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Reação em cadeia da polimerase (PCR) para a detecção do genoma de *Treponema pallidum* em amostras de sangue e fluido cerebroespinal de recém-nascidos em diferentes situações de risco para sífilis congênita – uma amostra do sul do Brasil

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Orientador: Prof. Dr. Renan Rangel Bonamigo

Co-orientador: Dr. Mauro Cunha Ramos

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Resumo

Introdução: A transmissão vertical da sífilis continua sendo um grave problema de saúde pública. Atualmente utilizam-se critérios clínicos e laboratoriais que, em conjunto, sugerem ou não o diagnóstico de sífilis congênita (SC). A multiplicidade de testes solicitados indica que a sensibilidade e a especificidade destes são aquém do ideal e refletem uma interpretação relativa dos resultados. É necessária a busca por testes com maiores sensibilidade e especificidade que auxiliem a tomada de decisão clínica. **Objetivos:** Desenvolver a técnica da reação em cadeia da polimerase (PCR) para identificação do DNA de *Treponema pallidum* em amostras de sangue e líquido cefalorraquidiano (LCR) de recém-nascidos em diferentes situações de risco para SC e verificar a positividade da técnica nestes grupos. **Materiais e Métodos:** Em estudo transversal, com grupo de comparação, conduzido entre agosto de 2019 e fevereiro de 2020 no Hospital Santa Casa de Misericórdia de Porto Alegre, foram coletadas amostras de sangue e LCR de recém-nascidos expostos verticalmente à sífilis, com indicação de investigação para SC conforme protocolo institucional. O grupo de comparação foi composto por recém-nascidos sem história de exposição à sífilis, mas com outras indicações clínicas para coleta dessas amostras. Todas as amostras coletadas foram submetidas ao exame de PCR convencional. **Resultados:** Foram incluídos 57 pacientes (35 expostos à sífilis e 22 como grupo comparativo). A PCR foi positiva em 57,6% das amostras de sangue e 66,7% das amostras de LCR nos pacientes expostos à sífilis. Neste grupo, não

houve relação entre a positividade da PCR e a positividade de seu RPR, a positividade do RPR da mãe e/ou o tratamento materno prévio. Verificou-se positividade da PCR em duas amostras de sangue e cinco amostras de LCR no grupo de comparação. **Conclusão:** Embora a PCR pareça ser um método promissor para compor a avaliação de casos suspeitos de SC, há necessidade de continuidade dos estudos sobre a técnica e a importância de seu uso.

Palavras-chave: Sífilis congênita, PCR, Diagnóstico

Abstract

Introduction: The vertical transmission of syphilis remains a serious public health problem. Currently, there are many clinical and laboratorial criteria that, together, suggest or not the diagnosis of congenital syphilis (CS). The need for multiple tests for the diagnosis of CS reveals that their sensitivity and specificity are less than ideal and reflect a relative interpretation of their results. Therefore, it is necessary to search for tests with greater sensitivity and specificity that can assist clinical decision-making. **Aim of study:** To develop the technique of polymerase chain reaction (PCR) for the identification of *Treponema pallidum* DNA in blood and cerebrospinal fluid (CSF) samples from newborns in different risk situations for CS. **Materials and methods:** In a cross-sectional comparative study conducted between August 2019 and February 2020 at Hospital Santa Casa de Misericórdia in Porto Alegre, blood and CSF samples were collected from newborns vertically exposed to syphilis, who had indication to investigate CS according to the hospital protocols (exposed group). The comparative group consisted of infants with no history of exposure to syphilis who had other clinical indications for collection of these samples. All biological samples collected were submitted to conventional PCR. **Results:** Fifty seven patients were included (35 in the exposed group and 22 in the comparative group). The PCR analysis was positive in 19 of the 33 blood samples (57.6%), and 22 of the 33 CSF samples (66.7%) in the exposed group. When comparing maternal treatment or positive RPR results of mothers or infants with PCR positive results, no relationship was found. There were also positive PCR results in 2 blood samples and 5 CSF samples in the comparative group.

Conclusion: Although PCR can be a promising method to compose the evaluation of suspected cases of CS, there is still need for further studies on the technique and the importance of its use.

Keywords: Congenital syphilis, PCR, Diagnosis

Lista de abreviaturas

IST: Infecção sexualmente transmissível

OMS: Organização Mundial da Saúde

VDRL: Venereal Disease Research Laboratory

RPR: Rapid Plasma Reagin

FTA: Fluorescent Treponemal Antibody

FTA-Abs: Fluorescent Treponemal Antibody adsorbed

TPHA: *Treponema pallidum* Hemagglutination Assay

TPPA: *Treponema pallidum* Particle Agglutination

EIA: Enzymatic imunoassay

CIA: Chemiluminescent immunoassay

PCR: Reação em Cadeia da Polimerase

DNA: Ácido desoxirribonucleico

RIT: Rabbit infective test

ELISA: Enzyme-Linked Immunosorbent Assay

EUA: Estados Unidos da América

LCR: Líquido cefalorraquidiano

HIV: Human Immunodeficiency Virus

ULBRA: Universidade Luterana do Brasil

UFCSPA: Universidade de Ciências da Saúde de Porto Alegre

PPG: Programa de Pós Graduação

HCSA: Hospital da Criança Santo Antônio

ISCMPA: Irmandade Santa Casa de Misericórdia de Porto Alegre

STI: Sexually transmitted disease

CS: Congenital syphilis

CSF: Cerebroespinal fluid

CDC: Centers for Disease Control and Prevention

SD: Standard deviation

LBW: Low birth weight

FGR: Fetal growth restriction

NB: Newborn

SUMÁRIO

JUSTIFICATIVA

A transmissão vertical da sífilis é um evento passível de prevenção, entretanto continua sendo um grave problema de saúde pública nos países subdesenvolvidos. É possível constatar isto considerando o fato de que no Brasil, entre os anos de 2018 e 2019, observou-se um aumento de 3,8 vezes na incidência de sífilis congênita no país, que passou de 2,4 para 9,0 casos novos por mil nascidos vivos. No mundo, a prevalência da sífilis em gestantes e seus desfechos desfavoráveis foi de aproximadamente 988 mil casos no ano de 2016. Isto revela a falência dos sistemas de saúde em diagnosticar e tratar adequadamente as gestantes portadoras de sífilis.

Para o diagnóstico da sífilis congênita, atualmente, são utilizados critérios clínicos e laboratoriais que, em conjunto, sugerem ou não o diagnóstico. A multiplicidade de testes solicitados indica que a sensibilidade e a especificidade destes se encontram aquém do ideal e refletem uma interpretação relativa dos resultados.

Por este motivo, faz-se necessária a busca por testes com maiores sensibilidade e especificidade e que possam auxiliar a tomada de decisão clínica.

Neste estudo, é avaliada a hipótese de que as crianças com PCR positivos para a detecção do genoma do *T. pallidum* em amostras de sangue e líquido cefalorraquidiano sejam portadores de sífilis congênita, considerando como “padrão-ouro” o atual fluxograma para o diagnóstico de sífilis congênita

e de neurossífilis, determinado pelas “Diretrizes brasileiras para o diagnóstico e controle da sífilis congênita”, do Ministério da Saúde do Brasil.[9]

É importante também avaliar o comportamento da PCR para a detecção do genoma de *Treponema pallidum* em amostras de sangue e fluido cerebrospinal em recém-nascidos em diferentes situações de risco para sífilis congênita, com o intuito de encontrar métodos diagnósticos seguros que permitam o diagnóstico mais preciso da sífilis congênita e que também auxilie a tomada de decisão clínica em relação ao tratamento destes recém-nascidos.

OBJETIVOS

Objetivo geral

Avaliar frequência da positividade da PCR para a detecção do genoma de *Treponema pallidum* em amostras de sangue e líquido cefalorraquidiano, em recém-nascidos sob diferentes situações de risco para sífilis congênita.

Objetivos específicos

Desenvolver uma técnica de PCR para a detecção do DNA de *Treponema pallidum* em amostras de sangue e líquido cefalorraquidiano, a partir de técnicas descritas na literatura.

Determinar a frequência de sinais clínicos de sífilis congênita ao nascimento em recém-nascidos em diferentes situações de risco para sífilis congênita.

Verificar possíveis fatores de risco para a ocorrência de sífilis congênita e de neurossífilis em recém-nascidos com diferentes situações de risco para sífilis congênita, considerando aspectos epidemiológicos, histórico de tratamento e acompanhamento pré-natal.

CONSIDERAÇÕES ÉTICAS

O projeto foi aprovado pelas Comissões de Ética em Pesquisa com Seres Humanos da Universidade Federal de Ciências da Saúde de Porto Alegre (UFCSPA) (parecer 2.547.348) e do Hospital da Criança Santo Antônio (HCSA) (Centro Coparticipante), sob o número 3.432.633.

Os autores da pesquisa assinaram o Termo de Compromisso para utilização de dados e a Declaração de manuseio de materiais biológicos.

Os pacientes que concordaram em participar do estudo assinaram o Termo de Consentimento Livre e Esclarecido.

ARTIGO REDIGIDO EM INGLÊS

Polymerase Chain Reaction (PCR) for *Treponema pallidum* genome in blood samples and cerebrospinal fluid of newborns in different risk for congenital syphilis - a sample from south of Brazil

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Introduction

Syphilis is a sexually transmitted infection (STI) caused by the *Treponema pallidum* spirochete bacterium which can be sexually or vertically transmitted.[1] It is a systemic disease, exclusive of humans and curable with appropriate antibiotic treatment.

Recently, the Brazilian Ministry of Health published a report revealing a 3.8-fold increase in the incidence of congenital syphilis (CS) between 2010 and 2018.[2] These data reflect a serious public health problem in underdeveloped and developing countries, since vertical transmission of syphilis is an absolutely preventable event.

The prevalence of syphilis in pregnant women and its unfavorable outcomes worldwide was estimated as 988.000 cases in 2016. It resulted in 143.000 abortions or fetal deaths, 14.000 neonatal deaths, 41.000 premature or low birth weight and 109.000 infected newborns who developed clinical complications due to syphilis infection.[3]

This burden of morbidity and mortality, however, is not proportionally distributed, with evident overload in middle and low income countries.[4] In those countries where investment in health is lower, access to health services is more difficult, including access to antenatal care.

Currently, for the diagnosis of congenital syphilis, clinical and laboratory criteria are used, which together suggest or not the diagnosis. The multiplicity of tests requested indicates that their sensitivity and specificity are less than ideal, and national and international institutions establish scenarios in which

the infection is considered present, probable or possible. For this reason, it is necessary to search for new tests that can assist clinical decision-making.

Considering the current epidemiological importance of maternal-fetal syphilis and the scarce publication about diagnostic tests for congenital syphilis, the aim of this study was to investigate CS using a molecular assay in samples of whole blood and cerebrospinal fluid (CSF) of newborns, and also to evaluate the factors related to mother-fetus exposure.

Methods

Settings

This cross-section study was conducted between August, 2019 and February, 2020, and involved newborns who were admitted at the Obstetrics Center of Hospital Santa Casa de Misericórdia de Porto Alegre (ISCMPA), Brazil.

The study was approved by the Research Ethics Committee of the Federal University of Health Sciences of Porto Alegre and Committee of Hospital Santa Casa de Misericórdia de Porto Alegre. Written consent for publication was obtained from all the relatives' patients. The epidemiological and clinical data, and complementary exams results were obtained from their medical records.

During the study period, a total of 57 newborns were studied, of whom 35 composed group 1 – consisted of newborns exposed to syphilis - and 22

composed group 2 (the comparative group), consisted of newborns not exposed to syphilis. Group 1 was made up of newborns exposed to syphilis, whose mothers had clinical and/or serological diagnosis of syphilis during pregnancy. Group 2 was made up of newborns whose mothers returned negative results in treponemal serological tests for syphilis during pregnancy, and had no history of syphilis exposition in the past, but the infants had an indication for blood and CSF collection for other reasons (such as early neonatal sepsis, congenital toxoplasmosis infection or herpes). Two patients were excluded because they did not fulfill the inclusion criteria for the comparative group: they had clinical indication to investigate exposition to toxoplasmosis, but their mothers had history of syphilis treatment before pregnancy, the reason why their treponemal tests were positive during prenatal and delivery, but nontreponemal tests were negative.

The working definition of CS and maternal syphilis cases applied in the present study are based on the recommendations of the Centers for Disease Control and Prevention (CDC) and Brazilian Health Ministry[5,6,7]. Maternal syphilis was assumed when during pregnancy, delivery or postpartum: (a) there was clinical signs of syphilis and a reactive test (treponemal and/or nontreponemal, with any title); (b) women were asymptomatic and had a reactive test (treponemal or nontreponemal, with any title) and no prior treatment record, and (c) women had a treponemal and a nontreponemal test reactive, regardless of symptomatology and previous treatment for syphilis.[7] Congenital syphilis was defined as any case of child borne by a mother with clinical and/or serological diagnosis of syphilis conducted at birth, who was not

treated or had inappropriate treatment during prenatal care. The diagnostic criteria for CS based on four different scenarios according to CDC is shown in chart 1.The Brazilian Ministry Health flowchart for diagnosis of CS is very similar to the CDC's guidelines, except that PCR is not available in the clinical context, only for research purposes.[6,7]

Chart 1. Diagnostic criteria for Congenital Syphilis, according to CDC (2015)[5]

Scenario 1: Proven or highly probable congenital syphilis	Any neonate with: a) an abnormal physical examination that is consistent with congenital syphilis **or**; b) a serum quantitative nontreponemal serologic titer that is fourfold ¶ higher than the mother's titer **or**; c) a positive darkfield test or PCR of lesions or body fluid(s).
Scenario 2: Possible Congenital Syphilis	Any neonate who has a normal physical examination and a serum quantitative nontreponemal serologic titer equal to or less than fourfold the maternal titer and one of the following: a) mother was not treated, inadequately treated, or has no documentation of having received treatment **or**; b) mother was treated with erythromycin or a regimen other than those recommended in these guidelines (i.e., a nonpenicillin G regimen)††**or**; c) mother received recommended treatment <4 weeks before delivery.
Scenario 3: Congenital Syphilis less likely	Any neonate who has a normal physical examination and a serum quantitative nontreponemal serologic titer equal to or less than fourfold the maternal titer and **both** of the following are true: a) mother was treated during pregnancy, treatment was appropriate for the stage of infection, and treatment was administered >4 weeks before delivery **and**; b) mother has no evidence of reinfection or relapse.
Scenario 4: Congenital Syphilis unlikely	Any neonate who has a normal physical examination and a serum quantitative nontreponemal serologic titer equal to or less than fourfold the maternal titer and **both** of the following are true: a) mother's treatment was adequate before pregnancy **and**; b) mother's nontreponemal serologic titer remained low and stable before and during pregnancy and at delivery (VDRL <1:2; RPR <1:4).

¶The absence of a fourfold or greater titer for a neonate does not exclude congenital syphilis; †† A women treated with a regimen other than recommended in CDC guidelines should be considered untreated.

On the clinical-epidemiological variables, the following data were collected from both groups: city of origin, maternal age, newborn sex gender and birth weight, gestational age, number of medicals during the prenatal period, first prenatal medical appointment, prenatal serology tests, adequate treatment of the mother and partner, use of illegal drugs, consumption of alcohol and tobacco, coinfection with human immunodeficiency virus (HIV), exposure to other infectious microorganisms during pregnancy (*Toxoplasma gondii*, *Herpes simplex virus*), and other chronic diseases.

The newborn infants were considered preterm when the gestation length was less than 37 weeks, and the classification for fetal growth was based on the birth weight/gestational age index and curves recommended by World Health Organization in 1996.[8] Other clinical signs consistent with CS were considered when mentioned in the medical records.

All the patients' mothers selected for the present study were screened using the nontreponemal Rapid Plasma Reagin test (RPR) test and quimioluminescent treponemal test at the moment of delivery in the ISCMPA laboratory. Samples of blood and cerebrospinal fluid (CSF) were collected from newborns who had recommendations for laboratorial analysis of these biological samples according to the standard clinical protocols of Hospital. The purpose of non-maleficence in this study was applied by the use of biological samples that would already be collected from newborns according to hospital clinical routines, to avoid submitting infants to other unnecessary procedures or interventions.

Children who had been exposed to vertical syphilis (exposed group) were evaluated with complete blood count, serum non-treponemal test (RPR), CSF analysis for RPR, cell count, glucose and protein, and long bone radiographs.

Children who had not been exposed to vertical syphilis during the pregnancy (comparative group) were evaluated according to their clinical indication (ex. early and late neonatal sepsis, vertical exposition to toxoplasmosis or herpes virus).

Conventional PCR was performed in all biological samples of whole blood and CSF collected. We developed the PCR technique to be used based on the description made by Palmer *et al*.[9]

After DNA extraction, two primers were used to encode the membrane protein of 47KDa *T. pallidum* gene (KO3: 5' GAAGTTTGTCCCAGTTGCGGTT-3'; KO4: 5'CAGAGCCATCAGCCCTTTTCA 3'). These primers were described to amplify a fragment of 260 bp. The amplification conditions were: 95°C for 2 minutes, followed by 35 cycles of 95°C for 20 seconds, 62°C for 20 seconds, and a final extension of 72°C for 20 seconds. The reaction amplified fragments of 260 bp, and these products were analyzed by electrophoresis using a 2% agarose gel. (Figure 1) Positive control included *T. pallidum* DNA extracted from genital ulcers of patients who were diagnosed with primary syphilis. Negative control was composed by a solution with only the mixture of the reagents, free of DNA.

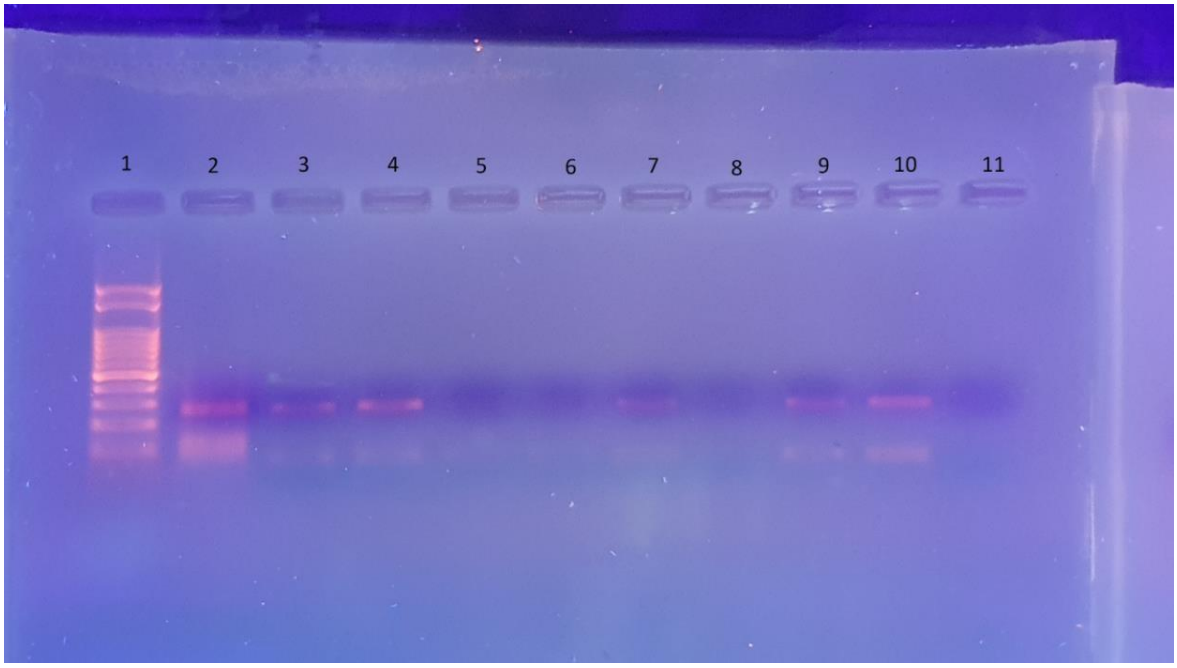

Figure 1. Representative image of an electrophoretic analysis on agarose gel 2% of the PCR products obtained with specific primers for *T. pallidum* from blood samples extracted from dried blood spot. 1: molecular size marker 100pb. 2: positive control; 3, 4, 7, 9 e 10:positive samples for *T. pallidum;* 5, 6 e 8: negative samples for *T. pallidum*; 11: negative control.

Statistical analyses

Demographic and clinical variables were described. Qualitative variables were described as absolute numbers and percentages. Quantitative variables were described as mean and standard deviation. The relationship between the results of PCR in biological samples and the clinical variables were described in its frequency by percentage. Data storage and analysis were performed using EPI Info 7.

The sensivity and specificity of the test were not calculated because there is no gold standard in clinical practice that can validate the PCR assay.

Results

The mean age of women was 26,45 (±SD: 5.87) years, and the mean age of both groups were similar. Table 1 presents these data and the set of data related to the findings described below.

There was only one patient - from the group 1 - who had not attended to prenatal care. Most of the women had six or more medical appointments during prenatal in both groups. The two groups were also similar in terms of the beginning of prenatal care, more than 50% of them started in the first trimester of pregnancy.

Comparative group had significant more stillbirths and consequently lower birth weight[7]. About 41% percent of newborns were premature in the group 2 and the mean birth weight for this group was 2688.09 (±SD:879.47) grams. On the other hand, 8.5% of the newborns were premature in the group 1 and the mean birth weight for this group was 3557.14 (±SD: 384.66) grams. Regarding classification for fetal growth based on the birth weight/gestational age index, three newborns were considered small for gestational age, one from group 1 and two from group 2.

Tobacco was the most commonly drug used during pregnancy, and the total number of smoking patients was similar in both groups. The use of alcohol was observed in two patients of the comparative group, while the use of illicit drugs was reported by three patients of the group 1. The use of these substances during pregnancy does not appear to be related to prematurity, low birth weight (LBW) or fetal growth restriction (FGR) in this sample.

HIV infection was observed at the same frequency in both groups (one patient in each group). Of note, only the patient in the group 2 did not treat HIV during pregnancy.

Two patients from the group 1 had history of previously exposition to syphilis, treated in the past. They were considered to be re-exposed to syphilis during the current pregnancy, the reason why their newborns were investigated for congenital syphilis with complementary exams.

Mothers from the group 2 were more likely to have chronic diseases. The most common were gestational diabetes mellitus (7%), followed by hypothyroidism (5%). The other chronic conditions observed less frequently were chronic arterial hypertension, obesity, sickle cell trait and epilepsy. Five patients (22,7%) from the group 2 had at least one of these conditions, while three patients (8,6%) from the group 1 had one or more of these conditions. The presence of chronic diseases does not seem to be directly related to stillbirths, LBW or FGR.

All newborns in the group 1 had CSF investigated because of vertical exposure to syphilis. One of them was also exposed to toxoplasmosis during pregnancy. In the group 2, five newborns had indication for CSF analysis due to vertical exposure to toxoplasmosis and one because of exposure to *Herpes simplex* virus during delivery. Most part of newborns in the comparative group was investigated because of early neonatal sepsis, and some of them were preterm. Stillbirths in the group 2 were related to maternal conditions as: prelabor rupture of membranes (3), preeclampsia (1), group B streptococcal

colonization (1), adramnia (1) and HIV without treatment (1). In the group 1 there were three preterm births: one patient did not have any chronic disease or obstetric condition, one patient presented gestational diabetes mellitus and one patient whose newborn had clinical signs of early congenital syphilis.

A total of 55 samples of CSF and 54 of whole blood were collected for this study. Two patients from the group 1 had only blood samples collected due to the technical impossibility of performing lumbar puncture or error in the storage samples, and three patients (two from the group 1 and one from the group 2) had only CSF collected.

Table 1. Socio-demographic and delivery variables, sexual, reproductive and health characteristics, and variables related to prenatal care. ISCMPA, 2019–2020

Variable	**NB exposed to syphilis (group 1)**		**NB comparative (group 2)**		
	N / n	% / SD	**N / n**	% / SD	Average
Age (mean in years)	26,7	SD: 5,96	26,0	SD: 5,85	26,45
Prenatal care	34 / 35	97,1%	22 / 22	100%	98,2%
Pre-natal care initiated in					
First trimester	16 / 34	47%	13 / 22	59,1%	51,8%
Second trimester	10 / 34	29,5%	06 / 22	27,3%	28,6%
Third trimester	02 / 34	5,9%	00 / 22	0%	3,6%
Missing data	06 / 34	17,6%	03 / 22	13,6%	16%
N° medical appointments					
No prenatal care	01 / 35	2,8%	00 / 22	0%	1,8%
1 to 5 medicals	08 / 35	22,8%	05 / 22	22,7%	22,8%
6 or more medicals	20 / 35	57,2%	15 / 22	68,2%	61,4%
Missing data	06 / 35	17,2%	02 / 22	9,1%	14%
Gestational age					
Term	32 / 35	91,5%	13 / 22	59,1%	79%
Preterm	03 / 35	8,5%	09 / 22	40,9%	21%
Post term	00 / 35	0%	00 / 22	0%	0%
Birth weight / gestational age index					
SGA	01 / 35	2,9%	02 / 22	9,1%	5,2%
AGA	33 / 35	94,2%	20 / 22	90,9%	93%
LGA	01 / 35	2,9%	00 / 22	0%	1,8%
Birth weight (mean in grams)	3557,14	SD: 384,66	2688,09	SD: 879,47	3037,50

Tobacco use (yes)	05 / 35	14,2%	04 / 22	18,2%	15,8%
Alcohol use (yes)	00 / 35	0%	02 / 22	9,1%	3,5%
Ilicit drugs use (yes)	03 / 35	8,5%	00 / 22	0%	5,2%
HIV infection (yes)	01 / 35	2,9%	01 / 22	4,5%	3,5%
History of syphilis treated in the past (yes)	02 / 35	5,7%	00 / 22	0%	3,5%
Other chronic diseases					
Gestational diabetes mellitus	02 / 35	5,7%	02 / 22	9,1%	7%
Hypothyroidism	01 / 35	2,9%	02 / 22	9,1%	5,3%
Other	01 / 35	2,9%	03 / 22	13,6%	7%
Motivation for CSF investigation					
Exposure to syphilis	35 / 35	100%	00 / 22	0%	61,4%
Exposure to toxoplasmosis	01 / 35	1%	05 / 22	22,7%	10,5%
Early neonatal sepsis	00 / 35	0%	15 / 22	68,2%	26,3%
Other	00 / 35	0%	02 / 22	9,1%	3,5%

NB: newborn; SGA: small for gestational age; AGA: appropriate for gestational age; LGA: large for gestational age

All CSF samples from group 1 were analyzed for cell count, protein, glucose and RPR. The RPR analyses were non-reactive in all CSF samples in this group. Protein, glucose and white blood cells were also normal for all samples in both groups. Some patients had isolated increases in red cell count in CSF due to incidental trauma during lumbar puncture.

Whole blood samples were collected from patients from the group 1 for RPR analysis. Additionally, blood was also collected from the comparative group patients for specific analysis according to their clinical indication. (Table 1)

PCR analysis was positive in five CSF and two whole blood samples in the group 2. A total of five patients had at least one sample positive for PCR. It is noteworthy that two of these patients had both CSF and whole blood positive for PCR analysis.

Regarding the group 1, PCR analysis was positive for 22 (66,7%) CSF and 19 (57,6%) whole blood samples. Fourteen patients had both CSF and whole blood samples positive for PCR (subgroup 1a). Eight patients had only positive PCR for their CSF samples (subgroup 1b), five had positive PCR only for whole blood samples (subgroup 1c) and eight patients had both CSF and whole blood negative results for PCR analysis (subgroup 1d). (Figure 2)

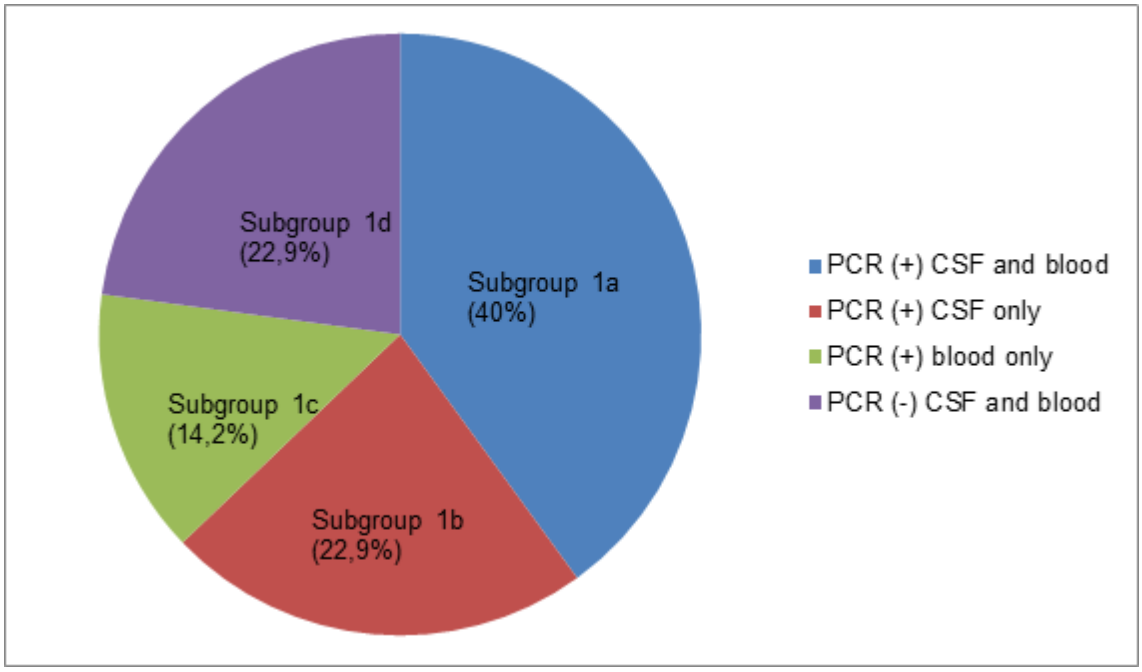

Figure 2. Classification of the group 1 patients (exposed to syphilis), according to their PCR results in biological samples

Twenty five newborns in the group 1 had reactive RPR results in whole blood samples. All of them had RPR titers equal to or less than their maternal

titers. (Table 2) Ten newborns and six women from this group had nonreactive RPR results for these samples. There was no relation identified between RPR titers (maternal and neonatal) and positivity in PCR assay.

Most women had documented penicillin treatment during pregnancy (74,3%) in all subgroups. On the other hand, only 20% of the sexual partners had documentation of penicillin treatment during pregnancy. (Table 2) It is noteworthy that women who had less than six medical appointments during prenatal care were less likely to treat syphilis during pregnancy.

Long bone radiographs were more likely to have normal findings in subgroups 1a, 1b and 1d. Only subgroup 1c had 60% of abnormal radiographic exams, and the findings were suggestive of congenital syphilis, but not specific. Symmetric localized demineralization of the medial portion of the proximal long bone metaphysis and metaphyseal lucent bands may occur in congenital syphilis but can also appear in neonatal hyperparathyroidism and osteomyelitis. The missing data was related to radiographs that did not have been evaluated and reported by radiologists. (Table 2)

Table 2. Group 1 characteristics sorted by subgroups according to PCR results in biological samples

	PCR (+) CSF/blood Subgroup 1a	PCR (+) CSF Subgroup 1b	PCR (+) blood Subgroup 1c	PCR (-) CSF/blood Subgroup 1d	Total
Newborn RPR n (%)					
> maternal	4 (28,5%)	4 (50%)	3 (60%)	3 (37,5%)	14 (40%)
< maternal	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)
equal to maternal	10 (71,5%)	4 (50%)	2 (40%)	5 (62,5%)	21 (60%)
Long bone radiography n (%)					
Normal	7 (50%)	5 (62,5%)	1 (20%)	3 (37,5%)	16 (45,7%)
Abnormal	2 (14,3%)	1 (12,5%)	3 (60%)	3 (37,5%)	9 (25,7%)
Missing	5 (35,7%)	2 (25%)	1 (20%)	2 (25%)	10 (28,6%)

Documented treatment during pregnancy n (%)					
Penicilin	10 (71,5%)	6 (75%)	4 (80%)	6 (75%)	26 (74,3%)
Other	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)
No treatment	4 (35,7%)	2 (25%)	1 (20%)	2 (25%)	9 (25,7%)
Documented sexual partner treatment n (%)					
Penicilin	2 (14,3%)	1 (12,5%)	2 (40%)	2 (25%)	7 (20%)
Other	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)
No treatment	12 (85,7%)	7 (87,5%)	3 (60%)	6 (75%)	28 (80%)
Newborn treatment n (%)					
Penicilin G Crystalline	10 (71,5%)	5 (62,5%)	4 (80%)	6 (75%)	25(71,5%)
Peniclin G Benzathine (single dose)	4 (28,5%)	2 (25%)	1 (20%)	0 (0%)	7 (20%)
No treatment	0 (0%)	1 (12,5%)	0 (0%)	2 (25%)	3 (8,5%)

Nearly 72% of the newborns exposed to syphilis were treated with intravenous penicillin for 10 days in hospital. Twenty percent of them were treated with one dose of intramuscular penicillin G benzathine and 8,5% did not receive any treatment. (Table 2) One of these patients belonged to subgroup 1b and two from subgroup 1d. The subgroup 1b patient did not receive any treatment because both maternal and newborn RPR results in the blood were non-reactive. Also, the mother was treated with three doses (7.2 million UI) of penicillin G benzathine during pregnancy and the others complementary exams were all normal. One of the patients from subgroup 1d did not receive any treatment for the same reason. Both patients had outpatient follow-up after hospital discharge. The other patient from subgroup 1d evaded from the hospital and did not wait for the exam results. For this reason, this newborn did not treat congenital syphilis. This was the same patient who did not attend to prenatal care and also didn’t treat syphilis during pregnancy. All of newborn’s

complementary exam results were normal.

One subgroup 1b patient had clinical signs of early congenital syphilis: hepatosplenomegaly, jaundice and nasal discharge. In complementary exams thrombocytopenia was observed, with normal levels of hemoglobin and elevated white blood cell count. This patient had four prenatal appointments, but did not treat syphilis during prenatal. Their RPR had higher titers, the mother's nontreponemal test was 1:128 and the newborn's was 1:32. The newborn also had elevation on serum bilirubin and C-reactive protein levels. Blood cultures were negative. He was born prematurely (gestational age was 35 weeks) and with low birth weight (2150 grams), but he did not have FGR according to birth weight/gestational age index. During hospitalization this child developed seizures. This patient had a positive PCR result for the CSF sample, but his whole blood sample was negative. His long bone radiography was missing, and he was treated with intravenous penicillin G crystalline for 10 days.

Discussion

In this study, there was equivalence between the newborns exposed to syphilis (group 1) and the comparative newborns (group 2) related to the number of prenatal appointments and the beginning time of the follow-up. This information is consistent with the data observed by Benzaken et al,[10] who found out that although there is a high prenatal coverage in the city of Porto Alegre, there is still a portion of the population that is more susceptible to infection with

syphilis during pregnancy. Notably, women in the case group 1 who had less than five medical appointments were less likely to treat syphilis during pregnancy.

The groups were also similar in maternal age, HIV infection, use of tobacco and alcohol. Women in the group 1 were more likely to use illicit drugs during pregnancy, which may reflect the relationship between the exposure to syphilis and social vulnerability.

The fact that only 20% of sexual partners have been investigated and/or treated for syphilis shows the need to develop policies that seek to include and maintain the partner in the prenatal care. This may be the reason why, although most women were treated for syphilis during prenatal, therapeutic failure was identified during the follow-up. Some therapeutic failures can also be explained by inadequate penicillin regimen for the syphilis clinical stage of mothers.

The only newborn who had clinical signs of congenital syphilis, had no report of penicillin treatment during prenatal care and presented (both neonate and the mother) higher RPR titers. This infant developed seizures during hospitalization, which could not be explained by any other reason than congenital syphilis, which leads us to believe that this infant had clinical signs of neurosyphilis. It is interesting to note that, although the analysis of proteins, glucose and cell count of CSF were normal, as well as negative RPR, the DNA of *T. pallidum* was detected in this CSF sample. Currently, the major difficulty for the diagnosis of neurosyphilis lies in the fact that there is no exam with high sensitivity to predict this condition in newborns. The sensitivity of VDRL in CSF

samples of neonates is 54% and its significance is not clear yet, since there may be false positives (related to maternal nontreponemal immunoglobulin antibodies that cross the placenta and diffuse into the fetal CSF or contamination of the CSF with blood from a traumatic lumbar puncture) and false negatives (neonates with initial nonreactive CSF VDRL may subsequently develop signs of neurosyphilis). Likewise, the sensitivities of pleocytosis (elevation of white blood cells in CSF) and elevation of CSF proteins are low, 38% and 56% respectively.[11] The literature estimates that CNS involvement by *T. pallidum* occurs in 40% of infants who have clinical, laboratory, or radiographic abnormalities of congenital syphilis, but is infrequent in infants without such manifestations.[11,12,13] Few studies have tested the PCR assay for CSF samples of newborns exposed to syphilis, and sensitivity ranged from 60 to 75% compared to RIT.[11,14,15] Michelow found positive PCR results in cerebroespinal fluid of newborns exposed to syphilis who had negative results for RIT, which may reflect false positive PCR test results or maybe that sample volume used for rabbit inoculation was insufficient.[11]

We used Elute filter paper cards for storage of blood samples in order to avoid contamination of blood samples previously handled in the ISCMPA laboratory, and because of the convenience of easy collection (the sample can be collected at the same time as “neonatal screening”) and easy storage. There is no such study using dried blood spots for PCR assay for *T. pallidum*. We based our hypothesis on several other studies that use dried blood spot to extract DNA of other biological agents such as hepatitis C and HIV.[16,17]

The presence of a small amount of circulating *T. pallidum* in the peripheral blood may explain why PCR was negative in the blood sample of the newborn with clinical signs of syphilis, once sensivity of this exam in this biological sample is highly variable.[11,14]

This was the first study to compare the PCR results in samples of newborns exposed to syphilis to a comparative group. Whereas the gold standard flowchart currently used for the diagnosis of congenital syphilis does not assure the infection, the existence of a comparative group allow us to assess the possibility of false positive PCR results, or that the current gold standard diagnostic flowchart may be missing exposed and/or infected infants, although this second hypothesis is less likely considering the sensitivity of RPR and rapid test for acquired syphilis is 98% and 94,5%, respectively.[18] The other few studies about this subject compared PCR results to the rabbit infective test (RIT) or the results of PCR newborn samples to their mothers' PCR results.[11,14,19]

Since there is no standardized technique PCR for detection of *T. pallidum* DNA, many studies tried to develop the most suitable technique for different biological samples. It seems that nested PCR is more sensitive compared to others, but further studies are necessary to confirm this hypothesis.[20] Besides that, sensitivity of PCR also depends on the biological sample and the target gene. We have developed a conventional PCR-based technique described by Palmer in 2003 for genital ulcer samples.[9] We have found that conventional PCR targeting *tpp47* was positive in 66,7% (22/33) of

CSF and 57,6% (19/33) of whole blood samples in neonates exposed to syphilis. On the other hand, also five CSF and two whole blood samples were positive in the comparative group, which cannot be explained by exposure to syphilis because all of these mothers had nonreactive treponemal and nontreponemal tests at the time of delivery, except if we considered the tests of these mothers as false negative and / or there was traces of treponemal antigens in patients already treated and negative in their tests. It could also be explained by a possible contamination of the samples when collecting, storing or performing the PCR. The phenomenon of immunological window - where the patient is in the early stages of the infection and there was no time yet for the immune system to produce detectable antibodies - could also explain these PCR results, although this hypothesis is less likely.

Thus, despite PCR may be a promising method to compose the evaluation of suspected cases of CS, there is still a need to continue studies on the technique and the importance of use.

Finally, this article sheds light on an often neglected topic, which still has little published data and presents an overview of clinical variables of interest.

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CONCLUSÕES

O estudo atual buscou desenvolver a técnica de Reação em Cadeia da Polimerase (PCR) para detecção do DNA do *Treponema pallidum* em amostras de sangue e líquido cefalorraquidiano (LCR) de recém-nascidos expostos à sífilis em comparação com um grupo de recém-nascidos sem esta exposição. Consideramos importante a existência do grupo para comparação, uma vez que a exposição vertical à sífilis não é capaz de confirmar a infecção por sífilis congênita (SC), sendo necessários diversos exames com baixa sensibilidade e especificidade que, em conjunto, sugerem ou não o diagnóstico de SC. Não houve relação direta entre os resultados destes exames com a positividade do PCR nas amostras biológicas nesta população estudada.

Considerando que não há padronização da técnica, desenvolvemos nosso exame de PCR convencional baseados em publicações de outros autores que desenvolveram suas técnicas em estudos realizados anteriormente, utilizando o gene *tpp47* como alvo. Este gene e o *polA* foram os que demonstraram maiores especificidade nos estudos já realizados.

A existência de um grupo comparativo, sem história de exposição prévia à sífilis e com testes não treponêmicos e treponêmicos não reagentes nos leva a acreditar que os resultados de PCR positivos encontrados nestas amostras são falso positivos, e que há necessidade de aprimorar a técnica de PCR para auxiliar no diagnóstico mais preciso da SC e na decisão do tratamento ou não dos recém-nascidos expostos.

CONSIDERAÇÕES FINAIS E PERSPECTIVAS

A busca por testes diagnósticos com maiores sensibilidade e especificidade para identificação da infecção por *T. pallidum* em recémnascidos expostos à sífilis continua sendo um desafio, especialmente porque não há muitos estudos acerca deste tema.

Vislumbra-se na PCR uma técnica promissora, que já se mostrou sensível para a detecção do *T. pallidum* em swabs de úlceras de sífilis primária. Entretanto, é preciso aprimorá-la para outros tipos de amostras biológicas, como sangue e líquido cefalorraquidiano, onde a concentração do treponema é menor.

A técnica molecular utilizada neste trabalho foi a de PCR convencional baseada em um trabalho descrito por Palmer *et al*., em 2003, que desenvolveu esta técnica para identificação do treponema em swabs de cancro primário. É possível que esta não seja a técnica mais adequada para as amostras de sangue e líquido cefalorraquidiano, e por este motivo o estudo tem como perspectiva submeter as amostras biológicas a uma técnica molecular diferente (PCR em tempo real) e comparar os resultados das duas técnicas futuramente (artigo a ser escrito, comparando as duas técnicas).