include cases according to strict criteria: newborns had to present with signs and symptoms compatible with GBS infection; at least one of the following biological samples had to be sent for culture: blood, urine, or cerebrospinal fluid; availability of residue volumes of the samples sent for cultures, or of others collected on the day of hospitalization, prior to antibiotic therapy prescription, to be analyzed by PCR; favorable outcome after GBS empiric treatment. In only one newborn GBS infection was confirmed by cultures, while infection was only presumptive in the other three patients (they fulfilled inclusion criteria but were GBS-culture negative). From a total of 12 biological samples (5 blood, 3 CSF and 4 urine specimen), eight were tested by culture methods (2/8 were positive), and 8 were tested by PCR (7/8 were positive), and only 4 samples were simultaneously tested by both methods (1 positive by culture and 3 by PCR). In conclusion, although based on a restricted number of neonates and samples, our results suggest that the proposed nested-PCR might be used to diagnose GBS sepsis as it has successfully amplified the three types of biological samples analyzed (blood, urine and cerebrospinal fluid), and was more sensitive than culture methods as PCR in urine confirmed diagnosis in all four patients. Moreover, PCR has enabled us to use residue volumes of urine samples collected by non invasive, non sterile methods, what is technically adequate as GBS is not part of the normal urine flora, thus avoiding invasive procedures such as suprapubic bladder puncture or transurethral catheterization. At the same time, the use of urine instead of blood samples could help preventing newborns blood spoliation.

Key words: GBS, neonatal sepsis, early-onset neonatal sepsis, neonatal infection, PCR, nested-PCR

INTRODUCTION

Group B *streptococcus* (GBS) remains the most common cause of early-onset sepsis, meningitis and pneumonia in newborns (3), albeit a considerable reduction of cases in developed countries due to the nearly universal use of intra-

partum prophylaxis for maternal GBS carriage (12). Broth culture, the current laboratory gold-standard is highly specific, but lacks sensitivity especially in neonates who are normally receiving antibiotics prior to hospitalization and culture sampling. More recently, PCR has proved to be a useful diagnostic tool due to its increased sensitivity and specificity to detect either broad-

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range bacterial agents associated to neonatal sepsis (12), or more specifically GBS (10).

The aim of this study was to validate a nested-PCR and to determine whether residue volumes of urine collected by a non invasive, non sterile method could be used to confirm neonatal GBS sepsis.

MATERIAL AND METHODS

Blood, CSF and urine cultures were performed in Todd-Hewitt broth supplemented with antibiotics (5). DNA extractions from blood were performed according to a protocol described elsewhere (2). CSF and urine DNA were extracted from 200 µL of samples using QIAamp DNA blood mini kit (Qiagen, Valencia, CA) (1). The first and the second PCR were performed in 50 µL of total volume containing 20 µL of DNA from blood, urine or CSF samples, 100 mM of Tris-HCl and 500 mM of KCl; 2.5 units of Taq DNA polymerase (Amersham Biotech, USA), 200 µM of dNTP (Amersham), 2.5 mM of MgCl₂; and 0.4 µM of each primer from the major GBS surface antigen (SAG). The outer primers were designed in our laboratory: CFB-Sb (sense) 5'-ATGATGTATCTATCTGGAAGTCTAGT G-3' and CFB-Ab (anti-sense) 5'-CGCAATGAAGTCTTTAATTTTC-3' while the inner primers were described elsewhere (7,8): An initial denaturation step of 5 minutes at 95°C was followed by 40 cycles of 1 minute at 95°C, 1 minute at 50°C and 1 minute at 72°C. A final extension time of 5 minutes at 72°C has ended amplifications (M.J. Research PT-150), in both rounds. Nested-PCR produced DNA fragments of 259 bp and 153 bp which were visualized in 2% ethidium bromide-stained agarose gels (Sigma, USA). In each experiment, negative controls (sterile water instead of genomic DNA), DNA from a non-infected individual, as well as a positive control (GBS-DNA from cultures) were included. All preventive measures were applied in order to minimize the risk of contamination with GBS-positive samples or pre-amplified products. The nested-PCR was able to detect as few as 1 femtogram of GBS-DNA after the second round of amplification (1.0 CFU is equivalent to 0.01 pg) (6).

RESULTS

All four newborns received penicillin or penicillin-derived antibiotics to treat GBS sepsis and evolved favorably. A total of 12 samples (5 blood, 3 CSF and 4 urine specimen) from the four newborns were tested either by culture (n=8), or by PCR (n=8). Amplification of some of the biological samples tested is shown in Fig. 1.

Table 1 summarizes culture and PCR results of the four patients.

In patient number 1 GBS infection was confirmed by blood and CSF cultures (no urine sample sent for culture). Although we could not obtain blood to perform PCR in this case,

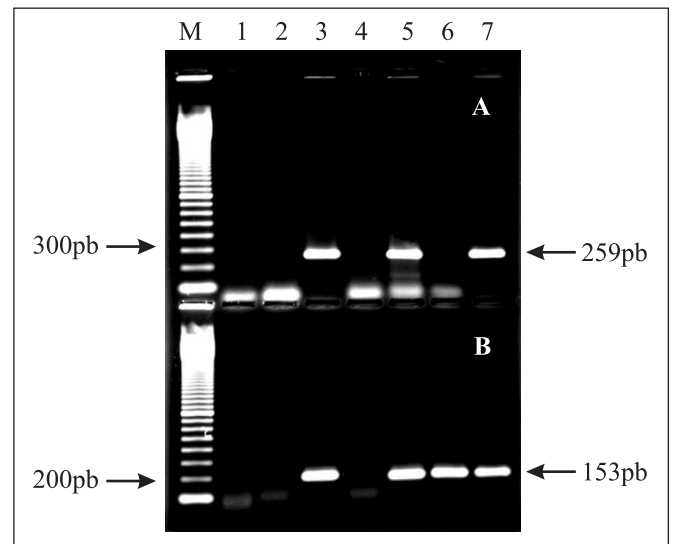


Figure 1. Electrophoresis of the nested-PCR amplification products in 2% agarose gels. A – first round. B – second round. M - Molecular weight marker - 100 bp (Amersham), lane 1 – negative control (sterile water instead of DNA); lane 2 – human GBS-free DNA (negative control); lane 3 – CSF of patient 1; lane 4 – CSF of patient 2; lane 5 – blood of patient 3; lane 6- urine of patient 4; 7- positive control (GBS-DNA). Samples of lanes 3 and 5 were positive from the first round, while sample of lane 6 was positive only after the second round of amplification.

Table 1. Description of patients, biological samples, culture and nested-PCR results. NT= not tested (no residual volume of sample available). Eight samples (B1, C1, B2, C2, U2, B3, B5 and C3) were sent for culture, and 8 samples (C1, U1, B2, C2, U2, B4, U3 and U4) were analyzed by PCR. Four samples were simultaneously analyzed by culture methods and PCR (C1, B2, C2, U2, represented in bold).

| Newborn | Biological Sample | GBS-Culture | Nested-PCR |
|---------|-------------------|----------------------|----------------------|
| 1 | blood | B1 - positive | NT |
| | CSF | C1- positive | C1 - positive |
| | urine | NT | U1- positive |
| 2 | blood | B2- negative | B2- positive |
| | CSF | C2- negative | C2- negative |
| | urine | U2 - negative | U2- positive |
| 3 | blood | B3- negative | B4- positive |
| | CSF | NT | NT |
| | urine | NT | U3- positive |
| 4 | blood | B5 - negative | NT |
| | CSF | C3- negative | NT |
| | urine | NT | U4 - positive |

amplifications yielded positive results in one aliquot of the same CSF sample sent for culture, and in one urine sample collected on the day of hospitalization, before antibiotic therapy was prescribed. In patient number 2 who presented with negative blood, CSF and urine cultures, it was possible to test the same three biological samples, and PCR resulted positive in blood and urine, but negative in CSF. In newborn number 3, one blood sample was sent for culture and resulted negative (no CSF or urine sample were sent for culture), while PCR was positive in another blood sample and in one urine specimen, both collected on the day of hospitalization, prior to antibiotic therapy. In newborn number 4, blood and CSF cultures were negative (no urine sample was sent for culture), while PCR was positive in one urine sample, obtained on the day of hospitalization, before antibiotics prescription (other blood samples and CSF aliquots were not available).

Considering the biological samples irrespective of the patients (see Table 1), eight were tested by culture methods (4 blood, 3 CSF and 1 urine specimen, corresponding to B1, C1, B2, C2, U2, B3, B5 and C3) resulting in 2 positive detections, while 8 were tested by PCR (2 blood, 2 CSF and 4 urine specimen, corresponding to C1, U1, B2, C2, U2, B4, U3 and U4), yielding 7 positive detections. In only four instances the same biological materials were simultaneously analyzed by culture and PCR (1 blood, 2 CSF and 1 urine specimen, corresponding to C1, B2, C2 and U2) resulting in 1 culture-positive and 3 PCR-positive samples.

DISCUSSION

Only a few studies have analyzed biological samples from neonates to detect GBS by molecular techniques, and in the majority of cases using stored biological material instead of freshly obtained specimen (4,9). We decided to follow very restrictive inclusion criteria of patients in the present study even though they had significantly decreased the number of cases enrolled, in an attempt to circumvent the possibility of GBS-culture negativity due to previous administration of antibiotics to the newborns rather than to other etiology of infections, although PCR performance could also be affected, probably in a lesser extent. These criteria have also faced the difficulty to find residue volumes of biological samples available for PCR analysis, taken into account that they should had been taken on the same day of hospitalization, prior to antibiotic administration.

In neonatology, the amount of biological samples required to perform life-supporting exams, as well as others to investigate the etiology of infections constitutes a limiting factor for laboratory diagnosis. Moreover, culture methods are almost as expensive as molecular techniques, time consuming and sometimes lack sensitivity, e.g. in neonates who have previously received antibiotics. It is true that a negative PCR cannot rule

out GBS infection either, however, PCR has been reported to be more sensitive (6,7,11), as occurred in the present study. The proposed nested-PCR was able to detect GBS-DNA in blood, CSF and urine, thus being suitable to analyze these three types of biological samples. Besides, the nested-PCR was more sensitive than culture methods as it has confirmed diagnosis in the four newborns studied, while cultures were negative in three (presumptive GBS sepsis cases). It is also important to emphasize that nested-PCR was able to confirm GBS infection in residue volumes of urine collected by non invasive, non sterile method, what is technically adequate considering that GBS is not part of the normal urine flora. At the same time, the use of urine instead of blood samples could help preventing newborns blood spoliation.

RESUMO

Diagnóstico de sepse neonatal causada pelo estreptococo do grupo B por meio de dupla amplificação de amostras residuais de urina

O estreptococo do grupo B (GBS) constitui a causa mais freqüente de sepse neonatal precoce. O teste de referência continua sendo o isolamento em cultura, apesar de apresentar problemas de sensibilidade. O objetivo do presente estudo foi validar uma técnica de dupla amplificação e determinar a possibilidade do uso de amostras residuais de urina colhidas por método não invasivo, não estéril, para a confirmação da sepse por GBS em recém-nascidos. As amostras foram amplificadas com primers do principal gene de superfície do GBS. A insuficiência de volume de material biológico para a realização de exames para suporte de vida, além de outros necessários à identificação do agente etiológico de infecções é muito freqüente em recém-nascidos. Mesmo assim, decidimos definir critérios bastante rigorosos para a inclusão de pacientes na casuística: os recém-nascidos deveriam apresentar sinais e sintomas compatíveis com infecção pelo GBS; deveriam ter tido ao menos uma amostra enviada para cultura, podendo ser sangue, urina ou líquido; disponibilidade de volumes residuais dessas amostras, ou de outras colhidas no dia da hospitalização, antes da introdução da antibioticoterapia, de forma a possibilitar a análise por PCR, e evolução favorável com a antibioticoterapia empírica. Em apenas um dos quatro recém-nascidos a infecção foi confirmada por cultura, enquanto nos outros três casos a infecção foi considerada presuntiva (pacientes preencheram os critérios de inclusão, mas o GBS não foi isolado). De um total de 12 amostras dos quatro pacientes (5 de sangue, 3 de líquido e 4 de urina), 8 foram testadas por cultura (2 foram positivas), 8 foram testadas por PCR (7 foram positivas), e apenas 4 pelos dois métodos simultaneamente (1 positiva por cultura e 3 por PCR). Concluímos que apesar do número restrito de pacientes e de amostras testadas, os resultados apresentados sugerem que

a amplificação proposta poderia ser usada para o diagnóstico de sepsse pelo GBS, uma vez que a amplificação foi possível nos três tipos de materiais biológicos testados (sangue, urina e líquido), e a PCR foi mais sensível que as culturas por ter conseguido confirmar a infecção na urina dos quatro pacientes, usando volumes residuais de amostras colhidas por método não invasivo, não estéril, o que é tecnicamente adequado uma vez que o GBS não faz parte da flora normal da urina, evitando procedimentos invasivos, tais como a punção supra-púbica da bexiga ou a cateterização transuretral. Ao mesmo tempo, o uso de urina em lugar de sangue ajuda a prevenir a espoliação sangüínea dos recém-nascidos.

Palavras-chave: Estreptococo do grupo B, sepsse neonatal, sepsse neonatal precoce, infecção neonatal, PCR, dupla amplificação

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