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Maternal-fetal interface: The contribution of histopathological analysis, molecular detection and indirect immunofluorescence of *Treponema pallidum pallidum* in placental tissue and umbilical cord

Interface materno-fetal: A contribuição da análise histopatológica, detecção molecular e imunofluorescência indireta na detecção do *Treponema pallidum pallidum* no tecido de placenta e cordão umbilical

Interfaz materno-fetal: La contribución del análisis histopatológico, la detección molecular y la inmunofluorescencia indirecta en la detección de *Treponema pallidum pallidum* en tejido de placenta y cordón umbilical

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Abstract

Treponema pallidum can be transmitted to the fetus through the placenta and cause congenital syphilis (CS). Despite that, few studies have focused on the diagnostic approaches used to analyse congenital syphilis. The aim of this study is to compare the diagnosis methods of CS based on the *nested* PCR-polA (*n*PCR), direct immunofluorescence (IF), and histopathology of diverse types of biological specimens obtained from the maternal-fetal interface. A cohort of 103 women and their new-born infants was investigated through the analysis of tissue (placenta and umbilical cord), peripheral blood from the mother and infant, and total blood from the umbilical cord by the methods. The women were

determined as having syphilis or not based on a clinical-serological diagnosis. Overall, 29.1% of the women investigated were diagnosed as having syphilis, with 70% being classified as recent cases, and 30% as being in the latent phase. The IF and *n*PCR procedures were equally effective for the detection of *T. pallidum* in the tissue of the placenta and umbilical cord, with a positive correlation being observed between the histopathological triad for CS and the identification of the spirochetes. The *n*PCR was more sensitive for the detection of the treponeme in the samples from the neonates and the umbilical cord. Equivalent results were obtained for the detection of *T. pallidum* using the *n*PCR and IF approaches. These techniques provided a valuable addition to the serological diagnosis and histopathological findings, and presented considerable potential for the epidemiological monitoring of SC.
Keywords: *Treponema pallidum*; Congenital syphilis; Fluorescent Antibody Technique; PCR.

Resumo

O *Treponema pallidum* pode ser transmitido ao feto pela placenta e causar sífilis congênita (SC). Apesar disso, poucos estudos têm enfocado as abordagens diagnósticas para analisar a interface materno-fetal. O objetivo deste estudo foi comparar os métodos de diagnóstico de SC baseados na *nested* PCR-polA (*n*PCR), imunofluorescência direta (IF) e histopatologia de diferentes tipos de espécimes biológicos obtidos da interface materno-fetal. Uma coorte de 103 mulheres e seus recém-nascidos foi investigada por meio da análise de tecidos (placenta e cordão umbilical), sangue periférico materno e infantil e sangue total do cordão umbilical pelos métodos citados. As mulheres foram determinadas como portadoras ou não de sífilis com base no diagnóstico clínico-sorológico. No geral, 29,1% (30/103) das mulheres investigadas foram diagnosticadas como portadoras de sífilis, sendo 70% classificadas como casos recentes e 30% em fase latente. Os procedimentos de IF e *n*PCR foram igualmente eficazes para a detecção de *T. pallidum* no tecido da placenta e cordão umbilical, sendo observada correlação positiva entre a tríade histopatológica para SC e a identificação das espiroquetas. O *n*PCR foi mais sensível para a detecção do treponema nas amostras de sangue dos neonatos e do cordão umbilical. Os testes de *n*PCR e IF corroboraram os achados histopatológicos. Essas técnicas forneceram um valioso acréscimo ao diagnóstico sorológico e aos achados histopatológicos, e apresentaram considerável potencial para o monitoramento epidemiológico da sífilis materno-fetal.
Palavras-chave: *Treponema pallidum*; Sífilis congênita; Imunofluorescência; PCR.

Resumen

Treponema pallidum puede transmitirse al feto a través de la placenta y causar sífilis congenita (SC). Pocos estudios se han centrado en enfoques diagnósticos para analizar la interfaz materno-fetal. El objetivo de este estudio fue comparar os métodos de diagnóstico de SC basados en *nested* PCR-polA (*n*PCR), inmunofluorescencia directa (IF) y histopatología de diferentes tipos de muestras biológicas obtenidas de la interfaz materno-fetal. Se investigó una cohorte de 103 mujeres y sus recién nascidos mediante análisis de tejidos (placenta y cordón umbilical), sangre periférica materna y infantil y sangre entera de cordón umbilical utilizando los métodos antes mencionados. Se determinó que las mujeres tenían sífilis ou no con base en el diagnóstico clínico-serológico. En geral, el 29,1% (30/103) de las mujeres investigadas fueron diagnosticadas con sífilis, con un 70% clasificado como casos recientes y un 30% en fase latente. Los procedimientos IF y *n*PCR fueron igualmente efectivos para la detección de *T. pallidum* en tejido placentario y de cordón umbilical, con una correlación positiva entre la tríada histopatológica para SC y la identificación de espiroquetas. La *n*PCR fue más sensible para la detección de treponama en muestras de sangre dos neonatos y de cordón umbilical. Las pruebas *n*PCR e IF corroboraron los hallazgos histopatológicos. Estas técnicas proporcionaron umna valiosa adición al diagnóstico serológico y los hallazgos histopatológicos, y tenían un potencial considerable para el seguimiento epidemiológico de la sífilis materno-fetal.
Palavras-chave: *Treponema pallidum*; Sífilis congénita; Técnica del Anticuerpo Fluorescente; PCR.

1. Introduction

Syphilis is a curable sexually transmitted infection (STI) caused by *Treponema pallidum subspecies pallidum* (*T. pallidum*), transmitted to the fetus through the placenta (Lafond & Lukehart, 2006). Maternal-fetal syphilis is a serious public health problem (Santos et al., 2020; Peeling et al., 2017) that should be prioritized, given that, in untreated women, pregnancy may result in the infection of 50-100% of the fetuses, with 40% resulting in miscarriage, stillbirth or perinatal mortality (Wenhai et al., 2004).

Numerous published studies of adult syphilis are available (Vrbová et al., 2020; Martin et al., 2009; Casal et al., 2011), although relatively few have focused on the diagnostic approaches involving different biological specimens in congenital syphilis (CS) (Casal et al., 2013; Woznicová et al., 2007; Wu et al., 2006). The clinical-laboratorial diagnosis of CS is hampered by the fact that around half the infants are asymptomatic at birth, and the serological diagnosis detects only the

maternal infection, given that the dynamic nature of the transfer of the antibodies from mother to fetus, principally those of the IgG type, confounds both treponemic and non-treponemic tests. The specific IgM-type serological test for *T. pallidum* may provide false-positive results in infected neonates due to the production of type-IgM antibodies by the fetus in response to the maternal IgG, known as the rheumatoid factor (Clements et al., 2020; Herremans et al., 2010; Dobson et al., 1988; Sanchez et al., 1989).

Given the scarcity of adequate specimens for the direct detection of *T. pallidum*, techniques such as immunofluorescence, silver staining, and dark field microscopy have not been used for routine diagnosis. However, studies that have focused on these techniques used for the analysis of the placenta and umbilical cord have revealed not only the spirochetes themselves, but also major histopathological modifications in the women with a positive serological diagnosis for syphilis (Schwartz et al., 1995; Genest et al., 1996). On the other hand, a number of studies based on molecular tools have indicated that this approach is relatively effective for the detection of the pathogen in a number of different types of biological specimens, based on a number of different target genes, such as *polA*, *tp47*, *tmpC*, *bmp* (Vrbová et al., 2020; Martin et al., 2009; Casal et al., 2011; Casal et al., 2013; Woznicová et al., 2007; Wu et al., 2006; Buffet et al., 2007). In this context, and considering one of the case definitions is: "Microbiological evidence of infection by *Treponema pallidum* in a nasal secretion sample or skin lesion, child biopsy or necropsy, abortion or stillbirth", this study aims to analyse the use of placenta tissue and its annexes, as well as peripheral and umbilical cord blood as a specimen for the detection of *T. pallidum* by nested PCR, direct immunofluorescence and tissue histopathology

2. Material and Methods

2.1 Study population

In this study, we investigated a cohort of 103 women using the available samples of the placenta, umbilical cord, total blood of the umbilical cord, and the total blood of the mother and infant. The mothers and live neonates were evaluated using treponemic and non-treponemic procedures, with maternal and congenital syphilis being defined according to the criteria established in Casal et al. (2013). The study was approved by the Ethics Committee of the Nucleus of Tropical Medicine (protocol: 095/2005-CEP/NMT), Federal University of Pará, Brazil. Written consent for publication was obtained from all the patients or their relatives. Prenatal, clinical and treatment data were obtained from the patient records.

The study was conducted on 25,600 pregnant women admitted for delivery to the obstetric centre of Fundação Santa Casa de Misericórdia do Pará (FSCMPA, Brazil) between January 2006 to December 2008.

2.2 Specimen collection and histochemical analysis

The placentas and connected tissue (umbilical cord and fetal membrane) were collected following the birth. In each case, the umbilical cord was separated from the placenta and two 2-cm sections were removed. A longitudinal incision was made in the placental body for the removal of a 3 cm x 4 cm section from the area to which the cord is connected. These specimens were fixed in a buffered solution of 10% formalin for 48 hours. An automatic tissue processor was then used to produce the histological sections of 5µm on silanized slides, which were stained with haematoxylin-eosin (HE) and periodic acid Schiffer (PAS).

Fresh fragments of the placental tissue and umbilical cord were simultaneously clamped for the extraction of DNA, which was stored in sterile cryogenic tubes and frozen at -20ºC. The blood samples from the umbilical cord (3 mL), mother (3 mL), and neonate (1,5 mL) were collected with EDTA as the anticoagulant for the extraction of DNA. The plasma was obtained from the blood samples taken from the mothers (3 mL) and neonates (2 mL) in tubes without anticoagulant.

The parameters of Russell (1974) and Schwartz (1995) were used to evaluate the histological modifications of the placentas and umbilical cords collected during the present study.

2.3 Direct immunofluorescence (IF) of the tissue

The tissue collected from the placentas and umbilical cords fixed on slides was deparaffinized in absolute xylol for 5 minutes, dehydrated in sequential alcohol baths, and washed in phosphate-buffered saline (PBS, pH 7.2). The antigenic retrieval of the tissue samples was conducted in a bath in a citrate solution (pH 6.0) for three minutes in a microwave at its highest potency, and then blocked with an albumin/PBS (1:20) solution for 10 minutes, and finally washed with PBS. The anti-*Treponema pallidum* antibody (Biocare Medical, Concord, CA – USA) was added to the sections and incubated in a moist chamber for 2 hours at room temperature. The samples were then blocked and washed with albumin /PBS once again. A fluorescein isothiocyanate (FITC) labelled goat anti-rabbit IgG antibody (Santa Cruz Biotechnology, CA – USA) was diluted 1:100 in PBS containing 1% Tween 20 and 0.0001 µg of the Trypan Blue. This solution was added to the sections, which were then incubated in a moist chamber for 1 hour. The slides were then washed in PBS for 10 minutes and mounted in buffered glycerine for analysis under an immunofluorescent microscope. The number of spirochetes observed using immunofluorescence was estimated using a semi-quantitative scale: Rare = 1-2 spirochetes per field; Frequent = 3-5 spirochetes per field, and Abundant ≥ 6 spirochetes per field.

2.4 Serology

The serum samples were taken from the mothers and live neonates by VDRL (Wama Diagnóstica, São Carlos, SP, Brazil). All the samples were analysed using the FTA-Abs IgG, ELISA IgG, and FTA-Abs IgM assays described by Casal et al. (2013)

2.5 Preparation of the DNA, nested PCR **primers and amplification conditions**

The DNA of *T. pallidum* was detected using a nested PCR (*n*PCR) to amplify an external region of 291 bp described by Casal et al. (2013) and an internal region of 121 bp of the *polA* gene. The second primer pair was based on an internal region of the PCR product obtained in the first amplification of the *polA* gene and consisted of a 19-mer forward primer (5'-TTAAAAGAAGCGCTGCGCA-3') and a 22-mer reverse primer (5'-TACAACAGGAATCTTCGAGCGA-3'). The amplification conditions of second reaction: 94ºC for 1 min, followed by 35 cycles at 94°C for 1 min, 57°C for 45 s, and 72°C for 1 min.

2.6 Statistic

Differences between groups were evaluated using the test for two independent samples appropriate to the data set being analysed, that is, Chi-square (χ^2), G-test and Odds Ratio. McNemar's χ^2 was used to assess the level of agreement between the *n*PCR and IF methods for the detection of *T. pallidum* in the tissue of the umbilical cord and placenta. A proportions test (combined *p* value) was used for the comparison of the results obtained using different types of specimens and diagnostic procedures. All statistical analyses were run in the Bioestat software, version 5.0 (Ayres et al., 2007), and differences were statistically significant when *P* values were less than 0.05.

3. Results

3.1 Characterization of the study population

Overall, 29.1% of the women (30/103) were diagnosed serologically as having syphilis, of which, 70% (21/30) were classified with recent syphilis, and 30% (9/30) as having the latent form of the disease. The mean (±SD) age of the women was 23.4±5.3 years. In the present study, it was possible to investigate 4.1% (3/70), 22.4% (13/58), and 7.6% (4/52) of the cases notified by the FSMPA in 2006, 2007, and 2008, respectively.

The epidemiological parameters for the women analysed in the present study are presented in Table 1. A significant pattern was observed in relation to lethal outcome and a history of STD, with the probability of a lethal outcome to the pregnancy (stillbirth or neonatal death: OR = 11.67; 95% CI = 2.93-46.50) and a history of STDs in previous pregnancies (OR = 11.50; 95% CI = 3.31-39.93) being approximately 11 times higher than that in mothers without syphilis ($p < 0.05$).

Table 1. Epidemiological characteristics of the two groups (mothers with and without syphilis) analysed in the present study.

Parameter	**Mothers with syphilis n= 30**		**Mothers without syphilis n= 73**		**Test**	**(*p*)**
Type of birth	**n**	**%**	**N**	**%**		
Normal	21	70	42	57.5	χ^2	0.3386
Caesarian	9	30	31	42.5		
Lethal outcome						
Yes (stillborn or neonatal death)	10	33.4	3	4.1	G	0.0005
No (live birth)	20	66.7	70	95.9		
History of STDs						
Yes	12	40	4	5.5	G	<0.0001
No	18	60	69	94.5		
Civil status						
Single	8	26.7	33	45.2	χ^2	0.1273
Stable union	22	73.3	40	54.8		
Use of condoms						
Yes	3	10	4	5.5	G	0.6964
No	27	90	69	94.5		
Number of sexual partners over the last 2 years						
1 Partner	14	46.7	41	56.2	χ^2	0.5089
≥ 2 Partners	16	53.3	32	43.8		
Number of medical appointments						
1 – 2 medicals	8	26.7	20	27.4		
≥ 4 medicals	18	60	47	64.4	G	0.7473
No pre-natal care	4	13.3	6	8.2		
VDRL test during prenatal period						
Yes	25	83.3	58	79.5	G	0.8579
No	5	16.7	15	20.5		

Source: Authors.

3.2 Histopathology of the placenta and umbilical cord in cases of congenital syphilis

The results of the histopathological analyses are presented in Table 2 for the syphilis cases. The histopathological triad for congenital syphilis was observed in 43.3% (13/30) of the cases of maternal syphilis, most of which (84.6%) were recent

cases of the disease (11/13). All but one of the cases of necrotizing funisitis (87.5%) was observed in women with recent syphilis who presented the triad. Of the women with two histological modifications, 54.5% (6/11) had recent syphilis. One of these 11 women (9.1%) had acute funisitis, and four others (36.4%) presented mononuclear funisitis and funisitis of the plasmatic cells.

In women not diagnosed with syphilis, by contrast, 39.7% (29/73) exhibited no major histopathological modifications, although 35.6% (26/73) presented villitis and perivillitis with slight focal infiltration of the mononuclear cells and in some cases the plasmatic cells, while 20.5% (15/73) were characterized by immature villi. The remaining three individuals (4.1%) exhibited two different types of histological modification, that is, villitis and perivillitis with endarteritis and perivasculitis of the villi of the placenta and the veins of the umbilical cord. Overall, the proportions of these modifications – which are indicative of congenital syphilis – were significantly higher in the infected mothers in comparison with those without syphilis (G-test with Williams' correction = 19.5173; $p < 0.0001$).

3.3 Detection of *T. pallidum* by IF and nested PCR

The frequency of *T. pallidum* detection in mothers with syphilis varied considerably among the different types of specimens analysed (Table 3). All the samples of patients classified as negative by the serological testing (control group) were also negative for IF and *n*PCR.

The pairwise analysis of the tissue of the placenta and umbilical cord using IF and *n*PCR revealed that 60% (18/30) were positive for both samples and techniques, while 23.3% (7/30) were positive in both analyses but only for the placental tissue, and 13.3% (4/30) only for the *n*PCR analysis of the umbilical cord. The other five patients (16.7%) returned negative results for all tests.

Table 2. Distribution of the histopathological modifications of the placenta and umbilical cord in the specimens analysed in the present study, according to the infection phase of the mother.

Histopathology of the Placenta and Umbilical Cord		**Maternal syphilis stage**				**nPCR (+)**	
Number of modifications	**Type of modification**[†]	**Early (n=21)**		**Latent (n=9)**		**Placenta**[*f*] **(n=30)**	**Cord**[*f*] **(n=30)**
Three		**n**	**%**	**n**	**%**		
	[(1)/(2)/(3)/(3.1)]	1	4.8	1	11.1	2	1
	[(1)/(2)/(3)/(3.1)/(3.2a)]	7	33.3	1	11.1	7	6
	[(1)/(2)/(3)/(3.1)/(3.2b)]	1	4.8	0	0	1	1
	[(1)/(2)/(3)/(3.1)/(3.2c)]	2	9.5	0	0	2	1
Two							
	[(2)/(3)/(3.1)]	4	19.0	2	22.2	5	6
	[(2)/(3)/(3.1)/(3.2a)]	0	0	0	0	0	0
	[(2)/(3)/(3.1)/(3.2b)]	1	4.8	0	0	1	0
	[(2)/(3)/(3.1)/(3.2c)]	1	4.8	3	33.3	3	4
One							
	[2]	4	19.0	2	22.2	5	4

[†]**(1)** Immaturity of the placental villi (hypercellularity of the villi); **(2)** Villitis and perivillitis with mononuclear and plasmatic cells; **(3)** Endarteritis and perivasculitis of the placental villi, and **(3.1)** Endarteritis and perivasculitis of the veins of the umbilical cord, and/or **(3.2)** presence in the umbilical cord of **(a)** necrotizing funisitis, **(b)** acute funisitis or **(c)** mononuclear and plasmatic cells; [*f*]The nPCR detected *T. pallidum* in most cases of recent or latent maternal syphilis. Source: Authors.

Table 3. Detection rates of *T. pallidum* in tissue and blood samples collected from the female patients with syphilis analysed in the present study.

Type of sample	IF (spirochetes) (n/N)				nPCR (n/N)		
	A	**F**	**R**	**(-)**	**(+)**	**(-)**	**NT**
Placental tissue	2/30	10/30	13/30	5/30	26/30	4/30	-
Tissue of the umbilical cord	0	2/30	16/30	12/30	23/30	7/30	
Total blood of the umbilical cord	-	-	-	-	21/30	2/30	7/30
Total blood of the mother	-	-	-	-	15/30	15/30	-
Total blood of the newborn infant	-	-	-	-	9/30	2/30	19/30

IF: immunofluorescence; ***n*PCR:** nested PCR; **A:** Abundant; **F:** Frequent; **R:** Rare; **NT:** not tested. Source: Authors.

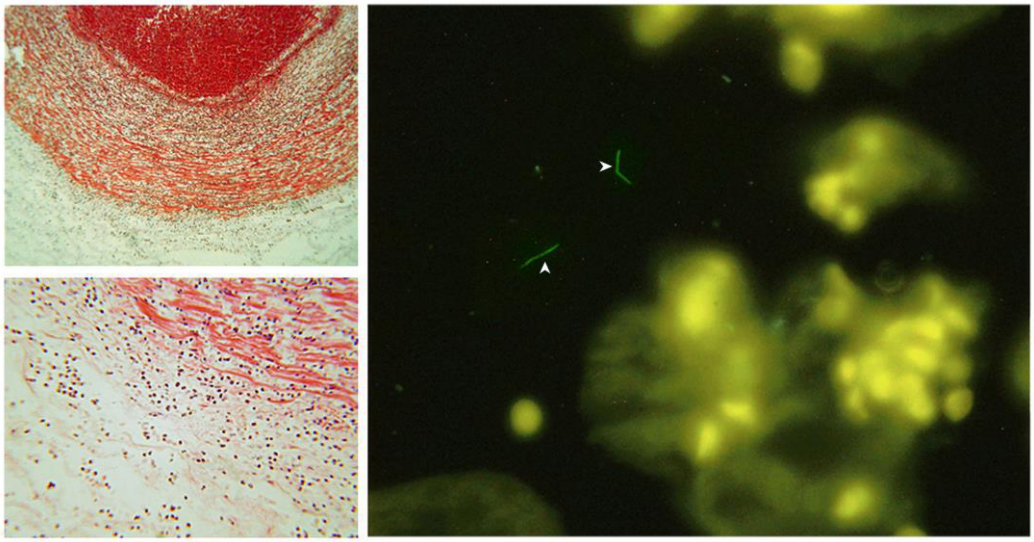
No significant difference was found between IF and *n*PCR ($p > 0.05$) for the detection of *T. pallidum* from the tissue samples obtained from the placenta and umbilical cord. However, the IF detected a significantly ($p = 0.0025$) higher frequency of the pathogen from placental tissue (83%) in comparison with that of the umbilical cord. No statistical difference was found between the diverse types of specimens for the *n*PCR approach ($p > 0.05$).

The results of the *n*PCR for the diverse types of blood samples were analysed using a proportions test, which found a significant overall difference (combined $p < 0.0029$), between the samples from the umbilical cord and the mother ($p < 0.01$). In general, *n*PCR detection rates for the tissue specimens and blood samples from the umbilical cord and neonate were higher than those for the maternal blood samples.

3.4 Relationship between the morphological modifications and the diagnostic tests

A significant positive correlation was found between the presence of the histopathological triad and the identification of *T. pallidum* by both IF (Pearson's $r = 0.3911$, $p = 0.0325$) and *n*PCR ($r = 0.4757$, $p = 0.0079$) in the women with syphilis (Figures 1 and 2). Considering both approaches, the spirochete was detected in 100% (13/13), 72.7% (8/11), and 71.4% (5/7) of the cases with three, two, and one histological modification, respectively. In these cases, the pathogen was found primarily in the intervillous space of the placenta, Wharton's jelly, and the adjacent region of the vascular endothelium of the umbilical cord. Most of the cases of *n*PCR (+) with two or three histological alterations were from the group of women with recent syphilis (Table 3).

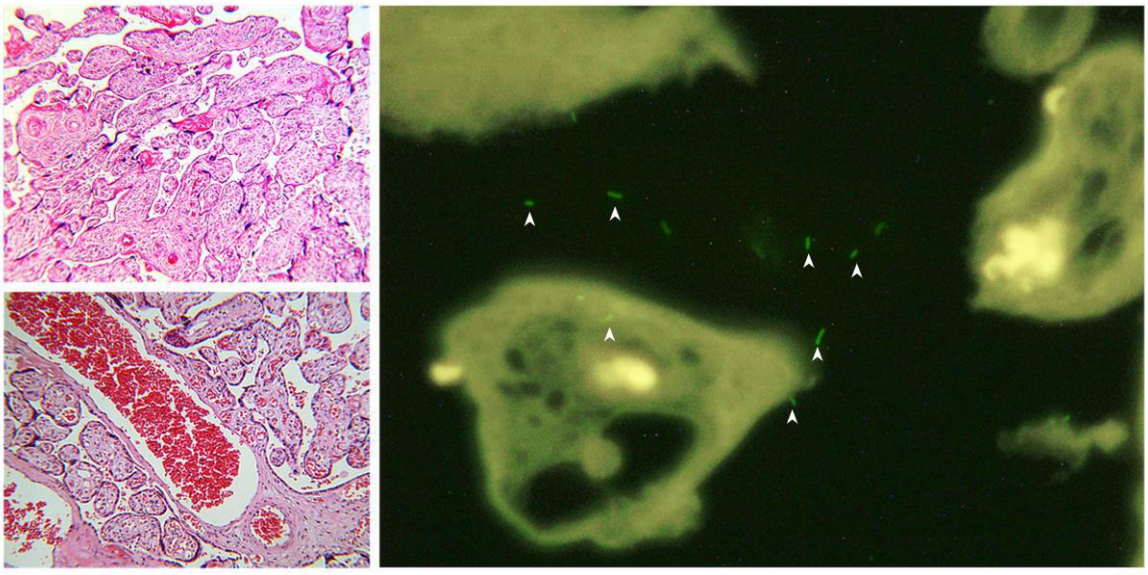
Figure 1. Endarteritis (A) (200x) and perivasculitis (B) (400x) of the veins of the umbilical cord and detection of *Treponema pallidum* (arrowhead) by the immunofluorescence of the tissue of the umbilical cord (C) (1000x).



Source: Authors.

Concerning the outcome of the pregnancy, *T. pallidum* was detected by both IF and *n*PCR techniques in 75% (6/8) of stillborn infants, 100% (2/2) of those that died in the neonatal period, and 55% (11/20) of the live births. Six of the 10 cases of lethal outcome (60%) presented the histopathological triad, and five (83.3%) of these cases had necrotizing funisitis. By contrast, only 35% (7/20) of the live neonates presented the triad and 30% (6/20) had two histological modifications, of which 3 of the 8 (37.5%) presented necrotizing funisitis.

Figure 2. Immaturity of the placental villi (A) 100x; Villitis and perivillitis with mononuclear and plasmatic cells (A, B) 100x; Endarteritis and perivasculitis of the placental villi (C) 100x and detection of *Treponema pallidum* (arrowhead) by the immunofluorescence of the tissue of the placenta (D) 1000x.



Source: Authors.

4. Discussion

The present study found that the immunofluorescence indirect and *n*PCR techniques were equally effective for detecting *T. pallidum* in the tissue of the placenta and umbilical cord. The *n*PCR was also more sensitive to the presence of this pathogen in the tissue samples, and the blood samples from the umbilical cord and the neonate in comparison with the blood from the mother, indicating that the best specimens for the detection of the treponeme are those from the maternal-fetal interface. The use of umbilical and placental specimens is advantageous because this material is available following the birth and avoids the need for invasive procedures for the collection of samples. No previous studies appear to have investigated these types of specimens together for the diagnosis of congenital syphilis, although molecular techniques have been used to identify this bacterium in samples collected from the biopsy of skin lesions, cerebral-spinal fluid, total blood, plasma, and the placenta (Martin et al., 2009; Woznicová et al., 2007; Wu et al., 2006; Genest et al., 1996; Buffet et al., 2007).

An additional important finding is the pattern of histopathological modifications found in the specimens of the placenta and umbilical cord, given that the presence of Russell's (1974) triad, which is seen as a positive histological expression of congenital syphilis, was positively associated with the detection of this pathogen by the IF and *n*PCR, thus confirming the congenital infection (Genest et al., 1996; Russell & Altshuler, 1974). The absence of the triad does not exclude the possibility of infection, however, which requires the identification of the treponeme, given that infection was confirmed by IF in 72.7% of the cases that presented only two histological modifications, considered to be suspect cases. In addition, the presence of the treponeme was identified in 66.7% of the cases of a single modification, which would normally be evidence of an unspecified pathological process. It is important to remember that *T. pallidum* was detected by *n*PCR and/or IF in most of the cases of recent or latent maternal syphilis which presented one, two or three histopathological modifications.

In a study of the placental tissue of patients that were either seropositive or seronegative for syphilis using silver staining, PCR, and histopathology, Genest et. al. (1995) found that none of the negative cases presented the triad or the spirochete based on the direct detection method, a similar result to that of the present study. A comparison of the studies nevertheless reveals some disagreements. For example, the silver staining sensitivity for the detection of *T. pallidum* (50%) was considerably lower than that of the IF technique (83.3%) used in the present study. In addition, the histopathological triad was detected in only 10.5% of the patients with syphilis and none of the cases of miscarriage, while in the present study, the triad was observed in 43.3% of the women with syphilis, and in 60% of the cases of stillbirth and neonatal death.

In the present study, the risk of losing the fetus was 11 times higher in mothers with syphilis, emphasizing the role of this infection in the lethal outcome of the pregnancy. In general, women infected with *T. pallidum* may transmit this pathogen to the fetus at any stage of infection, leading to approximately 40% of miscarriage, stillbirth or neonatal death (Wenhai et al., 2004; Mario et al., 2007).

Acute (73.3%) and chronic (26.7%) villitis were observed in the placentas of the women with syphilis investigated in the present study (Genest et al., 1996; Sheffield et al., 2002). The inflammatory infiltration that predominates in the villi, intervillous spaces, and decidua has a focal distribution (63.35%) made up of lymphocytes, neutrophils, and plasmocytes, with the IF highlighting the treponeme primarily in the intervillous space. In the case of the umbilical cord, 53.3% of the syphilis cases presented funisitis, half of which were necrotizing, and the other half acute or present in mononuclear and plasmatic cells, as observed by Schwartz (1995), who recorded funisitis, predominantly of the necrotizing type, in 48% of the cases analysed. Some studies have reported elevated levels of necrotizing funisitis, especially in infected stillborn infants (Navarro & Blanc, 1974; Jacques & Qureshi, 1992; Craver & Baldwin, 1992; Heifetz & Bauman, 1994). A similar pattern was recorded in the present study, in which five of the eight recorded cases of necrotizing funisitis were observed in stillborn infants and neonatal deaths, which were positive by IF. While these studies have shown a certain relationship between these variables, they

also contradict the idea that necrotizing funisitis is a diagnostic lesion of syphilis, given that other types of infection, such as those caused by Herpes, may also present this disorder. However, the histological modifications observed in the control group were not significantly different from those in the syphilis group.

Maternal syphilis had a prevalence of 29.1% in the present study, with most cases representing the active phase of the pathogen, although only 66.7% of the new-born infants of these women were notified. It is important to emphasize that the ten neonates not diagnosed or notified during the study period represent part of a widespread problem in northern Brazil, that is, the chronic under-reporting of cases. Here, 70% of these cases of *T. pallidum* were detected by IF in the placental tissue, and 30% were positive through the *n*PCR of the samples of tissue and blood from the umbilical cord. At least half of these infants should have been notified, given that they were consistent with the criteria adopted for the definition of congenital syphilis in Brazil (BRASIL, 2019). However, the other half of the infants would probably not have been diagnosed, given that they were negative in the puerperal VDRL serological analysis, given the lack of a treponemic test, even though they were reactive, both to the IgG ELISA and the FTA-Abs IgG, and were classified as having latent syphilis.

In general, the women of the two study groups (with and without syphilis) appear to be exposed to similar conditions, given that most live in stable relationships, do not use condoms during their sexual encounters, have had more than one partner over the past two years, and of those who prenatal exams, most had at least four medicals and the VDRL test. However, a history of STD in earlier pregnancies associated with a lethal outcome was more common in the women with syphilis, constituting a risk factor for congenital syphilis. In an earlier study in Brazil, Lago (2004) found an association between congenital syphilis and history of stillbirth in women with syphilis.

The effectiveness of the identification of the spirochetes in the tissue (which represents direct evidence of congenital syphilis) was evaluated by IF, given the difficulty of using the rabbit infectivity test (RIT) for the routine diagnosis of infection. The detection of *T. pallidum* through the *n*PCR analysis of the diverse types of specimens was consistent with the results of the IF and the histopathological modifications observed in the women with syphilis. The results also confirm that the possibility of detecting the pathogen in the adult organism is much lower than for the conceptus, given the reduced concentration of treponemes in the maternal circulation (Woznicová et al., 2007), given that the *polA* gene was found in 81.8% of the neonate blood samples, but in only 50% of the maternal samples.

Equivalent results were obtained for the detection of *T. pallidum* using the *n*PCR and IF approaches. The best specimens for the detection of this pathogen were those derived from the tissue of the placenta and umbilical cord, total blood from the umbilical cord, and peripheral blood from the new-born infant. The *n*PCR and IF techniques provided a valuable addition to the serological diagnosis and histopathological findings and proved to be a highly accurate alternative approach with the potential to become an accessible tool for the complementary diagnosis of congenital syphilis. The use of these techniques should contribute to the effectiveness of diagnoses and therapies and improve epidemiological monitoring.

Due to the lack of skin lesions and nasal secretion in newborns, as well as the excellent results of the histopathological analysis of the placenta and umbilical cord tissue associated with the demonstration of the pathogen by the IF and *n*PCR in this research, it is suggested that these specimens can be used in the investigation of microbiological evidence of *T. pallidum* infection for SC.

The present research was limited in terms of the sample number of women with syphilis and their respective live newborns, given that the study did not allow the analysis of specimens from cases of abortion, stillbirth, or stillbirth, requiring a broader investigation of the binomial.

5. Conclusion

Congenital syphilis is a serious public health problem, and its clinical and laboratory diagnosis is complicated due to the absence of specific signs and symptoms and the dynamics of the passage of antibodies from the infected mother to the baby. Thus, the definition of CS cases in maternity hospitals, whether of newborns, stillbirths, or abortion, is based on maternal information regarding inadequate treatment or treatment not performed during prenatal care. Based on this situation, the present study shows the importance of using placenta tissue and umbilical cord, abundant specimens at the time of delivery and non-invasive collection, as a sample for case definition as microbiological evidence, when associated with histopathological analysis with a of the treponemal methods used, that is, IF and/or *n*PCR to confirm the infection.

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