Prenatal diagnosis of congenital rubella infection in São Paulo

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Summary

Objective: rubella during the early stages of pregnancy can lead to severe birth defects known as congenital rubella syndrome (CRS). Samples collected from pregnant women with symptoms and suspected of congenital rubella infection between 1996 and 2008 were analyzed.
Methods: a total of 23 amniotic fluid samples, 16 fetal blood samples, 1 product of conception and 1 placenta were analyzed by serology and RT-PCR.
Results: all patients presented positive serology for IgG / IgM antibodies to rubella virus. Among neonates, 16 were IgG-positive, 9 were IgM-positive and 4 were negative for both antibodies. Of the 25 samples analyzed in this study, 24 were positive by RT-PCR. Changes in ultrasound were found in 15 (60%) of 25 fetuses infected with rubella virus. Fetal death and miscarriage were reported in 10 (40%) of the 25 cases analyzed. The rubella virus was amplified by PCR in all fetuses with abnormal ultrasound compatible with rubella. Fetal death and abortion were reported in 10 of 25 cases analyzed.
Conclusion: this study, based on primary maternal rubella infection definitely confirms the good sensitivity and specificity of RT-PCR using amniotic fluid and ultrasound. The results showed that molecular assays are important tools in the early diagnosis of rubella and congenital rubella syndrome.

Keywords: ultrasonography, prenatal, rubella syndrome, congenital, amniotic fluid, diagnosis, RT-PCR, serology.

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Introduction

Rubella virus (RV) usually causes a mild exanthematous disease frequently accompanied by adenopathy and occasional arthralgia. However, infection during pregnancy, particularly during the first trimester, can lead to severe birth defects known as congenital rubella syndrome (CRS).[1] Congenital rubella infection causes fetal death and a spectrum of birth defects known as congenital rubella syndrome. The syndrome affects multiple organ systems and common abnormalities include deafness, congenital heart disease, cataracts and mental retardation.[2] Traditionally, detection of fetal viral infection relies on isolation and culture of virus or maternal and cord blood seroconversion.[3] Viral culture and subsequent identification from mother and fetuses and/or amniotic fluid has been the gold standard. This method is slow, taking between 1 to 3 weeks to obtain a positive result. Maternal serology may also lack sensitivity and can be difficult to interpret. Advances in diagnostic techniques such as the polymerase chain reaction (PCR) allows rapid detection and it is a highly sensitive method. Recently, the diagnosis of congenital rubella infection is based mainly on the detection of rubella virus in amniotic fluid (AF) by RT-PCR[4,5] or detection of rubella virus specific IgM antibody in fetal blood.[6,7]

Rapid and accurate identification of the causative virus is increasingly important and can guide prenatal management as well as identify the need for long-term follow-up. In addition, in many countries, clinically recognized maternal rubella during the first 8 weeks of gestation is an indication for therapeutic abortion due to the high incidence of congenital defects. This is why there are only

a few PD studies in which rubella RNA is available. Infection prior to conception does not present a risk to the fetus, but when primary rubella infection occurs in the first 12 weeks of pregnancy, rubella virus will cross the placenta and induce generalized and persistent fetal infection in about 80% of cases.[3,8] Spontaneous abortion may take place in up to 20% of cases when rubella occurs in the first 8 weeks of pregnancy.[9,10] During the past 10 years, nationwide epidemics of rubella occurred approximately every 5 years in São Paulo, Brazil, namely in 1995, 2000 and 2007.[1,11] To reduce rubella transmission and prevent CRS, a nationwide campaign to vaccinate women and men against rubella was initiated in Brazil in 2001 and 2008. The World Health Organization (WHO) has estimated that more than 100,000 cases of CRS occur in developing countries every year; this represents a considerable social and economic burden. This study presents a series of CRI cases based on an integrated analysis of clinical features, serological and molecular investigation. Furthermore, all cases were submitted to clinical and laboratory analyses and a complete follow-up involving neonatal care in pediatric units by otorhinolaryngologists, ophthalmologists and cardiologists was carried out, thus providing a unique opportunity for the study of intrauterine transmission of rubella and its consequences to the fetus and newborn.

Methods

Patients

Forty patients with abnormal ultrasound findings, clinical signs and serological evidence of rubella, were studied prospectively between 1996 and 2008 at the Fetal Medicine Unit, University of São Paulo. No termination of pregnancy was performed since Brazilian law does not allow it. Maternal age from 16 to 39 years (mean: 25 years). Gestational age at the time of acute rubella was based on the last menstrual period (LPM) and the first trimester ultrasound scan using the crown-rump length measurement. The diagnostic strategy was based on seroconversion and/or the detection of rubella virus-specific IgM. Newborn follow-up was based on the presence of structural malformations, rubella antibodies at birth and at 3 months of age, echocardiographic alterations, brainstem evoked response audiometry, and ophthalmic pathology. Among the pregnant women, 20 had symptoms, 15 had fever, exanthema and lymphadenopathy; 2 had fever and exanthema, and 3 had exanthema and lymphadenopathy. This study was previously submitted to and approved by the Ethics Committee on Research with Human Beings at University of São Paulo (623/CEP).

Samples

The first maternal serum had been collected in the Clinical Hospital of the University's School of Medicine and the second sample after 15 days of the first collection. Newborn samples were obtained from umbilical cord blood at 3 months of age. Amniocentesis was offered to all 40 patients with clinical signs of rubella before 12 weeks of gestation, but only in 23 cases, amniocentesis was performed. The samples were obtained at 21 weeks of gestation and 6 weeks after the onset of clinical signs of rubella. A total of 23 amniotic fluid samples, 16 fetal blood samples, 1 product of conception and 1 placenta were analyzed (Table 1). All samples were stored at -80°C until use.

Serological assays

Detection of IgG and IgM specific antibodies to rubella virus was performed using a commercial enzyme immunoassay (Siemens, Marbuy, Germany) according to the manufacturer's instructions.

RNA extraction and reverse transcription

Viral RNA was extracted either directly from clinical specimens or from viral culture. Samples (200 µL) and inoculated cell cultures (300 µL) were extracted using Tri reagent (Molecular Research Center, Inc., Cincinnati, OH, USA) according to the manufacturer's protocol. Placental tissue was processed using the viral RNA Mini Kit (Invitrogen™ Life Technologies, Carlsbad, CA, USA). RV RNA was detected by RT-PCR as described previously.[4] The expected lengths of RT-PCR products synthesized using R2 and R7 (nt 8807–8991) and R11 and R8c (nt 8826–8968) primer pairs were 185 and 146 bp, respectively. All reactions were performed using positive and negative controls.

Results

Maternal and newborn serology

All pregnant women studied had positive serology results for IgG and IgM to rubella virus in the first or second sample (Table 1). In 5 of the subjects, a second sample could not be collected (np). Of the 16 newborns that had serum samples, 11 (68,75%) were IgM-positive and 5 (31,2%) were IgM-negative. Serology could not be performed in 9 of the newborn due to fetal death or miscarriage.

RT-PCR

Of the 25 samples analyzed in this study, 24 were positive by RT-PCR. The amniotic fluid samples negative on RT-PCR were confirmed by virus isolation. As a control,

TABLE 1 Serological and PCR analysis in 25 pregnant women and newborn and outcome

Pregnant								Newborn		
		Serology				RV detection		Serology		Outcome
Case/year	Gestational of week symptom	IgM		IgG				IgM	IgG	
		1° sample	2° sample	1° sample	2° sample	Sample	RT-PCT			
01/1996	11.2	pos	pos	neg	pos	AF	pos	pos	pos	Live
02/1996	11.9	pos	pos	neg	pos	AF	pos	pos	pos	Live
03/1996	10.9	pos	pos	neg	pos	AF	pos	pos	pos	Live
04/1996	11	pos	pos	neg	pos	AF	pos	pos	pos	Live
05/1998	11.5	pos	pos	pos	pos	AF	pos	pos	pos	Live
06/1999	11.4	pos	pos	pos	pos	AF	pos	pos	pos	Live
07/1999	12.0	pos	pos	neg	pos	AF	pos	np	np	Miscarriage
08/2000	4.4	pos	np	pos	np	AF	pos	np	np	Fetal death
09/2000	7.5	neg	pos	neg	pos	AF	pos	pos	pos	Live
10/2000	4.1	pos	neg	pos	pos	AF	pos	np	np	Fetal death
11/2000	11.2	neg	pos	neg	pos	AF	pos	pos	pos	Live
12/2000	1.4	pos	np	pos	np	AF	pos	np	np	Fetal death
13/2000	11.4	pos	pos	pos	pos	AF	pos	pos	pos	Live
14/2000	2	pos	pos	pos	pos	AF	pos	np	np	Fetal death
15/2000	5	pos	np	pos	np	AF	pos	np	np	Fetal death
16/2000	11.4	neg	pos	neg	pos	AF	pos	neg	pos	Live
17/2000	11.7	pos	pos	neg	pos	AF	pos	pos	pos	Live
18/2000	8.2	pos	pos	pos	pos	AF	pos	neg	pos	Live
19/2000	10.8	pos	neg	pos	pos	AF	pos	neg	pos	Live
20/2000	10.3	pos	pos	neg	pos	AF	pos	neg	neg	Miscarriage
21/2001	7.4	pos	neg	pos	pos	AF	pos	np	np	Fetal death
22/2001	11.5	pos	neg	pos	pos	AF	pos	pos	neg	Live
23/2001	4.5	pos	pos	pos	pos	AF	neg	neg	pos	Live
24/2002	4	pos	pos	np	np	POC	pos	np	np	Fetal death
25/2008	12	pos	pos	pos	np	Placenta	pos	np	np	Fetal death

pos: positive; neg: negative; POC: product of conception; AF: amniotic fluid; np: no processed

the 25 samples in this study were submitted to PCR for other viruses such as parvovirus B19, herpes virus simplex 1 and 2, and cytomegalovirus. Twenty-four samples were negative for these viruses, only one (case 23) was positive for cytomegalovirus.

Evaluation of ultrasound

Alterations were found in several follow-up evaluations by ultrasound in 15 (60%) of the 25 fetuses infected with rubella virus. Miscarriage and fetal death were reported in 10 (40%) of the 25 cases analyzed (Table 2). Rubella virus was amplified by PCR in all fetuses showing ultra sound alterations compatible with rubella: growth restriction, hepatomegaly, hydrocephaly, ventricular septal defect, intracranial calcifications, oligohydramnios, with more than one abnormality in some cases (Table 2). Although rare in congenital rubella, in one case (case 7) the cranial cap was absent, with cerebral tissue floating in amniotic fluid.

TABLE 2 Results of the analysis of the ultrasound and newborn of the 25 cases analyzed

Case	Ultrasonographic examination	Newborn follow-up
1	Without alterations	Live/cataract
2	Decrease AF, growth restriction	Live
3	Hydrocephaly	Live/speech impairments
4	Insufficient tricuspide	Live
5	Decrease AF, hepatomegaly	Live
6	Without alterations	Live
7	Cranial cap absent	Miscarriage
8	Growth restriction	Fetal death
9	Without alterations	Live/bilateral deep deafness
10	Hydrocephaly, growth restriction, ventricular septal defect	Fetal death
11	Without alterations	Live/ventricular septal defect
12	Growth restriction	Fetal death
13	Atrial septal defect, aortic stenosis	Live/arthritis 6 months
14	Fetal death	Fetal death
15	Hepatomegaly, hydrocephaly, intracranial calcifications	Fetal death
16	Without alterations	Live
17	Without alterations	Live
18	Oligohydramnios, arterial canal patency, aortic stenosis	Live
19	Without alterations	Live
20	Without alterations	Miscarriage
21	Ventricular septal defect	Fetal death
22	Without alterations	Live
23	Without alterations	Live
24	Growth restriction	Fetal death
25	Hydrocephaly, growth restriction, ventricular septal defect	Fetal death

In case nº 25, fetus showed ultrasound abnormalities: echogenic fetal bowel, growth retardation, oligohydramnios, placentomegaly, pericardial effusion, cardiomegaly, hepatomegaly, and ascites. Fetal death occurred at the 29th week of gestation. Rubella virus was amplified from placenta and histological diagnosis of the placenta showed active chronic villitis and chorioamnionitis, villous fibrosis, focal calcifications, and thrombotic vasculopathy with avascular villi and hemorrhagic endovasculitis.

Newborn follow-up

In the total group of live newborn, 10 newborns did not show change in the ultrasound during the prenatal, but after birth, one newborn showed bilateral deep deafness and other ventricular septal defects. There were 6 cases in which virological analyses of the amniotic fluid samples were positive while the newborn showed no signs of congenital rubella syndrome (cases 6, 16, 17, 19, 22 and 23). These results indicate that not all infected babies developed the congenital disorder. Out of 15 live newborn with alterations in ultrasound, 4 lived without sequela, and 3 presented alterations after birth: speech impairments and arthritis (Table 2).

Discussion

Historically, rubella and congenital rubella syndrome have been an important public health challenge in Brazil. Despite the fact that rubella vaccination was initiated in 1992, regular outbreaks of the disease have been reported since the beginning of 1995 and have continued until 2007.[12,13] Between 1999 and 2008, a large number of rubella cases were reported throughout the country, with the highest incidence among adults and a steady increase in the number of CRI and CRS cases.[11,14] The impact of CRS in life and in the economy of the country was high; therefore, it became necessary to accelerate control and, ultimately, eliminate rubella virus transmission. CRI cases may play an important role as virus-shedders and in the continuation of rubella transmission within the community. Therefore, identifying them to prevent fur-

ther rubella transmission is also important in the context of rubella elimination.[15]

The clinical diagnosis of acute rubella infection in pregnancy is extremely difficult. The rash is not very specific or particularly apparent, and most infectious cases are subclinical.[2,3] Therefore, demonstration of seroconversion and presence of high IgM titers is the primary mode of diagnosis of acute rubella in pregnancy. Unfortunately, booking serum samples are often not available. The risk of congenital infection may usually be estimated by establishing the gestational age at the time of maternal infection. However, diagnosis of intrauterine infection is difficult because often maternal serology is inconclusive especially when infection occurs between the 13th and 20th weeks of gestation.[2] Direct methods of diagnosing fetal infection by PCR are therefore essential for early diagnosis as shown by another report.[1] Our results showed that all pregnant women studied had positive serology results for IgG and IgM to rubella virus in the first or second sample. In the present cases, prenatal diagnosis was performed after 21 weeks of gestation and the time elapsed between the onset of maternal infection and the procedure was longer than 6 weeks, reducing false-negative results.

The humoral immune response of infants with CRS differs from that of naturally infected or immunized children.[2] Laboratory confirmation is based on the detection of rubella specific IgM and IgG antibodies and low avidity antibody. Fetal blood is not taken for this purpose until about 22 weeks of gestation, when fetal IgM becomes detectable; however, virus-specific IgM levels may be low at this time and therefore false-negative results may be obtained. Rubella is slower than in postnatal infection and low avidity can be detected for a longer period.[2,3,16] The present study confirms the above findings by means of IgM negative in the sera of the four newborns infected. However, these newborns that had no detectable IgM were positive for RV RNA by PCR assays. Among these cases, one newborn showed alterations by ultrasound, arterial canal patency and aortic stenosis another miscarriage. It has also been reported that false-positive IgM results can be obtained with sera from patients with other acute viral infection, such as measles, parvovirus B19, cytomegalovirus and Epstein-Barr virus infection.[17] Our finding that one newborn with rubella IgM reactivity was diagnosed with cytomegalovirus infection supports this observation. It becomes evident that unspecific stimulation of immune cells or cross-reacting antibodies can also be found in the fetal system.

Concerning the PCR results, rubella was detected in all amniotic fluid samples of mothers of infected fetuses. Macé et al.[5] report involving 45 pregnant mothers of rubella-infected fetuses, (83%) were rubella-positive. Furthermore, results similar to these were reported by Bosma et al.[4] and Jin et al.[18] PCR is a rapid and sensitive molecular method and it has not been applied systematically to CRS cases. It can be used in conjunction with serology for the diagnosis of acute infection and also for the diagnosis of pre and post-natal CRS since the serological results can be inconclusive.[3,4,5,19] However, detection of rubella viral RNA in AF which may be obtained from amniocentesis at about 12 weeks of gestation allows confirmation of fetal infection. The time between maternal infection and fetal sampling is an important consideration in prenatal diagnosis. The optimal sampling interval between maternal infection and fetal sampling should be 6 to 8 weeks for amniotic fluid and fetal blood.[5]

The consequences of rubella during pregnancy may be the birth of a child with congenital anomalies, which may be severe, and multiple birth of a child apparently normal or spontaneous abortion.[20] Rubella virus generally establishes a chronic nonlytic infection in the fetus and has the potential to infect any organs.[21,22] The rubella virus teratogenic process most probably begins when placental infection occurs during maternal viremia, leading to dissemination of the virus throughout the fetus. In addition, tissues from different organs of fetuses with CRS revealed teratogenic effects, such as reduced cell size and number compared with control, which was suggestive of mitotic inhibition or perhaps due to rubella induced chromosomal breaks.[21] Even when the fetus is infected, it survives in most cases; the pregnancy continues and premature births or stillbirths can occur.[3] In our results, the most common ultrasonographic prenatal rubella infection findings were heart and amniotic fluid anomalies. Hydrocephaly, although rare in congenital rubella, was observed in tree cases. Absence of the skullcap with cerebral tissue floating in the amniotic fluid was diagnosed in case nº 7 and the virus was detected in the placenta, liver, and central nervous system. Miller et al.[9] also report two fetuses with the same alterations. A ventricular septal defect was observed in two cases and an atrial septal defect associated with aortic stenosis was diagnosed in one case. Case nº 25 also showed several changes in placenta due to viral replication. As a result of prenatal diagnosis, it is clear that not all infected fetuses developed CRS. The simplest hypothesis implies that the decisive parameter is the replication level of the virus in the fetus, i.e. when virus replication in the fetus surpasses a certain critical level, the fetus would then develop CRS. If this does not occur, the fetus would remain undisturbed. It is noteworthy that rubella genome was de-

tected in the amniotic fluid, POC and placenta due to the infection of the mothers and also from a newborn that died with severe congenital disorders. In addition, antibodies IgG against rubella virus were transmitted from the mother to the fetus, while IgM antibodies were not transmitted but produced in the fetus from around 20 weeks of gestation. However, these antibodies could not eliminate the virus from the fetal body although they had neutralizing activity. Elimination of the virus from the infected tissue is dependent on cell-mediated immunity. At around 1 year of age, its ability becomes completely mature and thus the body is able to eliminate the virus. In contrast, in the case of infected eye lens, the virus cannot be completely eliminated from the lens. Once the virus enters the lens, it replicates and persists without elimination.[3]

In Brazil there are not many reports about CRI, because the diagnosis of infection with rubella virus during pregnancy or congenital rubella syndrome is performed only after the birth of the child. In some countries, the risk of disease in the first trimester justifies termination of pregnancy without prenatal diagnosis, which is not allowed in Brazil, because Brazilian law does not allow therapeutic abortion.

Resumo

Diagnóstico pré-natal de infecção congênita por rubéola, em São Paulo, Brasil.

Objetivo: a rubéola, durante os primeiros estágios da gravidez, pode levar a graves defeitos congênitos, conhecidos como síndrome da rubéola congênita (SRC). Amostras de gestantes com sintomas e suspeitas da rubéola congênita foram coletadas entre 1996 e 2008.
Métodos: um total de 23 amostras de fluido amniótico, 16 amostras de sangue fetal, um produto da concepção e uma placenta foram analisados por sorologia e PCR.
Resultados: todas as gestantes apresentaram sorologia positiva para IgG/IgM para o vírus da rubéola. Entre os recém-nascidos, 14 apresentaram anticorpos IgG positivos e 11 foram os anticorpos IgM positivos. Das 25 amostras analisadas neste estudo, 24 eram positivas por RT-PCR. Alterações na ultrassonografia foram encontradas em 15 (60%) dos 25 fetos infectados com o vírus da rubéola. Morte fetal e aborto espontâneo foram reportados em 10 (40%) dos 25 casos analisados. O vírus da rubéola foi amplificado por PCR em todos os fetos que apresentaram alterações na ultrassonografia, compatíveis com a rubéola. Morte fetal e aborto foram relatados em 10 dos 25 casos analisados.
Conclusão: os resultados mostraram que os ensaios moleculares são ferramentas importantes para o diagnóstico precoce da rubéola e da síndrome da rubéola congênita.

Palavras-chave: ultrassonografia pré-natal; síndrome da rubéola congênita; líquido amniótico; diagnóstico, RT-PCR; sorologia.

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