Rev. Inst. Med. Trop. Sao Paulo
56(1):77-80, January-February, 2014
doi: 10.1590/S0036-46652014000100012

Enterococcus faecium AND *Enterococcus faecalis* IN BLOOD OF NEWBORNS WITH SUSPECTED NOSOCOMIAL INFECTION

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SUMMARY

Enterococci are Gram-positive cocci saprophyte of the human gastrointestinal tract, diners who act as opportunistic pathogens. They can cause infections in patients hospitalized for a long time or who have received multiple antibiotic therapy. *Enterococcus faecalis* and *Enterococcus faecium* are the most common species in human infections. To evaluate the possibility of rapid detection of these species and their occurrence in the blood of newborns with suspected nosocomial infection, blood samples were collected from 50 newborns with late infections, admitted to the Neonatal Care Unit of the University Hospital Federal de Mato Grosso do Sul (UFMS-HU), from September 2010 to January 2011. The samples were subjected to conventional PCR and real time PCR (qPCR) to search for *Enterococcus faecium* and *Enterococcus faecalis*, respectively. The PCR results were compared with respective blood cultures from 40 patients. No blood cultures were positive for Enterococci, however, eight blood samples were identified as genomic DNA of *Enterococcus faecium* by qPCR and 22 blood samples were detected as genomic DNA of *Enterococcus faecalis* by conventional PCR. These findings are important because of the clinical severity of the evaluated patients who were found positive by conventional PCR and not through routine microbiological methods.

KEYWORDS: *Enterococcus faecium*; *Enterococcus faecalis*; Prematurity; PCR.

INTRODUCTION

Neonatal sepsis is the most frequent nosocomial infection in neonatal intensive care units and its incidence is increasing due to the increased survival of infants with very low birth weights[13,15]. The greatest risk factors for late onset sepsis in neonates include low birth weight, prematurity, long periods of hospitalization and invasive procedures[3]. The late sepsis usually manifests as septicemia and pneumonia, occurring 72 hours after birth[20], caused by pathogens found in the nosocomial environment, including enterococci among the main etiological agents[3,11].

The genus *Enterococcus* is comprised of gram-positive cocci in pairs or short chains[6,9], considered saprophytes of the human gastrointestinal tract. They can survive on inanimate objects such as thermometers and stethoscopes, as well as on the hands of health professionals for long periods[18].

Symptoms of neonatal infection are not specific, even for the different agents, with a need for more sensitive and specific microbiological and molecular tests[10].

In a study in Spain by FERNANDEZ *et al.,* 2004[2], 95 episodes of bacteremia caused by *Enterococcus faecalis* were documented, 83.2% with nosocomial origin, 85.3% associated with invasive procedures, 9.5% in neonates and 41.1% that had previously received broad spectrum antibiotics. In Brazil, a study by TITZE-DE-ALMEIDA *et al.* 2004[19], with results of phenotypic and molecular analysis showed rates of around 95% in both *Enterococcus faecalis* and *Enterococcus faecium*.

Blood culture is the gold standard for diagnosis of sepsis, but has low sensitivity and the results are available in no less than 48 to 72 hours[9]. The PCR methods are shown to be fast and specific[8,14], but when we compare qPCR with conventional PCR, it is known that even though it consists of a more stringent and sensitive technique, in practice it demands higher costs than conventional PCR.

Thus, the objective of this study was to identify through the technique of qPCR and conventional PCR, respectively, the presence of *Enterococcus faecium* and *Enterococcus faecalis* in the blood of newborns, suspected of infection and admitted to the Intensive Care Unit.

MATERIAL AND METHODS

The study included infants who were suspected of nosocomial

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infection, admitted to the Neonatal Intensive Care Unit, University Hospital, Federal University of Mato Grosso do Sul, with at least 72 hours of admission or those with clinical worsening or a change of antibiotics, in a five month follow up, from September 2010 to January 2011.

This research was initiated only after approval by the Ethics Committee on Human Research of the Federal University of Mato Grosso do Sul (Protocol 1520).

The peripheral blood samples were collected with all accuracy aseptically as usually taken for routine under medical request. Samples for blood cultures were sent to the Central Laboratory of the HU-UFMS, where they were processed and analyzed by the automated system, BACTTEC™ FX (BD, New Jersey, USA) and the results compared with those obtained by conventional PCR.

The DNA extraction was performed using the GE Health Care extraction Kit and the blood samples of 50 infants, identifying *Enterococcus faecium* by qPCR and *Enterococcus faecalis,* by conventional PCR.

For qPCR reaction, the reagents kit from: SYBR Green PCR Biosystems (Applied Biosystems, Warrington, UK) was used. Designed oligonucleotide *primers* selected within the PBP5 (penicillin-binding protein) genome region were FAEFOR-1 = 5'-ggt acaacccgattacttcgtcccat-3' and FAEFOR-2 = 5'-ggtacaacccgattactttgt cccat-3'; FAEREV-1 = 5'–tctgccgtctacttcttggatggt-3' and FAEREV-2 = 5'-tctgccgtctacttcttgaatggt-3', to obtain a specific target fragment of 94 bp.

The thermocycling conditions for qPCR were those standardized in the ABI Prism 7000 unit (52 °C / 2 min, 95 °C / 10 min, 45 cycles of: 95 to 60 °C / 15 ° C / 15 s) with a final dissociation curve for each sample. *Primers* (1.5 pmol each), DNA (50-100 ng) and Syber Green PCR Master Mix (Applied Biosystems, Warrington, UK) were combined and qPCR carried out under conditions recommended by the manufacturer. All positive and negative tests had controls included. A human s26 normalizer was used as internal control. The cumulative CT values (cycle threshold) were collected in qPCR for each positive sample amplified in triplicate.

Standard conventional PCR was used for the detection of *Enterococcus faecalis*[12]. The specific oligonucleotides *primers* designed and selected to amplify an amplicon of 87 bp, within the target region EntC2 (enterocin peptide precursor) of the genome of *Enterococcus faecalis*, were FAECFOR (5'-gcaattactggtggaggacctgg-3') and FAECREV (5'-tccaattccttttgcaagacctgc-3'). *Primers* were designed based on sequences selected in the Genbank (program NCBI-BLASTn).

PCR consisted of: 9.75 µL of water sterile filtered, 1.5 µL 10x PCR buffer, 1.2 µL of dNTP mix (200 mM each), 0.4 µL of 50 mM $MgCl_2$, 0.5 µL of each primer (10 pmol / microl), 0.15 µL (5 U / microl) of Taq DNA polymerase (Ludwig Biotec, Alvorada, Brazil) and 1 µL of genomic DNA (200 ng) in a total volume of 15 µL reaction. The reaction was carried out in a MJ Research PTC 100 Thermal Cycler with the following program: 95 °C / 5 min, followed by 05 cycles of 94 °C / 1 min, 56 °C / 1 min, 72 °C / 1 min, followed by 40 cycles: 92 °C / 1 min, 60 °C / 1 min, 72 °C / 1 min, and final extension at 72 ° C / 4 min.

The amplified fragments were photographed after electrophoresis on 6% of poliacrilamide gel (Ludwig Biotec) stained with silver nitrate. ATCC 19433 was used as a positive control for *Enterococcus faecalis,* autoclaved ultrapure water was used as a negative control.

The comparison between the groups of newborns evaluated in relation to blood PCR was performed using the nonparametric chi-square contingency table with 2x2 cells and with a value greater than five. The other results of the variables evaluated in this study were presented as descriptive statistics or graphs. Statistical analysis was performed using the SigmaStat version 2.0, whereas there were significant differences for values of $p < 0.05$[16].

RESULTS

We evaluated 50 newborns of both genus; the mean gestational age was 31 weeks, and the mean birth weight was equal to 1.745 kg.

With respect to invasive procedures, all newborns in which we detected the presence of genetic material of bacteria studied were subjected to some type of vascular catheter. The use of mechanical ventilation and parenteral nutrition represent important features among these patients.

Of the 50 patients studied, 40 had blood culture and none of the cultures observed *Enterococcus faecalis* or *Enterococcus faecium*. For the other ten blood cultures, the results were not included in the medical record.

The results showed 16% (n = 8) positive for *Enterococcus faecium* in peripheral blood of the newborn evaluated by qPCR (Fig. 1) and 44% (n = 22) were positive for *Enterococcu faecalis*, by simple conventional PCR (Fig. 2). Among all the infants evaluated, 12% (n = 6) were positive for both types of bacteria, *Enterococcus faecalis* being more frequent ($p = 0.004$).

DISCUSSION

Prematurity and low birth weights are described as neonatal factors relevant to the development of late onset sepsis, since it is the main cause of admission of neonates in intensive care units[4]. We also observed prematurity (46%) as an important characteristic of this population.

In this study, among the blood samples in which at least one of the microorganisms described here was detected, the use of central venous catheters has been the procedure with the highest correlation with the presence of bacteria (54%), followed by mechanical ventilation (32%) and parenteral nutrition (32%).

This data agrees with HERRMANN *et al.*[4], who in 2008 also found that the use of invasive procedures such as venous catheters, parenteral nutrition and mechanical ventilation had an important relationship with the development of late neonatal sepsis.

Some practices may be adopted as mitigation measures for late sepsis. Knowing that the use of vascular catheters and mechanical ventilation are closely related to the presence of infection, time of use must be reduced with safe and hygienic measures in the handling of equipment, and proper

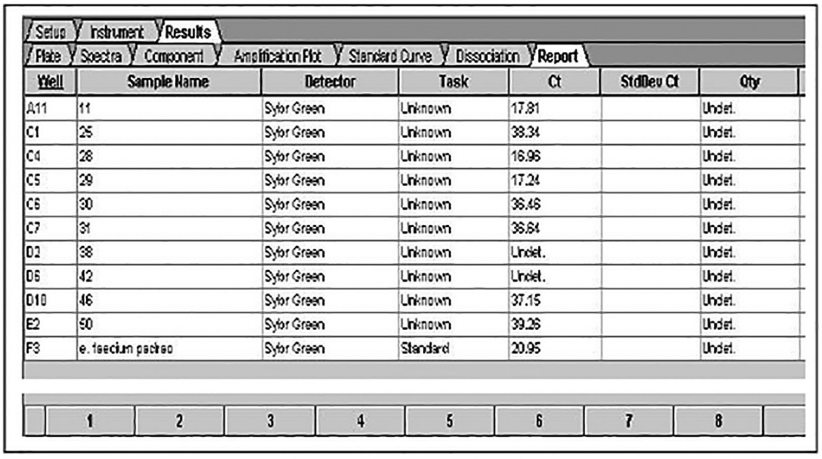

Fig. 1 - Samples positive for *Enterococcus faecium* by Real-Time PCR.

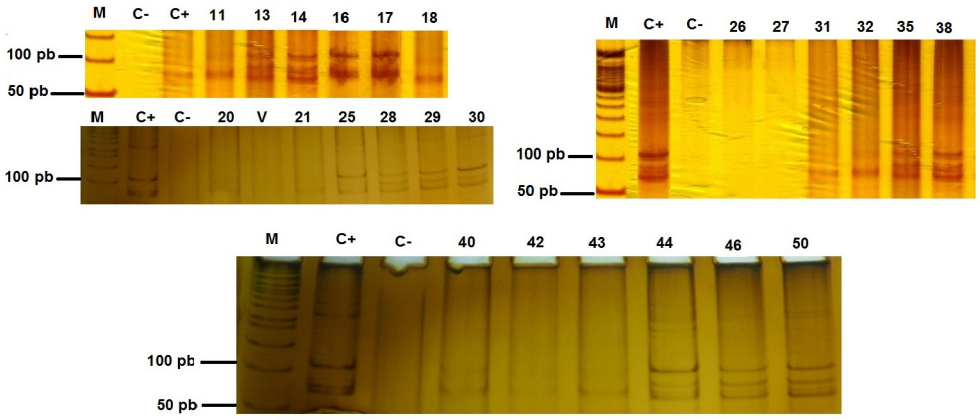

Fig. 2 - Conventional PCR for *Enterococcus faecalis* present in blood of newborns with suspected nosocomial infection. **(M)** 50 bp ladder marker (1µg); **(C+)** positive control for *E. faecalis*, ATCC 19433. **(C–)** negative control; 22 positive samples = **(11,13,14,16,17,18,20,21,25,28,29,30,31,32,35,38,40,42,43,44,46,50).**

hand washing are important measures in preventing infection nosocomial[4].

The use of parenteral nutrition has been associated with late infection and delaying the start of enteral feeding. Other authors suggest that the enteral route should be initiated as early as possible to reduce the time of parenteral nutrition, which is associated with a high risk of infection[4,7,11].

For genomic detection of bacteria *Enterococcus faecium* and *Enterococcus faecalis* in the blood of newborns with risk factors for infection, we used qPCR and conventional PCR, respectively, for being rapid and specific methods. We also compared these results with blood culture.

There was no positive result for the bacteria studied by means of blood cultures. Other studies have reported lower sensitivity of blood cultures[1,5,21], despite being the gold standard for diagnosis of sepsis, is a time consuming technique, whose results are ready in no less time than 48 to 72 hours and several authors have questioned its credibility[5].

Several methods for the molecular detection of *Enterococcus faecium* and *Enterococcus faecalis* have been reported as a powerful tool in the investigation of outbreaks of nosocomial infections[12]. Such techniques are distinguished by the rapidity and specificity of their results, which are essential for the prevention and control of transmission of infection, being all PCR based methods.

Further studies are needed to reveal in more detail the late onset of sepsis and its risk factors among newborns. Even though the presence of genomic DNA by conventional PCR and qPCR does not necessarily mean sepsis, possibly just colonization, adding to the patients symptoms and clinical observations, especially prematurity conditions, together lead the physician to take proper action or medical procedures in favor of children's treatment, avoiding deadly sepsis. High prematurity, associated with *Enterococcus faecium* and *Enterococcus faecalis* in the blood of newborns, reinforces the usage of conventional PCR and qPCR as additional tools for clinics.

CONCLUSIONS

The present finding is important due to the clinical severity of the evaluated patients who were positive by methods PCR and were not detected in routine microbiological methods. These methods were more effective compared to blood cultures that did not show any positive case for *enterococci*.

RESUMO

Enterococcus faecium e *Enterococcus faecalis* no sangue de recém-nascidos com suspeita de infecção nosocomial

Os enterococos são cocos Gram-positivos saprófitas do trato gastrointestinal humano, atuam como patógenos oportunistas. Podem causar infecções em pacientes: hospitalizados por um longo tempo ou que receberam antibioticoterapia múltipla. *Enterococcus faecalis* e *Enterococcus faecium* são as espécies mais comuns em infecções humanas. Para avaliar a possibilidade de detecção rápida dessas espécies e sua ocorrência no sangue de recém-nascidos com suspeita de infecção hospitalar, foram coletadas amostras de sangue de 50 recém-nascidos, com infecção tardia, internados na Unidade de Terapia Neonatal do Hospital Universitário da Universidade Federal de Mato Grosso do Sul (UFMS-HU), no período de setembro de 2010 a janeiro de 2011. As amostras foram submetidas a PCR convencional e PCR em tempo real (qPCR) para pesquisa de *Enterococcus faecium* e *Enterococcus faecalis*, respectivamente. Os resultados da PCR foram comparados com culturas de sangue respectivos de 40. Nenhuma hemocultura foi positiva para enterococos, no entanto, em oito amostras de sangue foi identificado DNA genômico de *Enterococcus faecium* através da técnica de reação em cadeia da polimerase em tempo real, e em 22 amostras de sangue, foram detectados DNA genômico de *Enterococcus faecalis*, através de PCR convencional. A descoberta é importante por causa da gravidade clínica dos pacientes avaliados que foram positivos por PCR convencional e não foram detectados na rotina por métodos microbiológicos.

ACKNOWLEDGEMENTS

To the team of NICU HU-UFMS for openness and collaboration in the blood samples and provided data and access to research patient's records.

FINANCIAL SUPPORT

This research was supported by the National Council for Scientific and Technological Development (CNPq) and Foundation for Development Support of Education, Science and Technology (FUNDECT). There was no conflict of interest between the authors of this research.

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Received: 25 January 2013
Accepted: 6 June 2013