ORIGINAL ARTICLE

Role of congenital rubella reference laboratory: 21-months-surveillance in Liguria, Italy

P. CANEPA[1 2], L. VALLE[2], E. CRISTINA[3], D. DE FLORENTIIS[2 4], V. PARODI[2], F. BANFI[2], M. ZANCOLLI[1 2], P. DURANDO[1 2 4], G. ICARDI[1 2 4], F. ANSALDI[1 2 4]

[1] Doctoral School in Clinical and Experimental Medicine, Course: "Vaccination Prevention", University of Genoa, Italy; [2] Department of Health Sciences, University of Genoa, Italy; [3] I.R.C.C.S. "G. Gaslini", Genoa, Italy; [4] U.O. Hygiene, "San Martino" University Hospital, Genoa, Italy

Key words

Rubella • Surveillance • Diagnosis

Summary

Introduction. *Rubella is generally a mild rush fever disease when acquired in childhood, but when infection occurs during the first months of pregnancy, high risk of trans-placental transmission to the foetus and of congenital anomalies exists. In November 2003, a National Plan for measles and congenital rubella elimination was approved in Italy. The aim was to reduce and maintain Congenital Rubella Syndrome incidence lower than 1 case per 100.000 live births/year by 2007. Since June 2006, Liguria Administrative Region recognized U.O. Hygiene, "San Martino" University Hospital, Genoa, as regional reference laboratory for diagnosis of rubella infection during pregnancy and post-partum.*

Methods. *Twenty-one-month virological-surveillance results between April 2007 and December 2008 were reported in terms of demographic data, risk factors, access reasons, clinical picture, vaccination, previous rubella disease, laboratory results of pregnant women and newborns.*

Results and conclusion. *Since the beginning of surveillance, 65 pregnant women with suspected virus infection and 18 newborns with suspected congenital rubella were followed up. The results of laboratory surveillance highlighted (i) the importance of an early screening, (ii) the suboptimal specificity of chemiluminescent assays, that often yield false positive IgM results and (iii) the fundamental role of second-level laboratory to confirm the serological diagnosis and to detect the virus by molecular techniques.*

Introduction

Rubella virus infection is sub-clinical in approximately 50% of cases and, when symptoms are present, they are usually quite mild, including inflammation of the retronucal lymph nodes and maculopapular rash, which may be preceded by mild catarrhal symptoms. The rash is immunologically mediated and coincides with the development of specific anti-rubella antibodies [1-3]. Rubella infection acquired during the first trimester of pregnancy can cause multifactorial foetal damages, that result from a combination of direct rubella virus cytopathic effect and on the induction of apoptosys. Moreover placental infection that occurs during maternal viraemia, causes focally distributed necrotic areas in the chorionic villae epithelium and in the endothelial cells of its capillaries [4].

Up to 90% of infants born from mothers infected during the first 11 weeks of gestation develop a pattern of birth defects called Congenital Rubella Syndrome/ Infection (CRS/CRI) [5], responsible, in early infancy, of glaucoma, pigmentary retinopathy, ventricular septal defect, pulmonary artery stenosis ecc. The risk of abnormalities decrease if the infection occurs after 12 week of gestation and after 16 weeks the incidence of foetal damage is less than 2%. Deafness and retinopathy are frequently the only manifestation of congenital infections in particular if it's subclinical.

The high public health relevance of rubella is due to the theratogenic effects resulting from virus infection during pregnancy. An high vaccination coverage could prevent the indigenous rubella virus circulation and so reduce the wide burden of the disease.

In 2002, World Health Organization (WHO) Regional Office for Europe developed the first strategic plan for rubella elimination and 3 years later a new plan was implemented with the aim to eliminate endemic rubella and reduce CRS incidence < 1 case per 100.000 live births by 2010 [8]. In Italy the National Plan for elimination of measles and congenital rubella, was approved on November 2003 [9, 10]. The goal of the plan was to eliminate measles and reduce the incidence of CRS cases as recommended by WHO, to reach national vaccine coverage of at least of 95% in 24 months children to ensure an high immunity level among women of childbearing age. Notification of rubella infection during pregnancy and CRS/CRI were mandatory since 1 January 2005. Furthermore, the national plan established a regional laboratory network with the aim to improve the diagnostic ability.

In this article, results of 21-month virological surveillance conducted in Liguria, an Italian region of 1,700,000 inhabitants and about 11,000 newborn/year, are presented.

Materials and method

Role of the laboratory

According to WHO recommendation the main objectives of rubella reference laboratory were (i) to develop protocols for the laboratory diagnosis of rubella and provide the necessary support, (ii) to provide resources and facilities for staff training and (iii) to confirm the clinical diagnosis of clinically suspected cases using IgM ELISA assays to help in early detection of CRI/ CRS.

Clinical diagnosis of rubella not always is possible, since the clinical signs are transient and can be confused with measles or parvovirus B19 infection [4], moreover 50% of rubella infections may be subclinical. Thus, laboratory confirmation by serological tests is essential. Detection of virus specific IgM in serum during pregnancy is the most commonly used method to confirm the infection occurrence.

The IgM detection chemiluminescent assays routinely used by first level laboratories present some limitations: firstly, specific IgM antibodies mostly persist for 6-12 weeks, and it's not possible to distinguish between a recent primary rubella infection or a re-infection; secondly, false positive results may occur due to cross-reacting IgM antibodies or to rheumatoid factor. Full clinical and epidemiological picture must be considered for the correct interpretation of the IgM detection assay.

Blood samples that result positive for IgM in early detection during pregnancy, should be sent to the Regional Reference Laboratory, where second level diagnosis is performed. It includes: (i) high specific immunoenzymatic assays for IgM detection and quantitation, (ii) test for IgG and IgG avidity evaluation, (iii) storage of all available consecutive samples for the evaluation of antibody kinetics and (iv) nucleic acid-based assay and culture for viral detection and isolation, to confirm if an infection has been recently acquired. In particular, RT-PCR to detect rubella virus directly from clinical material (pharyngeal swabs, amnios and urine) is highly sensitive and specific; sensitivity of detection of viral RNA in amniotic fluid varies from 87 to 100% [11, 12].

Diagnosis of CRS in newborn children focuses on virus isolation from urine samples and on the demonstration of virus specific IgM, which can be detected in almost 100% of infected infants 0-3 years of age and progressively decline to less than 50% at 12 months of age. Some infected newborns do not produce IgM at birth and IgM false negative results may occur despite the high specificity of immunoenzymatic assay. This could be the consequence of the fact that congenital infected neonates have high Rubella specific IgG titers of both self and maternal origin that tend to compete with IgM antibodies for binding. In these cases diagnosis is based on the evaluation of IgG kinetics and IgG avidity during the first months of life and by detection of viral RNA in clinical samples as urine and oral fluid by RT-PCR [13, 14].

Case definition

Rubella infection

All suspected cases of rubella during pregnancy and congenital rubella, that has been reported by physicians to the local health authorities, are classified into one of this categories:

(i) *suspected case.* Any generalized rash illness of acute onset; any positive result of rubella specific IgM in patient serum even in absence of clinical symptom; contact (person to person) with a confirmed patient or a person with a rash illness suspected of being rubella;

(ii) *probable case.* A case that meets the clinical case definition of acute onset, generalized maculopapular rush, temperature > 37,2°C and at least one of the following signs: lymphoadenopathy, arthralgia/ arthritis or congiuntivitis, also in absence of laboratory confirmation.

iii) *confirmed case.* A case that is laboratory confirmed or that meets the clinical case definition and is epidemiologically linked to a laboratory confirmed case [9].

Congenital Rubella

(i) *Suspected case:* Any infant less than one year of age in whom a health worker suspects CRS. A health worker should suspect CRS when an infant aged 0-11 months presents with heart disease and/or suspicion of deafness and/or one or more of the following eye signs: white pupil (cataract), diminished vision, pendular movement of the eyes (nystagmus), squint, smaller eye ball (microphthalmus), or larger eye ball (congenital glaucoma). A health worker should also suspect CRS when an infant's mother has a history of suspected or confirmed rubella during pregnancy, even when the infant shows no signs of CRS.

(ii) *Clinically confirmed case:* An infant in whom a qualified physician detects at least two of the complications listed in (a) below or one in (a) and one in (b): (a) Cataract(s), congenital glaucoma, congenital heart disease, loss of hearing, pigmentary retinopathy; (b) Purpura, splenomegaly, microcephaly, mental retardation, meningocephalitis, radiolucent bone disease, jaundice that begins within 24 hours after birth

(iii) *Laboratory confirmed case:* An infant with clinically confirmed CRS who has a positive blood test for rubella-specific IgM (almost 100% of such infants are positive at the age of 0-5 months; 60% are positive at 6-11 months; 50% at 12 months). Where special laboratory resources are available, the detection of rubella virus in specimens from the pharynx or urine of an infant with suspected CRS provides laboratory confirmation of CRS (60% of such infants shed rubella virus at the age of 1-4 months; 30% at 5-8 months: 10% at 9-11 months).

(iiii) *Congenital rubella infection (CRI):* If a mother has suspected or confirmed rubella in pregnancy, her infant should have a rubella-specific IgM blood test. An infant who does not have clinical signs of CRS but who has a positive rubella-specific IgM test is classified as having congenital rubella infection (CRI).

The diagnosis of rubella infection during pregnancy requires one or more of the samples: maternal blood,

foetal blood, urine, throat swab, amniotic liquid, liquor, saliva. Biological samples useful in case of CRS/CRI diagnosis are: blood, urine, throat swab, liquor, saliva

Laboratory investigations

Samples were collected from clinicians participating to the surveillance network and sent to the Reference Laboratory to confirm rubella virus infection in pregnant women if they have (i) generalized maculopapular rash, (ii) serologic test for immunoglobulin M (IgM) antibody positive, (iii) immunoglobulin G (IgG) seroconvertion and/or (iv) a link with a confirmed case (a contact of at least 15 minutes or face-to-face).
As regard as newborn children, laboratory diagnosis of CRS/CRI is required (i) for all infants born to mothers with suspected or confirmed infection and (ii) if there are clinical manifestations or anomalies consistent with congenital viral infection.
Laboratory diagnosis of congenital rubella syndrome is based on detection of specific immunoglobulin M and G (IgM and IgG) and evaluation of IgG avidity by means of enzyme-linked immunosorbent assay (ELISA), on viral growth by culture isolation and on detection of viral RNA using molecular test RT-PCR based.
In agreement with the WHO guidelines, in our protocol are reported criteria for:
(i) Diagnosis during pregnancy:
- IgM or IgG seroconversion;
- IgM in presence of clinical diagnosis and/or low avidity IgG;
- at least 4 fold increase of IgG titer between 2 serum samples (the first one collected no more than 7-10 days after a rash or after case contact and the second one at least 14 days after the first);
- viral growth on cell culture from urine, blood, throat swab or saliva collected from 7 days before until 14 days after the rash;
- RT-PCR positive test in samples collected from 7 days before until 14 days after a rash.

(ii) CRS:
- detectable IgM in foetal blood since 20th week of pregnancy;
- RT-PCR positive test or viral growth from foetal blood or amniotic liquid, preferably collected 6 weeks after seroconversion and/or at 22th week (this care reduces false-negative results).

(iii) CRI:
- IgM positive in the first month of life;
- persistent high IgG titer (if the newborn was uninfected, each month the IgG titer decrease of 50% and is undetectable since the 6th month);
- RT-PCR or culture isolation positive.

In Figure 1 is reported the algorithm adopted for the rubella infection and CRS/CRI diagnosis.

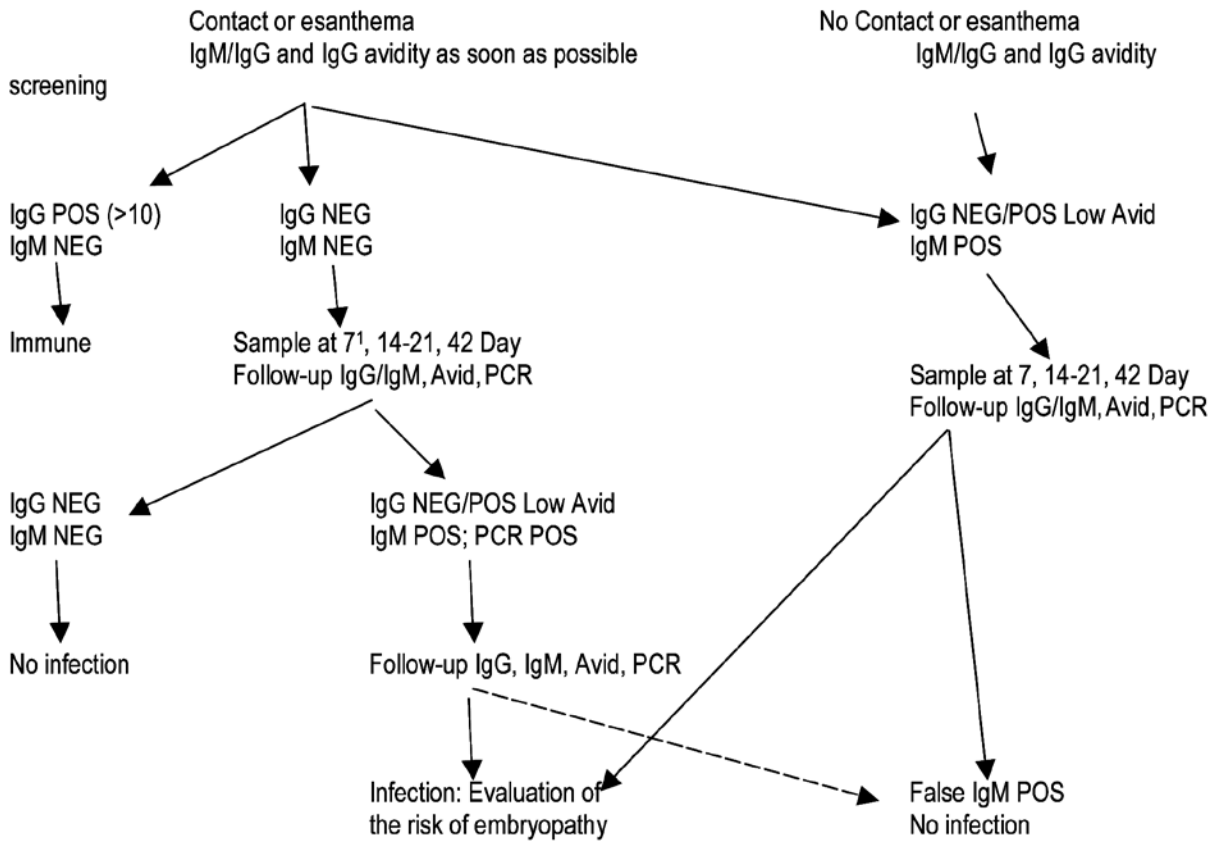
Reference laboratory assays

Rubella-virus specific IgM, IgG and IgG avidity were measured in serum samples using Enzygnost® Anti Rubella-Virus/ IgM and Enzygnost® Anti Rubella-Virus/ IgG (Dade Behring, Marburg, Germany), respectively, following the manufacturer's instructions.
Detection of rubella virus genomic RNA in amniotic fluid and urine was performed by ProDect RUBELLA and ProDect GEN.E.I.A (BSC Biotech S.p.A., Italy), following manifacturer's instructions. Briefly the amplimers were hybridised with single-stranded DNA probes specific for Rubella virus and applied on to the walls of microtitre wells coated with streptavadin–biotin.A DNA enzyme immunoassay was used to detect hybridised DNA; absorbance was measured photometrically.
Results confirmation all samples were tested by RT-PCR-Nested as previously described [14]. Traditional techniques were used for extraction (QIAamp Min Elute virus spin Kit, Qiagen, Milan, Italy) reverse transcription and PCR nested reaction; amplified products were electrophoretically separated on 2% agarose gel (Roche Diagnostics GmbH, Mannheim, Germany). Gel was stained with ethidium bromide and visualized under UV light.

Results

Eighty-four serum samples and 11 amniotic liquid samples from 65 pregnant women were tested for IgM and IgG detection with avidity evaluation and virus detection, respectively. All women had undergone rubella screening in other laboratories. Serum samples and urine from 18 newborn children with suspected infection, 10 born from mother followed by regional reference laboratory and 8 from women not previously tested by our centre, were analyzed; in particular, one of the children aged 7 months presented clinical manifestation consistent with CRS.
Demographic data, risk factors, clinical picture, vaccination, previous rubella disease, laboratory results of pregnant women and reasons of reference-laboratory access according to gestational week are reported in Table I.
IgM reactivity or weak reactivity at the serological screening performed at the first level laboratory was observed in 47 (72.3%) and 10 (15.4%) out of 65 mothers, respectively; 7(10.7%) seroconverted during pregnancy, 9 (13.8%%) had rash and only one referred a contact with a suspected case. The gestational week average was 14.7 ± 0.71 and 80% mother accessed to the second level laboratory between 5° and the 20° gestational week. It is noteworthy that 5 (10.7%) and 14 (29.8%) of the women, that resulted IgM reactive or weak reactive at the first level laboratory screening, performed the confirmation assay after the 21st gestational week and between 13 and 20th week, respectively.
Most of pregnant women (47, 72.3%) were asymptomatic. Four out of 65 (6.2%) patients referred fever and 9 (13.8%) presented rash associated or not with other symptoms such as fever, pharingodynia or artralgia (Tab. I).

Fig. 1. Flow-chart for pregnant women applied by regional reference laboratory.



Nineteen women (29.2%) referred to be vaccinated against rubella but only one of them provided a certification. Five (7.7%) pregnant women referred past rubella infection without laboratory confirmation. Globally, 41 (63%) women were aware of being susceptible to rubella during pregnancy.

Twenty (30.8%) pregnant women continued the follow-up in compliance with the flow-chart in Figure 1. Among the 45 pregnant women that interrupted the follow-up, one aborted at the 11th gestational week because of a confirmed Cytomegalovirus (CMV) infection, one, which had exanthema, spontaneously aborted, one voluntary aborted, 8 women completed the pregnancy.

Eleven women IgM positive at the first visit, accessed to the laboratory for rash, seroconvertion or contact with a confirmed case, and underwent to amniocentesis at 21st-23rd gestational week for rubella virus detection. Only one out of 11 amniotic fluid analysed resulted positive for rubella virus RNA by PCR and for rubella virus isolation on cell culture, supporting the diagnosis of acute rubella infection.

As regards newborns, 10 born from women followed by the reference laboratory during pregnancy were submitted to follow-up, that consist of monthly collection of blood and urine samples. Nine babies were normal with no evidence of CRI at birth and resulted negative either to IgM antibodies and viral molecular detection in urine samples. Only one of ten presented cardiac anomalies at birth, but laboratory results confirmed that such abnormalities were not correlated with CRS. Eight newborn from mother monitored by other laboratories were followed during the first months of life. One out of 8 presented abnormalities at birth with diagnosis of psychomotor retard, myoclonia, hypotonia and suspected auditory deficit but serological and molecular pattern resulted negative for rubella. One of the children resulted IgM positive without clinical signs of CRS showed a decrease of IgM titres during follow-up and a negative PCR result.

Discussion

To our knowledge this is one of the first studies that examines the role of a second level laboratory for rubella diagnosis in a Country, such as Italy, where the disease is under elimination.

The laboratory survey of congenital rubella performed in Administrative Region Liguria presents some limitations and shows some improper practices during pregnancy follow-up. First of all, the survey didn't have probably captured all the suspected cases of congenital rubella, according the case definition established by Health Ministry. Eversince, in Liguria, the 36.1% of women included in the age group 18-49 years is potentially

Tab. I. Demographic data, risk factors, access reasons, clinical picture, vaccination, previous rubella disease, laboratory results of pregnant women and reasons of reference-laboratory access.

Mother (n. = 65)		
	Age (year, mean ± SD)	30.74 ± 0.71
	Gestation period (week, mean ± SD)	14.7 ± 0.71
	Access Reason	
	Seroconvertion	7 (10.8%)
	IgM positivity at the first visit	48 (73.8%)
	Rash	9 (13.9%)
	Suspected or confirmed case contact	1 (1.5%)
	Clinical picture during pregnancy	
	Asimptomatic	47 (72.3%)
	Fever	4 (6.2%)
	Rash	6 (9.2%)
	Rash and fever	1 (1.5%)
	Rash and pharingodynia	1 (1.5%)
	Rash and artralgia	1 (1.5%)
	Artralgia	4 (6.2%)
	Retronucal lymphadenopathy	1 (1.5%)
	Vaccination	
	Certified	1 (1.5%)
	Referred by patient	18 (27.7%)
	No	29 (44.6%)
	Unknown	17 (26.2%)
	Previous Rubella	
	Lab-confirmed	0
	Suspected	5 (7.7%)
	No or unknown	60 (92.3%)
	Concomitant diseases	
	CMV	1 (1.5%)
	Follow-up: accesses	
	1	45 (69.2%)
	2	15 (23.1%)
	3 or more	5 (7.7%)
Children (n. = 18)	**Mother followed by reference lab**	
	Yes	10 (56.0%)
	No	8 (44.0%)
	First access (day, mean ± SD)	47.8 ± 70.0
	Follow-up: accesses	
	1	8 (44.4%)
	2	6 (33.3%)
	3 or more	4 (22.2%)

susceptible to rubella virus or are unaware of their immune status, the annual number of pregnancies is around 11,000 and the clinical specificity of the commercial rubella chemiluminescent IgM immunoassays tests routinely used by first level laboratories ranged between 93.9% and 96.1%, the expected annual accesses to the second level laboratory in Liguria should reach 155 patients [15-17]. Nevertheless during the 21-months of surveillance the accesses were only 65, showing that at least 100-120 women potentially resulted IgM-positive do not have a contact with reference laboratory. The reasons of the missed second level diagnosis could be the lack of rubella screening or the underestimation of the IgM-positivity relevance during pregnancy follow-up. It is not possible to evaluate the role of the two improper practices, but unpublished data showed a high coverage of rubella screening during pregnancy. In any case, it appears necessary to implement actions to catch-up women that have poor contact with health system by the involvement and the collaboration of various health professionals (pediatricians, gynaecologists, public health officers) and to provide appropriate rubella educational information. Furthermore, the lack of rubella cases detection has important public health effects making less sensible the survey system, postponing the alert signal. The reinforce of a warning system for early detection of rubella infection allows the adoption of all the activities useful to limit and stop viral spread and helping in achieving goals of rubella control and elimination.

Moreover, this study highlighted that a high percentage of pregnant women performed the confirmation laboratory screening after the 21st gestational week, later than recommended in the WHO guidelines. The correct timing of sampling together with complete information about the onset of clinical picture, vaccination status, risk factors for disease and epidemiological data obtained from patients, are needed for the correct interpretation of the laboratory results and to achieve an accurate diagnosis, minimizing anxiety for the patients, especially if pregnancy termination is considered. Screening test on pregnant women should be performed before 8th gestation week, because after this period it's very difficult to exclude a CRI in a suspected case. After infection or vaccination, specific IgM levels usually fall below the detection level within 2 months [18]. A IgM-negative and IgG-positive sample collected and analyzed by the second level laboratory after 8th gestation week in a pregnant previously resulted IgM positive or IgG negative at screening does not allow to exclude the infections in the first gestation weeks. IgM positive results at screening assay is quite frequent since heterotypic IgM antibody responses may occur in patients infected with other viruses, particularly EBV, and sera from these patients may give false positive results for rubella [15, 19-21]. Another of the most common causes for false positive reactions include the presence of rheumatoid factor, specific IgG, and non-specific, cross-reacting IgM in the samples. For these reasons, results from about 2% of serum samples tested for rubella IgM will be difficult to interpret, if and when the correct sampling time is respected [16].

At this regard, the determination of avidity for IgG may be useful as affinity is proportionally correlated with the age of the infection event. So a high avidity value that persist over the time allows us to exclude a recent infection.

Furthermore, about 70% pregnant women did not continue the follow-up so in many cases we were not able to obtain information about come measures. In the majority of cases the results of the first sample analyzed by the second level laboratory allowed us to exclude the chance of a previous infection and the follow-up completion represented an important feed-back for the evaluation of the laboratory performance.

In conclusion, results obtained in this study highlighted the need to maintain and implement rubella surveillance activities and to improve the management of pregnant women. At individual level clearly emerged the importance of the counselling offered to pregnant women helping them in deciding to continue or interrupt pregnancy and the need of providing appropriate rubella educational information. Public health goals to rapidly reach are high levels of rubella vaccination coverage through vaccination of susceptible persons, the surveillance together with reporting implementation so to allow an appropriate and rapid response when a case of rubella is identified.

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■ Correspondence: Paola Canepa, Department of Health Sciences, University of Genoa, via Pastore 1, 16132 Genoa, Italy - E-mail: paola.canepa@unige.it