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Determination of rubella virus-specific humoral and cell-mediated immunity in pregnant women with negative or equivocal rubella-specific IgG in routine screening

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ABSTRACT

Background: Immunity to rubella-virus (RV) is commonly determined by measuring specific IgG (RV-IgG). However, RV-IgG results may be different and even discordant, depending on the assay used. Cell-mediated immunity is not routinely investigated for diagnostic purposes.

Objectives: Our aim was to investigate humoral and cellular immunity of women with negative or equivocal RV-IgG before, and after post-partum vaccination.

Study design: A total of 186 pregnant women were included in the study. During pregnancy, humoral immunity was investigated with two RV-IgG immunoassays, an immunoblot and a T-cell mediated immunity test. In the post-partum vaccination period, measuring RV-IgM and RV-IgG avidity allowed us to determine whether women raised a primary or a secondary immune response.

Results: Before vaccination, 52.2% women, supposed to be susceptible, had positive anti-E1 RV-IgG indicating strong evidence of previous exposure to RV. All (100%) pregant women who had a positive immunoblot before immunization raised a secondary immune response to vaccination, and 96.8% who had a negative immunoblot before immunization, raised a primary immune response to vaccination. All women who raised a primary immune response after vaccination had negative anti-E1 RV-IgG and negative cell-mediated immunity.

Discussion: These results indicate that individuals can have evidence of protective immunity against rubella despite negative RV-IgG.

1. Background

Rubella has been eliminated or is becoming a rare disease in many countries where there are effective vaccination programs. However, in these countries, assessing immunity, particularly of childbearing age women, is of major importance in order to identify and immunize susceptible women.

Immunity to rubella virus (RV) is commonly determined by measuring specific-IgG (RV-IgG), and in many countries where immunity is now mainly due to vaccination, RV-IgG testing mainly relies on commercial immunoassays (CIAs). Irrespective of their format, all CIAs currently available are calibrated against the WHO International Standard and report results in IU/mL [1,2,4]. The use of IU implies that health professionals can assume that results obtained by different CIAs are comparable, and therefore expect that the interpretation (positive, negative, equivocal) of a single sample tested in different assays is

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identical. Unfortunately, as reported in several publications, RV-IgG results may vary depending on the assay used particularly for samples with low RV-IgG titers [5–8]. This may lead to confused clinical management of pregnant women considered immune or susceptible depending on the assay used. It may also lead to unnecessary re-vaccinations or unnecessary management in prenatal diagnosis units in case of RV-IgG seroconversion wrongly considered as primary infections.

2. Objectives

Our aim was to investigate both humoral and cell-mediated immunity of pregnant women with negative or equivocal RV-IgG titers, with confirmatory assays capable of identifying rubella-protein-specific immunity, avidity and cell-mediated immunity. Additionally, we investigated whether their immune response to post-partum vaccination triggered a primary or a booster response.

3. Study design

3.1. Study design

According to French recommendations, pregnant women who cannot provide documented evidence of their immunity against RV, are tested for RV-IgG in the first trimester of pregnancy. If seronegative a second test is performed at 20 weeks of gestation (WG) and if still negative, vaccination is offered in the post-partum period (trivalent measles-mumps-rubella vaccine).

In our study, during pregnancy, humoral immunity was investigated with two RV-IgG CIAs (Enzygnost anti-rubella virus IgG, Siemens HealthCare and LXL Rubella IgG, DiaSorin), and an immunoblot to explore E1-specific antibodies (recombBlot rubella IgG, Mikrogen GmbH). Cell-mediated immunity was investigated with a T-cell stimulation assay based on the γ−interferon (IFN-γ) response after stimulation of the peripheral blood mononuclear cells (PBMC) of the susceptible patient by RV (Fig. 1). In the post-partum vaccination period (5–10 weeks following rubella vaccination), only humoral immunity was investigated with the same two RV-IgG CIAs as well asRV-IgM and RV-IgG avidity, that allowed us to determine whether a primary or a secondary immune response occurred following vaccination [2,9–12].

This study was approved by AFSAPPS (12/02/2011) and the ethics committee (CPP, 12/06/2011): ID RCB 2011-A01445-36 (NCT02934295).

3.2. Study population

During a two-year period (2012–2014), pregnant women tested in two hospitals located in the Paris area without proof of rubella immunity induced by infection/vaccination were offered inclusion in the study if their routine RV-IgG screening test was negative or equivocal. Their immune status as susceptible to rubella and offer of post-partum vaccination was based upon the routine immunoassays.

3.3. T-cell stimulation assay

PBMC were separated from whole blood by Ficoll gradient and added to 96-well culture plates at concentrations of 10^6 cells/well in RPMI (Roswell Park Memorial Institute medium) complemented with 5% fetal calf sera, 1% L-glutamine, 1% penicillin and streptomycin. All PBMC cultures were prepared with fresh cells (blood collected less than four hours before Ficoll purification). For each patient's sample, rubella virus antigen (RAg) (Rubella virus Grade IV #6123, Viral Antigens, Memphis, USA) was added at a final concentration of 50µg/ml to triplicate wells. Phytohemagglutinin (PHA) was added in other triplicate wells as a positive control, and RPMI was added in triplicate wells as a negative control (non-stimulated cells (NS)). Cell cultures were incubated at 37 °C under 5% CO_2. Preliminary studies were performed with multiple rubella antigen concentrations (range 5 to 200 µg/ml) and several preparation and concentrations of cells per well (whole blood, PBMC range 10^4 to 10^7) from positive donors (healthy adults not pregnant with positive RV-IgG, n = 12). Supernatants from PBMC stimulated with RAg, PHA, or NS, were collected from wells after 46 to 48 h incubation, and stored at −70 °C until testing for IFN-γ. IFN-γ was chosen because it is released predominantly by specific cytotoxic T-cells and T helper 1 (Th1) cells within short incubation periods, whereas non-stimulated T-cells do not synthesize IFN-γ. Secretion levels of IFN-γ in response to each stimulation were determined by ELISA following the manufacturer's protocol (QuantiFERON® – TB Gold Cellestis GmbH, Europe). For each blood sample, we obtained three RAg stimulated

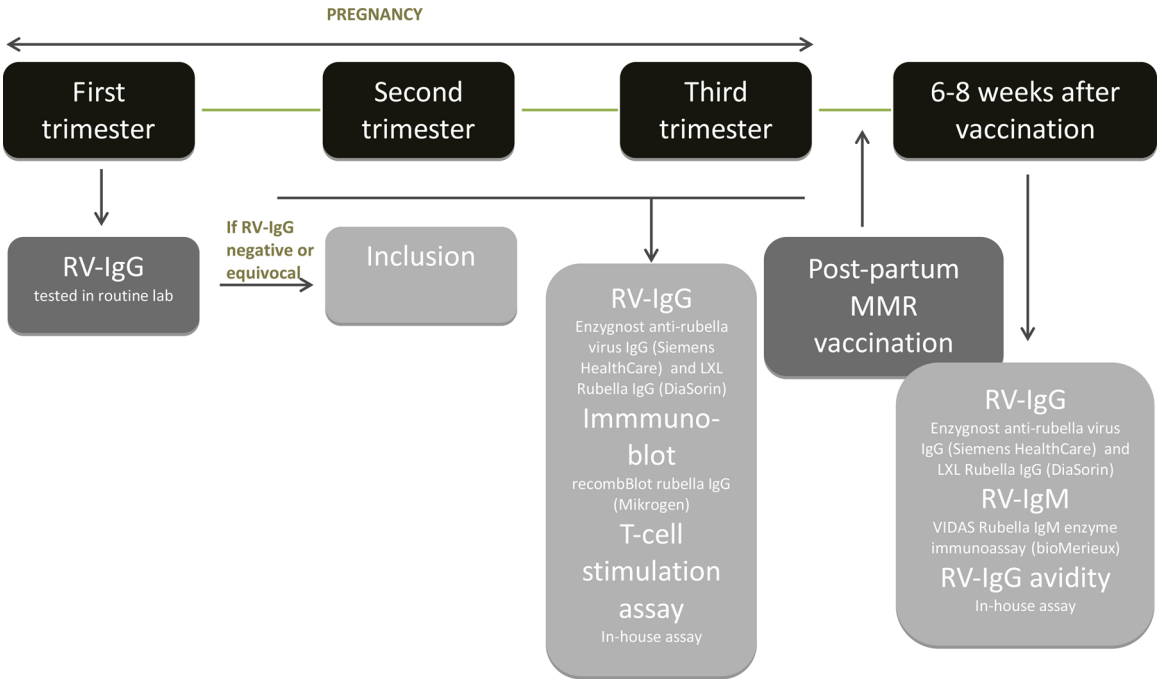

Fig. 1. Design of the study (RV-IgG: rubella specific IgG).

Table 1
Immune status of pregnant women before post-partum vaccination assessed in routine screening and confirmatory assays.

	Negative	Equivocal	Positive
RV Immune status during pregnancy before enrollment in the study	162 (87,1%)	24 (12,9%)	0
* Enzygnost (Siemens)	105/186 (56.4%)	20/186 (10.8%)	61/186 (32.8%)
* LXL (equivocal zone: 5–9.9 IU/mL) (DiaSorin)	150/186 (80.6%)	26/186 (14.0%)	10/186 (5.4%)
* LXL (equivocal zone: 9–11 IU/mL) (DiaSorin	175/186 (94.1%)	2/186 (1.1%)	9/186 (4.8%)
* Immunoblot (Mikrogen)	89/186 (47.8%)	NA	97/186 (52.2%)
* T-cell stimulation assay	54/141 (38.3%)	NA	87/141 (61.7%)

measures, three PHA stimulated measures and three NS measures. The mean concentration from each triplicate well was used for analysis. Median background levels from unstimulated control PBMC cultures were subtracted from the median rubella-induced response to calculate corrected secretion values. The assay was considered as valid if: INFγ (NS) < 8 IU/ml and IFNγ (PHA) > 0.5 IU/ml. T-cell stimulation was interpreted as positive for RAg if: IFNγ (RAg) – IFNγ (NS) > 0.35 IU/ml.

3.4. Immunoblot

A commercial immunoblot (recombBlot rubella IgG, Mikrogen GmbH, Germany) was recently shown to be a reliable reference assay for qualitative evaluations of CIAs [5,8]. Samples collected during pregnancy were tested with the immunoblot according to the manufacturer's instructions. This immunoblot allows detection of RV-IgG required for a reliable serological diagnosis, namely IgG anti-envelope (E1 and E2) and anti-capsid (C), and anti E1/E2 antigen complex. An immunoblot was scored positive when at least the anti-E1 band was observed. An immunoblot was scored negative if no bands were observed. All samples with positive immunoblots had an E1 band.

3.5. RV-IgG assays

All samples were tested for RV-IgG with two CIAs according to the manufacturer's instructions: Enzygnost anti-rubella virus IgG (Siemens HealthCare, Germany) (equivocal zone: 4–5.9 IU/mL), and LXL Rubella IgG (DiaSorin, Italy) (equivocal zone: 9–11 IU/mL or 5–9.9 IU/mL). Both assays report results in IU/mL.

3.6. RV-IgM assay

Samples collected in the post-partum vaccination period were tested for RV-IgM with VIDAS Rubella IgM enzyme immunoassay (bioMerieux, France) according to the manufacturer's specifications (equivocal zone: 0.8–1.2).

3.7. RV-IgG avidity

The RV-IgG avidity index was measured using our in-house urea wash method [13]. An avidity index below 45% is considered low, one between 45% and 75% is considered moderate, and one above 75% is considered high.

4. Results

4.1. Characteristics of the study population and history of rubella infection/vaccination

A total of 211 pregnant women with negative or equivocal RV-IgG were offered inclusion in the study and 186 accepted. History of rubella infection/vaccination was available for all women: 101 women reported neither history of vaccination nor rubella infection (54.3%), 9 reported a history of rubella infection in childhood (4.8%), and 76 (40.9%) reported a history of vaccination prior to current pregnancy. However, only 30 of 76 (39.5%) women could provide documented evidence of this immunization and all of those had only received one dose.

4.2. Immune status of women in routine pregnancy screening

Before inclusion in the study all women were screened for RV-IgG in their routine laboratory. Testing was preferentially performed with three CIAs: Architect Rubella-IgG (Abbott Diagnostics) (30.2%), Cobas 6000 Rubella-IgG (Roche Diagnostics) (14.3%) and LXL Rubella-IgG (DiaSorin) (12.3%). RV-IgG were negative in 162 cases (87.1%) and equivocal in 24 cases (12.9%).

At inclusion, all 186 women were retested for RV-IgG with Enzygnost and LXL assays. With Enzygnost assay, 105 women were RV-IgG negative (56.4%), 20 were equivocal (10.8%), and 61 were positive (32.8%). With LXL assay, using the 5–9.9 IU/ml cut-off, 150 women were RV-IgG negative (80.6%), 26 were equivocal (14.0%), and 10 were positive (5.4%). Using the 9–11 IU/ml cut-off, 175 women were RV-IgG negative (94.1%), 2 were equivocal (1.1%), and 9 were positive (4.8%) (Table 1).

Immunoblot results were available for all 186 sera and T-cell stimulation assay results were available for only 141 samples (because this assay is very difficult to standardize and its sensitivity is closely related to the delay between sampling and PBMC isolation) (Table 1). Eighty-nine samples (47.8%) were found negative with immunoblot, among which 44 (23.7%) were also negative with T-cell stimulation assay. Ninety-seven samples (52.2%) were found positive with immunoblot, among which 66 (35.5%) were also positive with T-cell stimulation assay. Overall, results of immunoblot and T-cell stimulation assay were concordant in 110 cases (78.0%). Interestingly, all samples with equivocal RV-IgG, irrespective of the assay used, were positive with immunoblot. All individuals that documented to have received one dose of rubella-containing vaccine were positivein the immunoblot assay.

Correlation between RV-IgG titres with the two CIAs performed in our laboratory and immunoblot results is presented in Fig. 2. All samples with RV-IgG below the equivocal cut-off of the assay, irrespective of the assay used, had negative immunoblot results.

4.3. Rubella-specific immune response to post-partum vaccination

As all 186 women, were considered susceptible with the first RV-IgG screening test performed before inclusion, all of them were offered and accepted vaccination. The median time between delivery and vaccination was 2.72 days (range 0–59 days). However, only 128/186 women attended to their post-natal consultation and were available for collecting the post-vaccination serum sample. The median time between vaccination and sample collection was 7.2 weeks (range 2.5–21.6); 7 women presented less than 5 weeks and 14 more than 10 weeks after vaccination.

Post-partum vaccination sera were tested for RV-IgG. Testing with the Enzygnost assay, 2 (1.6%) women were still negative and 126 (98.4%) were positive. Testing with the LXL assay: using the 5–9.9 IU/ml cut-off, 3 (2.3%) women were negative, 7 (5.5%) were equivocal, and 118 (92.2%) were positive; using the 9–11 IU/ml cut-off, 9 (7.0%) women were negative 2 (1.6%) were equivocal, and 117 (91.4%) were

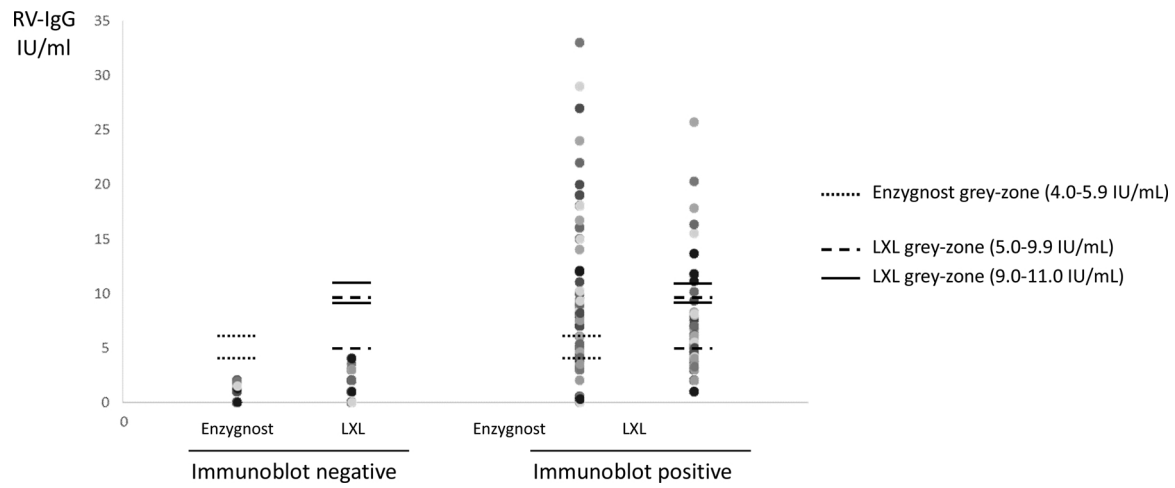

Fig. 2. Before vaccination: correlation between RV-IgG titers (Enzygnost (Siemens) and LXL (DiaSorin)) and Immunoblot result. Coloured bars indicate the respective grey-zones of CIAs (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

positive. Only one sample was negative with both assays. For the only sample negative for RV-IgG (collected only 3.4 weeks after vaccination), RV-IgM was very high indicating a primary immune response to vaccination.

Forty-four (54.7%) women had low avidity indicating a primary immune response to vaccination. Thirty-five (27.3%) women had moderate RV-IgG avidity, of which 16 had positive/equivocal RV-IgM indicating a primary immune response to vaccination. Nineteenwomen had negative RV-IgM and were classified as secondary immune response to vaccination, as RV-IgG avidity may not maturate to high avidity in vaccinated individuals [13]. Forty-eight women had high RV-IgG avidity indicating a secondary immune response to vaccination, and all had negative RV-IgM.

Overall, 61 (47.7%) women raised a primary immune response after vaccination (low RV-IgG avidity or moderate RV-IgG avidity and positive or equivocal RV-IgM), and 67 (52.3%) raised a secondary immune response after post-partum vaccination (high RV-IgG avidity or moderate RV-IgG avidity and negative RV-IgM) (Fig. 3).

4.4. Correlation between immune status before vaccination and rubella-specific immune response to post-partum vaccination

Concordant results between immunoblot and T-cell stimulation assay were compared to RV-IgM results, and to RV-IgG avidity results (Fig. 4). For the 24 samples with immunoblot/T-cell stimulation assay discordant results and for the 30 samples with only immunoblot result available before vaccination, the same correlation was performed relying on the immunoblot results only. All 65 (100%) pregnant women who had a positive immunoblot before immunization raised a secondary immune response to vaccination. Among the 63 pregnant women who had a negative immunoblot before immunization, 61 (96.8%) raised a primary immune response to vaccination (Table 2). There is therefore a clear correlation between the immunoblot result before vaccination and the humoral immune response after immunization (Fig. 4). Only 2 (3.2%) pregnant women with negative immunoblot before vaccination raised a secondary immune response to vaccination. One of these pregnant women came to the post-vaccination visit 18.1 weeks after vaccination, and for the second patient T-cell stimulation assay was positive. Among pregnant women who had a negative T-cell stimulation test before immunization, 27/36 (75%) raised a primary immune response to vaccination, and among those who had a positive T-cell stimulation test before immunization, 47/61 (77%) raised a secondary immune response to vaccination (Table 3).

5. Discussion

Our results highlight that 52.2% pregnant women, supposed to be susceptible, had positive anti-E1 RV-IgG, that all those who had a positive immunoblot before immunization raised a secondary immune response to vaccination, and that all women who raised a primary immune response after vaccination had negative anti-E1 RV-IgG and negative cell-mediated immunity.

One consequence of the rubella vaccination programs is a decline of circulation of RV, as well as the decline of the percentage of individuals

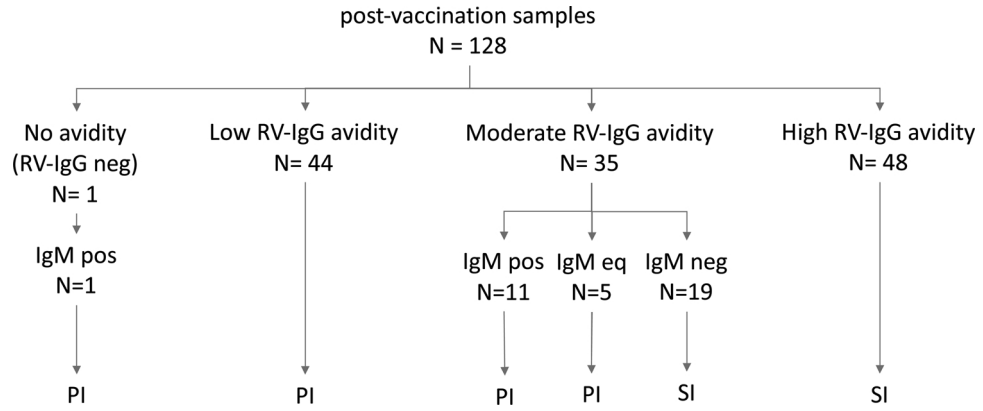

Fig. 3. Immune response to post-partum vaccination (pos = positive, eq = equivocal, neg = negative, low avidity < 45%, 45% < moderate avidity < 75%, high avidity > 75%, PI = primary immune response to vaccination, SI = secondary immune response to vaccination).

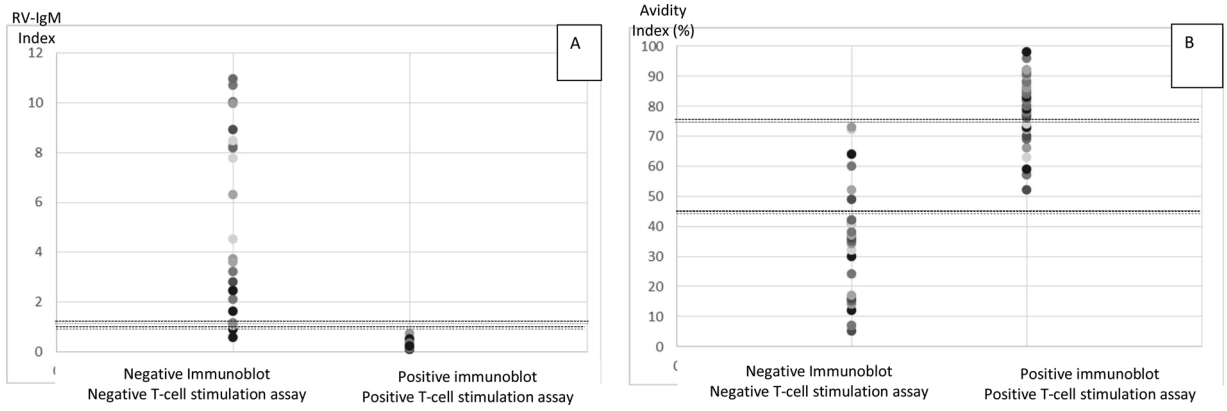

Fig. 4. Correlation between immune status before post-partum vaccination and the immune response to vaccination. Bars indicate the respective cut-offs of the assays.

A. Correlation of immunoblot/T-cell stimulation assay concordant results before post-partum vaccination and RV-IgM results in response to post-partum vaccination (Rubella IgM enzyme immunoassay, bioMerieux, France - equivocal zone: 0.8–1.2).

B. Correlation of immunoblot/T-cell stimulation assay concordant results before post-partum vaccination and RV-IgG avidity results in response to post-partum vaccination (in-house RV-IgG avidity index - low avidity < 45%, 45% < moderate avidity < 75%, high avidity > 75%).

that are naturally immune and the opportunities for post-vaccination natural boosters. Several studies conducted in countries that implemented vaccination decades ago showed that in the younger age groups, RV-IgG levels are lower, with a higher rate of “negative” or equivocal results, in vaccinated populations [14–21]. For example, in Finland, it was reported that all individuals vaccinated with 2 doses of MMR had RV-IgG ≥ 10IU/mL 6 months after second dose of vaccine, but 24% had levels below 10 IU/mL 20 years later (Enzygnost- Siemens assay) [22]. However, authors also report that avidity index didn’t change during the 20-year follow up and stayed high in 96% individuals [22]. Similarly, in a cohort of canadian pregnant women tested between 2009 and 2012, 10.1% of vaccinated individuals had levels between 5 and 10 IU/mL (in-house assay) [23,24]. Overall, a growing proportion of the populations is expected to have low levels of RV-IgG (even equivocal or negative), increasing significantly the difficulties for clinical interpretation of immune/non-immune status of these individuals [17]. This is expected to have an impact on public health programs which recommend follow up and vaccination t o women whose RV-IgG levels are equivocal or negative.

Similarly, to what was described in other studies [5,8], we report that 35.5% of the women considered susceptible at RV prenatal screening, were in fact RV seropositive according to immunoblot/T-cell stimulation concordant results, and up to 52.2% of them had positive anti-E1 RV-IgG. Such individuals have therefore strong evidence of previous exposure to RV, but the most important issue is: do these very low levels of RV-IgG (interpreted negative or equivocal with CIAs) protect against RV infection? This is why we investigated whether these “susceptible” women raised a primary or a secondary response when re-exposed to the RV (RA 27/3 strain). Our results indicate that 100% (65/65) women who had a positive immunoblot before immunization raised a secondary immune response to vaccination, and 100% women (57/57) who raised a primary response to vaccination had a negative immunoblot before immunization (Table 1). Moreover, in our study, none individual with positive immunoblot raised primary immune response to vaccination. Importantly our study provides evidence that immunoblot results are correlated to protective immunity and therefore could be used to confirm low level reactivity of CIAs. Indeed, cut-offs currently recommended for CIAs guarantee their specificity and ensure that all susceptible women are targeted for rubella vaccination [2,5], butour results show that considering equivocals as positives greatly improve sensitivity without having negative impact on specificity.

Several studies have shown that individuals with detectable RV-IgG below 15 IU/mL raise a secondary immune response following re-immunization and that viremia rarely occurs suggesting that these levels are sufficient to prevent viral infection [11,25–30]. Our study confirms that child-bearing age women with minimal detectable RV-IgG (having positive anti-E1 antibody detected by immunoblot) raise a secondary immune response following re-immunization. Few publications report cases of rubella reinfection in immune individuals but these were not attributed to low levels of RV-IgG [31–35]. Our main concern in this situation is the risk of transmission to the fetus and subsequent congenital rubella syndrome (CRS). However, all published CRS cases that occurred after vaccination were reported more than 20 years ago, and the mother had received the Cendehill vaccine [31,35–37]. Indeed, by 1979 all vaccines licensed in the USA and in Europe were replaced by RA27/3 which generally induces higher antibody titres and produces an immune response very close to natural infection compared the other vaccines [28,38,39]. RA27/3 vaccine is now the most widely used vaccine strain worldwide. To date, decreasing seroprevalence in highly vaccinated populations have not led to major outbreaks of rubella or an increased incidence of CRS which is a strong indication that low levels of RV-IgG may be sufficient for protection [40–44].

Concerning cell-mediated immunity, concordance between our T-cell stimulation assay and the immunoblot was not as good as expected (78%). Indeed, from a technical point of view, T-cell stimulation assay is much more difficult to standardize and we noticed that its sensitivity was closely related to the delay between sampling and PBMC isolation (the assay becoming invalid when this delay exceeded 4 h). However, in almost all individuals seronegative for RV-IgG, but with specific anti-E1 RV-IgG on the immunoblot, IFN-γ production was detected (indicating specific stimulation of T-cells). Based on the knowledge of the role taken by cell-mediated immunity in response to viral infection, and the

Table 2
Correlation between immunoblot result before post-partum vaccination and humoral immune response after vaccination.

	Negative Immunoblot before vaccination	Positive Immunoblot before vaccination	Total
Primary immune response to vaccination	61/63 (96.8%)	0/65 (0%)	61
Secondary immune response to vaccination	2/63 (3.2%)	65/65 (100%)	67
Total	63	65	**128**

Table 3
Correlation between T-cell stimulation assay result before post-partum vaccination and humoral immune response after post-partum vaccination.

	Negative T-cell stimulation result before vaccination	Positive T-cell stimulation result before vaccination	Total
Primary immune response to vaccination	27/36 (75.0%)	14/61 (23.0%)	41
Secondary immune response to vaccination	9/36 (25.0%)	47/61 (77.0%)	56
Total	36	61	97

absence of correlation between antibody and cellular immunity, the use of the IFN-γ secretion for the detection of cell-mediated immunity to RV in seronegative individuals provides valuable additional information regarding determination of the comprehensive immune status to RV.

Considering susceptible women who are in fact immune can lead to confused clinical management of pregnant women, unnecessarily (re)-vaccination, or have an impact on diagnosis. For example, a recent study in Texas (US) reported a significant incidence (6.8%) of rubella infection in pregnancy among 298 pregnant women (that had antenatal screening performed in different laboratories using unspecified assays), although rubella has been eliminated in the Americas since years [45]. This situation may lead to unnecessary anxiety for parents, termination of pregnancy and/or medico-legal complications. It also has an impact on seroprevalence determinations, which are particularly important in supporting the goal of eliminating rubella infection.

On one hand, our study along with previous ones shows that very low levels of RV-IgG may be sufficient for protection and that establishing new CIAs cut-offs could improve correlation with the true immune status of individuals. On the other hand, the high response rate to a single dose of rubella vaccine (≥95%) and the long-term persistence of protection in vaccinees do not support a routine requirement for a second dose of rubella vaccine, but based on the indications for a second dose of measles- and mumps-containing vaccines, a second dose of MMR is recommended in most countries [46]. However, as France has not be able to reach high coverage with two doses, women who received one dose could be considered as immune for rubella and not be screened anymore. Finaly, best would be that women were screened before pregnancy and immunized if necessary.

Previous communications of these information

- Oral communication at 18th annual meeting of European Society for Clinical Virology (ESCV), Lyon, 2013.
- As an invited speaker:
  - Standardization of Rubella Virus Immunoassays. **20thannual meeting of European Society for Clinical Virology**, Stresa (Italy), 2017
  - Investigating low or negative rubella IgG titers: performance of 8 commercial rubella IgG immunoassays and correlation with protection. **SoGAT meeting**, London, 2016.
  - Rubella Diagnosis and immune status. **ESCV Workshop "Serology in Clinical Virology"**, Trondheim (Norway), 2016.
  - Rubella: pitfalls in the diagnosis and the determination of the immune status. **20thannual meeting of European Society for Clinical Virology**, Edinburg (UK), 2015.
  - Report from Rubella IgG standardization working group. **WHO 13thGlobal Measles and Rubella Laboratory Network Meeting**, Geneva, 2015.
  - Lack of standardization of Rubella IgG assays. Is there any solution? **WHO 12thGlobal Measles and Rubella Laboratory Network Meeting**, Istanbul, 2014.

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Conflicts of interest

None.

Ethical approval

This study was approved by AFSAPPS (12/02/2011) and the ethics committee (CPP, 12/06/2011): ID RCB 2011-A01445-36 (NCT02934295).

Randomized controlled trial

Not applicable.

Author contributions

O. Picone, L. Grangeot-Keros, C. Vauloup-Fellous: design of the study

O. Picone, AG. Cordier S. Nedellec, A. Letourneau, M. Carbonel, JM. Ayoubi, A. Benachi : inclusion and follow-up of pregnant women

C. Vauloup-Fellous, E. Bouthry: developped the assays in the lab, coordination of lab work and wrote manuscript

E. Bouthry, Y. Bejaoui-Olhmann: performed all the tests on blood specimens

M. Brollo and E. Rouge: coordination of the study and collection of epidemiological information

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