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Original Article

Treponema pallidum PCR with blood and cerebrospinal fluid of newborns exposed and not exposed to syphilis during pregnancy

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Abstract

Introduction: Congenital syphilis (CS) has severe adverse outcomes, including abortion and death. Diagnosis of CS in asymptomatic newborns remains difficult. This study aims to evaluate an in-house polymerase chain reaction (PCR) on cerebrospinal fluid (CSF) and blood samples (BS) to identify *T. pallidum* DNA in newborns.

Methodology: We performed an exploratory cross-sectional study that included newborns exposed to syphilis during pregnancy (SEG) and non-exposed (SNEG) newborns, between 2019 and 2020. In-house conventional PCR for *T. pallidum* targeting the *tpp47* gene was used to analyze CSFS and dried blood spots.

Results: BS was obtained from 54 newborns (33 SEG/21 SNEG) and CSF from 55 newborns (33 SEG/22 SNEG). Twenty-five (71.4%) SEG newborns had reactive BS rapid plasmatic reagins (RPR), and all of them had RPR titers less than or equal to the corresponding maternal titers. All RPR CSF tests were negative. PCR for *T. pallidum* DNA was positive in 19/33 (57.6%) BS, and in 22/33 CSF. The only SEG newborn with clinical signs of early CS had a positive CSF PCR and a negative BS PCR. Conversely, among SNEG newborns, PCR was positive in 2/21 BS and 5/22 (22.7%) CSF.

Conclusions: *T. pallidum* DNA was identified using our PCR tests. The exposed group did not present abnormalities that would indicate CS. This prevented conclusions regarding sensitivity and specificity. Dried spot permitted bedside collection, easy transportation, and storage. Further research is needed to evaluate and improve the accuracy of CS low-cost PCR tests, especially for limited resource settings.

Key words: congenital; syphilis; diagnosis; molecular; PCR.

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Introduction

Syphilis is caused by *Treponema pallidum*, a spirochete that can be sexually, parenterally or vertically transmitted [1]. It is a multisystem chronic disease with potentially permanent adverse outcomes. Nonetheless, all complications of acquired as well as congenital syphilis can be prevented by appropriate and timely antibiotic treatment [2].

A meta-analysis of pregnant women with untreated or inadequately treated syphilis showed that 65.5% developed adverse outcomes: abortions or stillbirths in 25.6%; neonatal deaths in 12.3%; clinical syphilis in 15.5% of the newborns; and prematurity or low birth weight in 12.1%. Neonatal admission was necessary in 27.5% of live births [3].

Congenital syphilis (CS) has significantly increased in high-income countries during the last two decades [4]. Syphilis, however, is not proportionally distributed globally, and a disproportionate number of cases are in medium- and low-income countries [5].

The incidence of acquired syphilis among pregnant women in Brazil was estimated to be 20.8 for 1,000 live newborns in 2019. According to the Congenital Syphilis Epidemiological Bulletin published by the Brazilian Ministry of Health, there was a four-fold increase in the incidence of CS between 2010 and 2019, when the incidence was 8.2 cases per 1,000 live births [6].

The practical presumptive diagnosis of syphilis in pregnant women can be done with the utilization of treponemal, including point-of-care rapid tests, and nontreponemal tests. A recent metanalysis revealed that the rapid tests for syphilis had a sensitivity of 0.83 (95% CI: 0.58–0.98) and a specificity of 0.96 (95% CI: 0.89–

1.00), and the rapid plasma reagin (RPR) test had a pooled sensitivity of 0.75 (95% CI: 0.54–0.88) and a pooled specificity of 0.97 (95% CI: 0.96–0.99) [7]. Brazilian recommendations include testing in the first prenatal visit, ideally in the first trimester, and also in the third trimester, as well as at the time of delivery [8]. Nonetheless, the diagnosis of congenital syphilis and neurosyphilis is difficult, particularly in asymptomatic newborns [9] and failure to detect infection during the neonatal period can result in a missed opportunity to prevent permanent damage. On the other hand, overtreatment results in the adverse consequences of longer hospital stays as well as higher financial costs [10]. As such the diagnosis is so difficult that national and international recommendations propose the identification of different situations in which the infection of the newborn can be classified as possible, probable, or present. The latter is considered in three situations: 1) an abnormal physical examination that is consistent with congenital syphilis; or 2) a serum quantitative nontreponemal serologic titer that is fourfold higher than the mother's titer [11]; or 3) a positive darkfield test or polymerase chain reaction (PCR) of lesions or body fluids [11,12].

Nucleic acid amplification tests (NAAT) have established their importance in the management of sexually transmitted diseases, and many have become the gold standard in several situations. This is the case of the *T. pallidum* PCR for acquired and congenital syphilis that is already recommended as a definitive diagnosis of infection. Nonetheless, very few tests have been standardized and are commercially available. Prices are still unaffordable for most settings, especially ones in which high prevalence make these tests most needed.

Considering the individual and public health impact of congenital syphilis and the need of improving affordable diagnostic tools [13], this exploratory study aims to evaluate an in-house molecular test for the detection of *T. pallidum* genome in blood and cerebrospinal fluid (CSF) of newborns from mothers with and without a diagnosis of syphilis during pregnancy.

Methodology

This was a cross-sectional study conducted between August 2019 and February 2020 at the Neonatal Intensive Care Unit (NICU) of Santa Clara Hospital, a university hospital in Porto Alegre, Brazil. The study population included admitted newborns of mothers with serological diagnosis of syphilis during pregnancy (syphilis exposed group, SEG), and newborns of mothers who had repeated negative serology for syphilis during pregnancy and whose newborns were admitted in the NICU at delivery for other reasons, such as early neonatal sepsis, congenital toxoplasmosis, or herpes simplex (syphilis non-exposed group, SNEG).

Demographic, clinical, and epidemiological data were obtained from the hospital records, including maternal age, gestational age, and birth weight. Prenatal history included date of the first and total number of prenatal visits, results of serology tests for syphilis performed during prenatal care, and adequacy of syphilis treatment of the mother and her partner. Coinfection with human immunodeficiency virus (HIV), exposure to other infectious microorganisms during pregnancy (e.g., early neonatal sepsis, *Toxoplasma gondii,* herpes simplex virus), and other chronic diseases were recorded. Information regarding consumption of alcohol and tobacco and use of illegal drugs was also collected. Newborns were considered preterm when the gestation age was less than 37 weeks. The classification for fetal growth was based on the birth weight/gestational age index and on the curves recommended by the World Health Organization (WHO) in 1996 [14]. Clinical signs consistent with congenital syphilis were also documented when present.

Syphilis serological status of the mothers in the delivery room was confirmed by performing a nontreponemal test (rapid plasma reagins, RPR) and a treponemal test (chemiluminescence) according to the manufacturer's recommendations. Neonates in the SEG group were systematically evaluated with a complete blood count, a serum nontreponemal test (RPR), a CSF analysis which included a RPR test [15], cell counts, and glucose and protein quantification. Long bone X-rays were also performed. Children of the SNEG, were evaluated according to the clinical protocols of the hospital, which also routinely included collection of blood and CSF. Blood samples (BS) were collected using filter paper, and CSF was collected in sterile Eppendorf vials. The study was approved by the Ethics Committee of the hospital (number 3.432.633) and written informed consent was provided by all participants. Treatment and/or other necessary procedures were not delayed while awaiting PCR results.

After DNA extraction, the detection of *T. pallidum* was made by the use of a conventional PCR technique, using KO3 and KO4 primers that amplify a 260 bp segment of the *tpp47* gene (KO3: 5' GAAGTTTGTCCCAGTTGCGGTT-3'; KO4: 5'CAGAGCCATCAGCCCTTTTCA 3'), as described

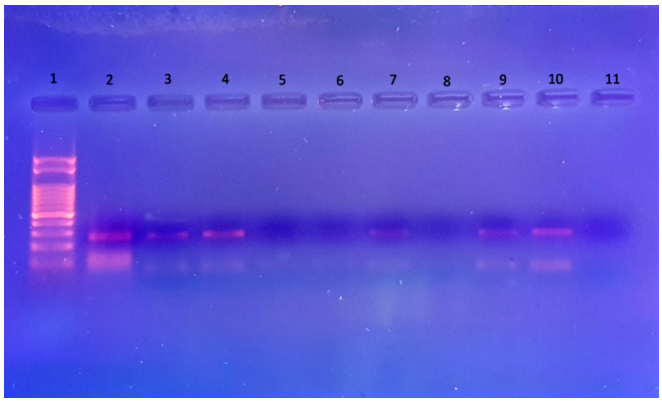
by Palmer *et al*. [16]. The samples were prepared for DNA amplification. A solution was prepared with 10X buffer, 1.5 mM $MgCl_2$, 200 µM of each dNTPs, 25 pmol of each primer and 1.25U of the enzyme *Taq* platinum DNA polymerase. Initial denaturation was performed at 95 °C for 2 minutes, followed by a denaturation at 95 °C for 20 seconds, 62 °C for 20 seconds and 72 °C for 20 seconds for 35 cycles, and a final extension at 72 °C for 5 minutes. Amplification was performed in a thermocycler MJ Research PTC 96 (MJ Research, Waltham, Massachusetts, USA). The PCR result was analyzed by electrophoresis in a 2% agarose gel containing 0.05% ethidium bromide and visualized under ultraviolet light. Positive controls (*T. pallidum* DNA) and negative controls (ultrapure water) were used as internal quality control of the PCR reaction (Figure 1).

Data storage and analysis were performed using EPI Info 7 (Centers for Disease Control and Prevention, Atlanta, Georgia, USA). Quantitative variables were described as means and standard deviations. The demographic, clinical and epidemiological variables are presented as percentages.

Results

There were 35 SEG newborns and 22 SNEG newborns. Sociodemographic characteristics of the mother, prenatal care related findings, delivery and

Figure 1. Representative image of an electrophoretic analysis on 2% agarose gel of the polymerase chain reaction (PCR) products obtained with specific primers for *T. pallidum* from blood samples extracted from dried blood spot.



1: molecular size marker 100 bp; 2: positive control; 3, 4, 7, 9 and 10: positive samples for *T. pallidum*; 5, 6 and 8: negative samples for *T. pallidum*; 11: negative control.

Table 1. Demographics and variables related to prenatal care and to the delivery by exposition to *T. pallidum* during pregnancy at the Neonatal Intensive Care Unit of Santa Clara Hospital, Brazil during 2019-2020.

Variable	SEG Newborns		SNEG Newborns	
	N / n	% / SD	N / n	% / SD
Maternal age (median in years)	25		25	
Prenatal care	34 / 35	97.1%	22 / 22	100%
Pre-natal care initiated in				
First trimester	16 / 34	47.0%	13 / 22	59.1%
Second trimester	10 / 34	29.5%	06 / 22	2.3%
Third trimester	02 / 34	5.9%	0 / 22	0
Missing data	06 / 34	17.6%	03 / 22	13.6%
Number of medical appointments				
No prenatal care	01 / 35	2.8%	0 / 22	0
1 to 5 medicals	08 / 35	22.8%	05 / 22	22.7%
6 or more medicals	20 / 35	57.2%	15 / 22	68.2%
Missing data	06 / 35	17.2%	02 / 22	9.1%
Gestational age				
Term	32 / 35	91.5%	13 / 22	59.1%
Preterm	03 / 35	8.5%	09 / 22	40.9%
Post term	0 / 35	0	0 / 22	0
Birth weight / gestational age index				
SGA	01 / 35	2.9%	02 / 22	9.1%
AGA	33 / 35	94.2%	20 / 22	90.9%
LGA	01 / 35	2.9%	00 / 22	0
Birth weight (mean in grams)	3557.14	SD: 384.66	2688.09	SD: 879.47
HIV infection (yes)	01 / 35	2.9%	01 / 22	4.5%
History of syphilis treated in the past (yes)	02 / 35	5.7%	00 / 22	0
Other chronic diseases				
Gestational diabetes mellitus	02 / 35	5.7%	02 / 22	9.1%
Hypothyroidism	01 / 35	2.9%	02 / 22	9.1%
Other	01 / 35	2.9%	03 / 22	13.6%
Motivation for CSF investigation				
Exposure to syphilis	35 / 35	100%	00 / 22	0
Exposure to toxoplasmosis	01 / 35	1%	05 / 22	22.7%
Early neonatal sepsis	00 / 35	0	15 / 22	72.7%
Exposure to herpes simplex	00 / 35	0	01 / 22	4.5%

NB: newborn; SEG: Syphilis Exposed Group; SNEG: Syphilis nonexposed group; SGA: small for gestational age; AGA: appropriate for gestational age; LGA: large for gestational age.

health status of the newborns are presented in Table 1. Maternal age ranged from 18 to 41 years, and the median was 25 years. All but one woman in the SEG group attended prenatal care. Of these, more than half started during the first trimester of pregnancy, and most of them had more than six visits.

One participant in each group had HIV infection, and the SNEG participant had not been treated for HIV during pregnancy. Two SEG mothers reported having been treated for syphilis before this pregnancy and were probably reinfected before or during the current pregnancy. SNEG mothers were more likely to have comorbidities. The most common was gestational diabetes mellitus (7%), followed by hypothyroidism (5%). The other chronic conditions observed less frequently were hypertension, obesity, sickle cell trait and epilepsy. Five SNEG mothers (22.7%) had at least one of these conditions, while three (8.6%) SEG mothers had two or more of these conditions. Most (74.3%) SEG mothers reported treatment for syphilis during their most recent pregnancy, but most did not have complete information about when and what drugs were prescribed. Moreover, only 20% of sexual partners had reportedly received treatment during this pregnancy.

Among the SNEG newborns, five had been exposed to *Toxoplasma gondii,* one to Herpes simplex virus during delivery, and 15 had suspected early neonatal sepsis. One SEG newborn had also been exposed to toxoplasmosis during pregnancy. There were three SEG preterm births: one of these newborns had manifestations of congenital syphilis, another was an infant of a diabetic mother, and the third had no additional identifiable risk factor. Nine (41.1%) SNEG newborns were premature. Overall, the mean birth weight for SNEG newborns was 2,700 (SD: ± 879.47) grams, and 3,600 (SD: ± 384.66) grams for SEG newborns. Fetal growth was based on the birth weight/gestational age index, and one SEG and two SNEG newborns were small for gestational age.

Of the SEG newborns, 71.4% were treated with intravenous penicillin for 10 days in hospital, 20% received one dose of intramuscular penicillin G benzathine and 8.5% received no treatment (Table 2). Clinical signs of early congenital syphilis were found in only one SEG patient. He was born prematurely (gestational age 35 weeks) with low birth weight (2,150 grams). During hospitalization he developed seizures and a physical exam revealed hepatosplenomegaly, jaundice, and bloody nasal discharge. He had normal hematocrit and hemoglobin levels but increased bilirubin and C-reactive protein levels. Blood cultures for other bacteria were negative. The mother had an RPR titer of 1:128, and his titer was 1:32. The RPR in the CSF was unavailable, and he was treated with intravenous penicillin G for 10 days. It is to be noted that the mother was not treated for syphilis during prenatal care, despite attending four prenatal visits.

Adequate BS were obtained from 54 newborns (33 SEG and 21 SNEG) and sufficient CSF was obtained

Table 2. Syphilis exposed group (SEG) characteristics by subgroups according to polymerase chain reaction (PCR) results in biological samples (n = 35).

	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
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 (3.5%)	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
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
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	7 (20%)
No treatment	0	1 (12.5%)	0	2 (25%)	3 (8.5%)

CSF: cerebrospinal fluid; RPR: rapid plasmatic reagins.

from 55 newborns (33 SEG and 22 SNEG). Twenty-five (71.4%) SEG newborns had reactive RPR tests in their BS, and all of them had RPR titers equal to or less than the corresponding maternal titers. CSF samples in both groups were analyzed for cell counts, protein, and glucose and demonstrated no abnormal results. RPR analyses were non-reactive in all CSF samples in the SEG group. A few patients in both groups had the presence of red cells in their CSF due to incidental trauma during lumbar punctures.

Among SEG newborns, PCR for *T. pallidum* DNA was positive in 19 of the 33 (57.6%) BS, and 22 (66.7%) of the 33 samples of CSF. Conversely, among SNEG newborns, two (9.5%) of 21 BS and 5 (22.7%) of 22 CSF samples were PCR positive. The only newborn with clinical signs of early congenital syphilis had a positive CSF PCR and a negative serum PCR.

When we combined the subgroups of newborns with positive PCR results and compared them with newborns whose PCR results were negative, we were able to calculate the *p* value of the variables presented (Table 3).

Discussion

Our exploratory study identified *T. pallidum* DNA using a conventional PCR which targeted *tpp47*. This technique was validated in a previous study of our group in approximately two thirds of BS as well as in two thirds of CSF samples of the newborns exposed to syphilis during pregnancy [17]. On the other hand, newborns from mothers who had repeated negative serology for syphilis during pregnancy had a positive *T. pallidum* PCR in one-tenth of the BS and one-fifth of the CSF samples. The only newborn with clinical signs of early congenital syphilis had a positive CSF PCR and a negative serum PCR. To our knowledge, this is the first study that compared infants exposed to syphilis during pregnancy to those who were not exposed. Enrolling non-exposed newborns who were hospitalized during the same period with conditions that required similar procedures as part of standard investigations (i.e., venipuncture and lumbar puncture) permitted establishing comparison without ethical constrains. Dried blood spots on filter paper have been used to obtain DNA of other biological agents such as hepatitis C and HIV as reported in previous studies [18,19]. Its use in our study was of much help and might support its adoption in the investigation of congenital syphilis, since samples can be collected at the bedside, easily transported, and stored, reducing the possibility of contamination of samples.

The characteristics of the exposed group, which did not present abnormalities that would permit to establish the unequivocal diagnosis of congenital syphilis as present (i.e., a serum quantitative nontreponemal serologic titer that is four-fold higher than the mother's titer or CSF abnormalities), prevented conclusions regarding sensitivity and specificity of our PCR test. In addition, the only symptomatic newborn who developed seizures, and in whom central nervous system (CNS) syphilis was highly probable, had no CSF abnormalities. The sensitivity of the PCR also

Table 3. Comparative analysis between newborns with positive and negative polymerase chain reaction (PCR) results in the syphilis exposed group (SEG).

	PCR (+)	PCR (-)	Total	*p* value
RPR in blood samples				
< Maternal	11 (78.6%)	3 (21.4%)	14 (100%)	
Equal to maternal	16 (76.2%)	5 (23.8%)	21 (100%)	1.0000
> Maternal	0	0	0 (100%)	
Rx of long bones				
Normal	13 (81.2%)	3 (18.8%)	16 (100%)	
Abnormal	6 (66.7%)	3 (33.3%)	9 (100%)	0.684
Missing	8 (80%)	2 (20%)	10 (100%)	
Documented treatment during pregnancy n (%)				
Penicilin	20 (76.9%)	6 (23.1%)	26 (100%)	
Other	0	0	0 (100%)	1.000
No treatment	7 (77.8%)	2 (22.2%)	9 (100%)	
Documented sexual partner treatment n (%)				
Penicilin	5 (18.5%)	2 (25%)	7 (20%)	
Other	0	0	0 (0%)	0.648
No treatment	22 (81.5%)	6 (75%)	28 (80%)	
Newborn treatment n (%)				
Penicilin G Crystalline	19 (76%)	6 (24%)	25 (100%)	
Peniclin G Benzathine	7 (100%)	0	7 (100%)	0.069
No treatment	1 (33.3%)	2 (66.7%)	3 (100%)	

RPR: rapid plasmatic reagins.

depends on the target gene and the types of biological samples. Studies using the *tpp47* gene have demonstrated sensitivities of 60-75% in CSF samples and 67-94% in blood or serum samples. Specificities ranged from 90-100% [20]. Also, the absence of a test with high enough sensitivity to predict congenital syphilis in newborns poses difficulties, since the sensitivity of the Venereal Disease Research Laboratory test (VDRL) in CSF samples of neonates is 54%, and sensitivities of pleocytosis and elevation of CSF proteins are also low, 38% and 56% respectively [21]. The literature estimates that CNS involvement by *T. pallidum* occurs in 40% of infants who have clinical, laboratory, or radiographic abnormalities of congenital syphilis and is infrequent in infants without these manifestations [21,22]. Unexpectedly, 2 blood samples and 5 CSF samples in the SNEG had positive *T. pallidum* PCR tests. These women could have been recently infected and had not yet developed detectable antibodies, but this is extremely unlikely. It would be more reasonable to consider these results to be false positives. Follow up of these patients could provide information but it was not part of the objectives of our study. Although we established strict internal quality controls and complied with all current laboratory protocols, contamination of samples is a remote possibility. Studies of PCR assays for CSF samples of newborns exposed to syphilis had a sensitivity ranging from 60–75% when compared to rabbit inoculation tests (RIT) [23,24]. Among them, Michelow *et al.* found positive PCR results in the CSF of newborns exposed to syphilis who had negative results for RIT, and he considered them false positives or due to an insufficient volume of material used for RIT [21]. Investigators have also tried to develop the most suitable technique for different biological samples, and considered that nested PCR is probably more sensitive when compared to others PCR methods. [25].

In our sample, only 3% of SEG women did not attend prenatal care. Brazil has increased coverage of prenatal care, but must still improve its quality [26]. Despite having attended prenatal care, these women came to the hospital with no or inadequate treatment for syphilis. Regardless of the availability of point-of-care tests for syphilis, as well as HIV, hepatitis B and C, in all Brazilian public health care centers, diagnosis and/or treatment of maternal syphilis is frequently overlooked, reinforcing the need of performing screening tests for syphilis in all pregnant women at the time of delivery, as recommended by the national protocols [27]. No mother or newborn infant should leave the hospital without documented serological status [11]. Absence of partners' treatment continues to be a problem in the country [28]. Only 1 in 5 partners were treated in our sample. Nearly 72% of the newborns exposed to syphilis in our sample that were admitted went through a thorough investigation with invasive procedures and staying at hospital for at least 10 days to receive intravenous penicillin.

The small sample size may be considered a limitation of this study. The coronavirus disease 2019 (COVID-19) pandemic hindered enrollment and collection of samples. Nonetheless, it permitted some important information, and, as an exploratory study, generated baseline data for sample size calculation for more conclusive studies.

The nested PCR technique appears to be more promising for the identification of *T. pallidum*, especially when dealing with capillary blood samples, as they may contain a low number of circulating bacteria making DNA detection more difficult. We hope that a convenient and ethical method of enrolment can be used by other groups from different resource scenarios, especially in scarce health resource countries, where investigation of congenital syphilis is of a much greater need.

Conclusions

Our exploratory study demonstrated that the utilization of an in-house PCR using CSF and BS permitted to identify *T. pallidum* DNA in the studied samples and that using dried spot blood sampling proved highly convenient. We believe that further pursuit of a standardized accurate low-cost PCR technique could greatly improve the quality of care for patients with congenital syphilis.

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