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PREMATURE RUPTURE OF MEMBRANE AND MOLECULAR AND MICROBIOLOGICAL PROFILE OF BLOOD OF NEWBORNS WITH SUSPECTED NEONATAL INFECTION

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ABSTRACT

Many term and preterm newborns with clinical signs of sepsis, whose mothers had premature rupture of membranes (PROM), have negative blood cultures, but in molecular biology research the presence of genomic DNA from different congenital bacteria has been observed. Thus, the association of molecular examination with rtPCR with different bacterial primers may help us in a more objective treatment. The samples 101 newborns with PROM were analyzed by rtPCR with primers for bacteria *Streptococcus agalactie, Escherichia coli* and *Klebsiella pneumoniae*. Blood culture results were obtained from newborn records. The mean PROM time in preterm and term pregnancies was 72.87 ± 101.85 hours and 48.70 ± 84.14 hours, respectively. The blood culture results were negative for the analyzed bacteria. No statistical association was seen of PROM time with the presence of bacterial genomic DNA (93.1%), but the clinical manifestations do suggest association with neonatal sepsis. *Escherichia coli* prevailed (64.3%), followed by *Streptococcus agalactiae* (19.8%) and *Klebsiella pneumoniae* (2.0%) by means of the Polimerase Chain Reaction (qPCR). Clinical and physiological findings suggest that PROM is associated with the clinical manifestations of sepsis and the presence of genomic DNA from pathogenic bacteria responsible for the occurrence of neonatal infection in preterm and term newborns.

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INTRODUCTION

Infections in the neonatal period are a major concern of neonatology because they are commonly responsible for a high morbi-mortality rate (Moreira *et al*., 2013; Silveira & Procianoy, 2012).

Congenital sepsis is a serious disease characterized by a systemic inflammatory response due to intrapartum transmission of pathogenic microorganisms (Camacho-Gonzales *et al*., 2013; Kacerovsky *et al*., 2014). Research has shown that premature rupture of membranes (PROM) is

associated with early and secondary neonatal sepsis and the pathogens of the birth canal (Camacho-Gonzales *et al*., 2013; Rodrigo-Trujillo *et al*., 2016). The same studies also report that the risk of fetal and neonatal sepsis increases according to the time of rupture of amniotic membranes (Camacho-Gonzales *et al*., 2013; Kacerovsky *et al*., 2014). Also, pathogenic vaginal microorganisms play an important role in the rupture of amniotic membranes and neonatal sepsis. Studies in Brazil have shown that the most common microorganisms found in neonatal sepsis are *Streptococcus agalactiae*, *Escherichia coli*, *Haemophilus influenzae* and *Listeria monocytogenes*, and that they can reach the fetus by the hematogenic route or migrate from the vaginal canal into the intra-amniotic cavity, colonizing the fetus and triggering the infectious process (Endale *et al*., 2017; Gonçalves *et al*., 2016); Hackenhaar *et al*., 2014; HERBST *et al*., 2007; Nomura *et al*., 2009; Patriota *et al*., 2014). Laboratory tests are important tools for the diagnostic confirmation of sepsis or other neonatal infections. The isolation of the pathogenic microorganism in any liquid or secretion of the organism is considered as gold standard and represents the most specific method to diagnose neonatal sepsis. However, according to the literature, microbiological tests have a considerably low sensitivity when considering the severity of the disease; in addition, the amount of blood used is difficult to collect, especially in case of newborns (1 mL), and the results are lengthy (5-7 days) (Brasil, 2017; Ferreira *et al*., 2013; LI Wang *et al*., 2015; Silva-Junior *et al*., 2016; Yamoto *et al*., 2016).

One of the reasons why empirical treatment is used is the delay in getting the blood culture results, but an inadequate coverage of the probable microorganism involved may also occur, causing antimicrobial resistance of the bacterium and nosocomial infection under the conditions of multiresistant gram-negative bacteria. The indiscriminate use of anti-fungal is also a factor of increased mortality (Endale *et al*., 2017; Patriota *et al*., 2014). The identification of bacterial DNA by molecular technique is the best way to quickly identify the microorganism in the blood. The main advantage of this technique, without a doubt, is safety and a treatment directed to the bacterium involved. Molecular tests have the CE label, but none of these assays is still approved by the US Food and Drug Administration (FDA) (AL-Zahrani *et al*., 2015; Yamoto *et al*., 2016). The PCR test will be diagnosed up to 24 hours, with good sensitivity, even when the targets are present in extremely low amounts; small amounts of whole blood (between 0.3 and 0.5 ml) are associated with the sepsis clinic (AL-Zahrani *et al*., 2015; Krupa *et al*., 2005; Yamoto *et al*., 2016).

MATERIAL AND METHODS

This is a cross-sectional observational study with newborns to mothers with PROM, regardless of gestational age, admitted to the Neonatal Intensive Care Unit (NICU) and the Neonatal Intermediate Care Unit of the Maria Pedrossian School Hospital of the Federal University of Mato Grosso do Sul (UFMS), Cândido Mariano Maternity Ward and Regional Hospital of Mato Grosso do Sul (HRMS) (Brazil) from March to August 2014. The study was approved by the Research Ethics Committee with Human Beings of the UFMS (number 1,151,725) and the Free and Informed Consent Term was signed. Mothers with PROM ≥ 12 hours met the inclusion criteria providing that their pregnancies were not term ones, they were not in labor and no dystocia was observed. The exclusion criteria were: newborns diagnosed with congenital malformation; those whose mothers or legal guardians did not agree to take part in the study; and neonates born to indigenous or quilombola (former slaves) mothers. The population consisted of 101 women and their respective preterm or full term neonates with PROM ≥ 12 hours. The evaluations of the gestational ages of the newborns were performed using the New Ballard Score (NBS) method (BALLARD AL-ZAHRANI *et al*., 1991). According to the gestational ages, the newborns were divided into two groups: term (≥38s) and preterm (<38s) provided that the mothers were not in active vaginal labor. The bacterial genomic DNA of the samples was submitted to the standard PCR protocol with 6% polyacrylamide gel visualization, following Jordan and Durso´s norms (2000), with modifications. This protocol suggests the use of the primer universal, whose sequence was 5' AACTGGAGGAAGGTGGGGAT-3 'and 5'-AGGAGGTGATCCAACCGCA-3', which only identifies the presence of genomic DNA from microorganisms, not identifying bacterial specificity (GALVES, 2013; JORDAN & DURSO, 2000; REIS, 2013). Blood cultures were performed by the automated BACTTEC TM FX system (BD, New Jersey, USA)[20] according to the hospital routine and the results were obtained from medical records. The bacterial genomic DNA of the samples was submitted to the standard PCR protocol with a6% polyacrylamide gel visualization, following Jordan and Durso´s norms, (2000) with modifications. The characterization of the PCR fragments of the studied microorganisms are set out in Table 1 and the procedures using these fragments were performed by qPCR using the SYBR Green PCR core reagents kit (PE Biosystems), processed on the ABI Prism 700- (Applied Biosystems) and AB Vii A7 (Applied Biosystems) apparatus, based on the protocol for qPCR developed by NUFIGEN (Grimberg *et al*., 1989). Positive and negative controls (no-template-PCR) were included in all assays. For each positive sample the value of Ct (treshold cycle) was determined. Autoclaved ultrapure water and purified total nucleic acid of the studied microorganisms were used as negative control. The chi-square, Mann-Whitney and descriptive statistical tests were used to evaluate the associations of the PROM with the identification of genomic DNA by the universal primer, the presence of genomic DNA of the bacteria by the qPCR analysis, the blood culture results

Table 1. Characterization of PCR fragments of the studied microorganisms

Initiators	etiological agent	PCR product (pb)
SAGAF1 = 5'-TTG CAG CCA GTT GAA GAT CGT-3' SAGAR2 = 5'-ATT CGT GGT GCT GCT GGT GG-3'	*Streptococcus agalactiae*	350
ECOF1 =5'-CTG GTC GAC GAC AAG ATG CA-3' ECOR2 = 5'-CTG GAA GAC GAG TAA TTC TC-3'	*Escherichia coli*	323
RW01 = 5'-AACTGGAGGAAGGTGGGGAT-3' DG74 = 5'-AGGAGGTGATCCAACCGCA-3'	Universal	380
KLEBFOR = 5'- GCA CTG CGT GGT GAT GTC GC-3' KLEBREV = 5'- TGT ACC GAC GGG CAA TCT TCA-3'	*Klebsiella pneumoniae*	82

of the newborn and the maternal data. All statistical analyses were performed using the SPSS Software, version 20.0, with a significance level of 5%.

RESULTS AND DISCUSSION

Regarding maternal data, the age of the mothers ranged from 14 to 43 years (23.71 ± 6.59 years). The gestational age varied from 25 to 42 weeks, and the mean gestational age in the preterm and term groups was 32.71 ± 3.53 weeks and 39.34 ± 1.15 weeks, respectively. All newborns selected for the study (n = 101) had an infection clinic and received antimicrobial therapy. There was no association (p> 0.05) of gestational age with variables Apgar score, gender and type of delivery. Neither was an association seen of maternal fever and Urinary Tract Infection (UTI) in preterm gestations with any of the bacteria analyzed in this study, regardless of the time between membrane rupture and delivery (p> 0.05) (Table 2).

were found positive for the presence of bacterial genomic DNA compared with qPCR, in which the genomic DNA of *Streptococcus agalactiae* (19.8% / n=20), *Escherichia coli* (64% / n=65) and *Klebsiella pneumoniae* (2.0 / n=2) were identified. This analysis suggests that the preterm group is statistically the most vulnerable to neonatal *Escherichia coli infection* (Table 3). The mean maternal age of the study population is in agreement with other studies (23.71 ± 6.59 years) (ENDALE *et al*., 2017; PATRIOTA *et al*., 2014), but it was not considered a risk factor for the development of PROM (HACKENHAAR, *et al*., 2017). Although the present study did not emphasize the statistical association of PROM with maternal urinary tract infection (UTI), it is important to emphasize that some pieces of research report this correlation, since UTI can trigger uterine activity and even stimulate changes in the synthesis and degradation of collagen membranes (LI WANG *et al*., 2015). The maternal infection may therefore precede the rupture.

Table 2. Association of genomic DNA with time of amniotic membrane rupture, maternal fever and ITU in pre-term and term newborns

membrane rupture time (hours)	Presence of bacteria *per* maternal symptom					
	Pre-term (G1)			term (G2)		
	Fever % n	ITU % n	p value(1)	Fever % n	ITU % n	p value(1)
Streptococcus agalactiae						
0 to 23 (T1)	14 (1)	71 (5)	0,28	0	100 (1)	-
24 to 47 (T2)	17 (1)	50 (3)	1,0	0	0	-
48 to 71 (T3)	0	0	-	-	-	-
≥ 72 (T4)	0	25 (1)	-	0	0	-
Escherichia coli						
0 to 23 (T1)	12 (2)	59 (10)	1,0	0	60 (3)	-
24 to 47 (T2)	0	38 (5)	-	0	0	-
48 to 71 (T3)	0	25 (1)	-	-	-	-
≥ 72 (T4)	4 (1)	35 (8)	0,35	0	0	-
Klebsiella pneumoniae						
0 to 23 (T1)	0	0	-	0	0	-
24 to 47 (T2)	0	0	-	100 (1)	100 (1)	-
48 to 71 (T3)	0	0	-	-	-	-
≥ 72 (T4)	0	0	-	0	0	-

T1: Amniotic membrane rupture time 1; T2: Amniotic membrane rupture time 2; T3: Amniotic membrane rupture time 3; T4: Amniotic membrane rupture time 4. G1: Newborns with gestational age ≤ 38 weeks. G2: Newborns with gestational age> 38 weeks. (1) Fisher's Exact Test.

Table 3. Classification of membrane rupture time (hours) in relation to the presence of bacteria in pre-term and term newborns

Variables	Gestational age		Total (n=101)	p value(2)
	Pre-term (G1)	Term (G2)		
	(n=84)	(n=17)		
	% (n)	% (n)	% (n)	
membrane rupture time (hours)				
0 to 23 (T1)	33,3 (28)	58,8 (10)	37,6 (38)	0,09
24 to 47 (T2)	20,2 (17)	17,6 (3)	19,8 (20)	0,81
48 to 71 (T3)	9,5 (8)	0,0 (0)	7,9 (8)	0,40
≥ 72 (T4)	36,9 (31)	23,5 (4)	34,7 (35)	0,43
bacteria				
Streptococcus agalactiae	22,6 (19)	5,9 (1)	19,8 (20)	0,22
Escherichia coli	67,8 (57)	47,0 (8)	64,3 (65)	0,17
Klebsiella pneumoniae	1,2 (1)	5,9 (1)	2,0 (2)	0,76
Bacterial DNA(2)	92,8 (78)	94,1 (16)	93,1 (94)	0,99

T1: Amniotic membrane rupture time 1; T2: Amniotic membrane rupture time 2; T3: Amniotic membrane rupture time 3; T4: Amniotic membrane rupture time 4. G1: Newborns with gestational age ≤ 38 weeks. G2: Newborns with gestational age> 38 weeks. (1) Chi-square test.

These variables were not associated in case of term pregnant women, since there were no mothers with fever or UTI, what made statistical calculations unfeasible. It was possible, though, to make the analysis of bacterial DNA between 24 and 47 hours (T2). *Streptococcus agalactiae, Escherichia coli* and *Klebsiella pneumoniae* were not identified by the blood culture technique. The results of the conventional PCR using the universal primer demonstrated that 93.1% of the 101 samples

Problems in the technique of isolation and identification of microorganisms and the use of maternal antibiotics may explain the negative results obtained by blood culture (GALVES, 2013; SILVA-JUNIOR *et al*., 2016; SILVEIRA & PROCIANOY, 2012), taking into account that the collection of the material followed all recommended techniques and standards. Some studies have shown that the blood culture technique in the analysis of other bacteria also yielded low

percentages of positivity (3%) for the presence of a microorganism associated with neonatal sepsis (Galves, 2013; krupa *et al*., 2005; Silva-Junior *et al*., 2016). Although blood culture is considered the gold standard for the diagnosis of neonatal sepsis, the rate of positive results obtained in other studies has been low (7%) (Camacho-gonzales *et al*., 2013; Galvez, 2013; Grimberg *et al*., 1989; Menezes *et al*., 2008; Polin, 2012; REIS, 2013), and this fact makes diagnosis difficult and may interfere with treatment and prognosis (Grimberg *et al*., 1989). Several studies have demonstrated that the PCR is a sensitive technique for identifying DNAs of etiological agents of neonatal infection, using less blood (300 µL) and producing faster results (Dutta *et al*., 2009; Labib *et al*., 2013; Tejada *et al*., 2008). The use of the universal primer in PCR has been an accurate and very sensitive tool in suspected sepsis diagnosis in infants (Patriota *et al*., 2014; Tejada *et al*., 2011). The results show that *Escherichia coli* was the predominant bacterium in newborns regardless of the gestational age and the time between premature membrane rupture and delivery, being therefore the main microorganism associated with infection in this study. The results are compliant with another related study (Stoll *et al*., 2011).

Currently, gram-negative microorganisms have been highlighted as etiological agents of neonatal sepsis, especially in premature and very low birth weight infants in developing countries (Reis, 2013; Tsai *et al*., 2012). The development of guidelines recommending intrapartum prophylaxis with antibiotics aimed at reducing the cases of neonatal sepsis caused by *Streptococcus agalactiae* has in recent years decreased the incidence of sepsis caused by the bacterium (Tsai *et al*., 2012). The results of this study have shown that the DNA identification of *Streptococcus agalactiae* was high, corroborating other recent studies in the same region, which also demonstrated high rates of identification of the genomic DNA of the same bacterium (Silva-Junior *et al*., 2016; Miglioli, 2009). However, it should be considered that these are risk groups with suspected sepsis, which suggests that the high prevalence of this bacterium is an important factor of neonatal sepsis in the region. *Klebsiella pneumoniae* was the pathogen with the lowest prevalence among the infants analyzed in the present study. In the previously mentioned research (SILVA-JUNIOR *et al*., 2016), using the heptaplex PCRtr technique, the low prevalence of *Klebsiella pneumoniae* (5.3% - n=8) was also observed in newborns with suspected neonatal sepsis. However, the incidence of *Klebsiella pneumoniae* in infants with neonatal sepsis reported in other scientific studies (Tejada *et al*., 2011; Tsai *et al*., 2012; Miglioli, 2009; Bercaite *et al*., 2012; Lopes *et al*., 2008; Tragante *et al*., 2008) ranged from 10.1% to 26.9%, a much higher value in comparison with that reported in this study. The results of the present work are in compliance with other studies (SILVA-JUNIOR *et al*., 2016; YANG *et al*., 2012; FALCIGLIA *et al*., 2012), demonstrating the frequency variation of the microorganisms identified in the different hospitals studied. However, both *E. coli* and *S. agalactiae* predominate in developing countries as microorganisms that cause neonatal sepsis.

Conclusions

The presence of genomic DNA of *Streptococcus agalactiae, Escherichia coli* and *Klebsiella pneumoniae* was detected by the qPCR technique, but statistical studies did not show the association of the presence of the bacteria DNA with the different times of membrane rupture. In the study population, the observed clinical signs do suggest a possible association, which implies the need to take into account those differences that can be misleading. Of the bacteria analyzed, *Echerichia coli* prevailed (64.3%), corroborating different authors (Tsai *et al*., 2012; Miglioli, 2009). *Streptococcus agalactiae, Escherichia coli* and *Klebsiella pneumoniae* were not identified by the blood culture technique. In detecting the genomic DNA of the analyzed bacteria, conventional PCR (universal primer) and qPCR proved to be more sensitive diagnostic techniques when compared with blood culture. However, the use of specific primers in qPCR associated with clinical signs allows for a faster, specific and probably better prognosis. Although the techniques of molecular biology are already used in other countries and even in some regions of Brazil, this technique is still scarcely used in MS. Further studies are suggested that demonstrate the safety and the agility provided by this technique, and the possible beneficial interference in the costs related to hospitalization time and antibiotics use.

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