

Immunoblot evaluation of rubella humoral immunity in Portuguese women with negative or low-positive rubella IgG

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ABSTRACT

Despite its low incidence in Europe, rubella's immune status is still routinely assessed using commercial immunoassays. However, some discrepancies have been observed between these tests and the Immunoblot.

The main objective of this study is to evaluate, by a commercial immunoblot, the immune status of a female population that had previously presented negative, low-positive, or equivocal rubella IgG with a commercial immunoassay. In total, 146 serum samples were reevaluated.

The results demonstrated that 81 % and 73 % of the samples, were positive by Immunoblot (Immunoblot interpretation criterion 1 and criterion 2, respectively).

Our results confirm previous studies, showing that Immunoblot can significantly reduce the false negatives for rubella in the female population. This application by avoiding unnecessary revaccinations represents a potential gain for health. However, it is pertinent to conduct an extended multidisciplinary discussion to analyse the pros and cons of this algorithm.

1. Introduction

Rubella is still considered a global public health problem despite decreasing incidence rates worldwide, following the implementation of vaccine programs. In Europe, where incidence is low, cases tend to happen in countries where vaccination rates are decreasing [1–3].

It has been established that vaccine-acquired antibody concentrations decrease over time to values lower than after natural infection [4]. One study observed that six months after taking two doses of Measles, Mumps, and Rubella (MMR) Vaccination, all individuals had IgG ≥ 10 IU/mL, but after 20 years, 24 % had values below 10 IU/mL [5]. In another study, the individuals who were seronegative or had low baseline titers, years after two doses of the rubella vaccine, had an anamnestic response to a third rubella vaccine dose, suggesting that there was an immune response to the first vaccination, which was followed by an antibody decrease over time in these individuals [6].

Commercial immunoassays currently available are calibrated with the WHO International Standard, resulting in IU/mL [7]. Values for

immunity are set above 10 IU/mL, but several publications have highlighted the lack of comparison of results between assays, which can lead to confusing clinical management for pregnant women who can be classified as immune or susceptible depending on the test used. This situation can also lead to unnecessary vaccinations of already immune women and the report of false seroconversions among people with low rubella virus IgG titers [8].

The immunoblot (IB) is considered a reference test for the serological evaluation of rubella, allowing the detection of antibodies specific to the rubella virus, namely anti-envelope antibodies (anti-E1 and anti-E2), anti-capsid (c), and the anti-E1/E2 antigen complex [7].

The main objective of this study is to evaluate the immune status of the rubella virus, using the IB as a reference, in a female population that had previously presented equivocal or negative results with two commercial immunoassays. Additionally we aim to associate the results obtained in the IB and the responses regarding vaccination status.

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2. Material and methods

2.1. Study population

All the adult females who went to the Germano de Sousa Laboratory Medicine Center (CMLGS) between April and May 2019 to determine their immune status for rubella were included if their results showed one of the following criteria: i) a negative IgG (< 9UI/mL), ii) an equivocal (9-10UI/mL), or iii) low positive (10-15UI/mL) with the Liaison[®] Rubella IgG immunoassay (DiaSorin, Georgia, US).

2.2. Clinical samples

One hundred and forty-six serum samples were tested. The blood was collected in tubes with a coagulation activator and separating gel (BD Vacutainer[®] SSTTM II Advance) and, after clot retraction, it was centrifuged at 3000 rotations per minute for 10 min to obtain the serum. Serum samples were stored at 4°C until analyzed (up to 48 h after collection).

2.3. Methodology used to determine IgG antibodies

All samples that were tested with the Liaison[®] Rubella IgG immunoassay and which gave negative or equivocal results (inclusion criteria) were then tested by the VIDAS[®]RUB IgG II immunoassay (BioMérieux, Lyon, France) and by an IB test, the recomBlot Rubella IgG (Mikrogen GmbH, Neuried, Germany).

1. Immunoassay tests:

The interpretations were made according to the leaflets of the different immunoassay tests:

- Liaison[®] Rubella IgG: positive >10UI/mL; negative <9UI/mL; equivocal zone 9-10UI/mL. In the present study, results between 10 and 15 IU/mL were considered “low positive”.
- VIDAS[®]RUB IgG II: positive >15UI/mL; negative <10UI/mL; equivocal zone 10-15UI/mL.

2. RecombBlot Rubella IgG :

This test allows the detection of antibodies against the structural proteins E1, E2 and c of rubella. The results were interpreted according to the following criterion (criterion 1): tests were considered positive when at least the anti-E1 band was visible, negative when none of the bands were observed (except the reaction control), and doubtful when the bands were not clearly discriminated [7].

To see the impact of different interpretations of the IB assay, another criterion (criterion 2) was used to compare the results of the Liaison[®] Rubella IgG immunoassay with the IB. This second criterion refers to the intensity of the E1 band, which must be equal to or greater than the intensity of the E2 band of the weakly positive control carried out in the same series [9].

Four samples with positive IgG (>15 IU/mL with the Liaison[®] Rubella IgG test) were also included as a positive control for the RecombBlot Rubella IgG test.

Vaccination status: Vaccination status was declared by the participants, but no evidence was provided.

2.4. Ethics consideration

The study was submitted and approved by the Ethics Committee of Nova Medical School | Faculty of Medical Sciences of the Universidade Nova de Lisboa (NMS-FCM). (N°16/2019/CEFCM).

3. Results

Our study included 146 female individuals, with an average age of 29, ranging from 16 to 57 years. Of the 146 samples, 143 (98 %) were

negative (<10IU/mL) by the screening test (Liaison[®] Rubella IgG), with the remaining 3 samples (2 %) showing positive results but with a low titer, between 10 and 15UI/mL. With the VIDAS[®] RUB IgG II test, of the 146 samples, 104 (71 %) were negative (<10IU/mL), 34 (23 %) had equivocal results (titer 10-15UI/mL) and the remaining eight samples (6 %) were positive (>15UI/mL).

With the IB and following the criteria assumed for this study, 118 (81 %) of the 146 samples were positive, 24 (16 %) were negative and four samples (3 %) were equivocal (difficulty reading the bands). When comparing the results obtained with Liaison[®] Rubella IgG and those from IB, 27 (19 %) samples had concordant results in both assays (24 (16 %) were negative and three (2 %) were positive) and the remaining 119 (82 %) were discordant. Of these, 119 were negative for the commercial immunoassay, 115 were positive for IB and four were equivocal (Table 1).

Comparing the immunoblot with the VIDAS[®] RUB IgG II test, 32 (22 %) samples had a concordant result in both assays (24 negative and 8 positive), while the remaining 114 (78 %) did not. Of these discordant samples, 34 (23 %) samples positive for IB had been equivocal for the commercial immunoassay, four (3 %) equivocal samples for IB had been negative in the commercial immunoassay and the remaining 76 (52 %) were positive for IB but negative for the VIDAS RUB IgG II test (Fig. 1).

All the 24 negative results by the IB were also negative with both commercial immunoassays. The results with Liaison[®] Rubella IgG ranged between <0.20 and 1.32 IU/mL, but with VIDAS[®] RUB IgG II the titer was always 0IU/mL.

According to criterion 2 for the interpretation of IB, 106 (73 %) of the samples were positive, 37 (25 %) negative and 3 (2 %) equivocal (Fig. 2).

The four samples with positive results in the Liaison[®] Rubella IgG test (results between 16 and 41.50 IU/mL) and VIDAS[®] RUB IgG II test (results between 16 and 45 IU/mL) were positive by the IB, according to both interpretation criteria.

Table 2 shows the IB results according to vaccination status. Overall, 60 (41 %) women said they were vaccinated, 20 (14 %) women said they were not vaccinated, and the remaining 66 (45 %) did not know or did not respond. Of those who reported being vaccinated, 59 (59/60) had a positive IB (only one woman who reported having been vaccinated had a negative IB result).

Among the 20 women who said they were not vaccinated, 11 cases had positive IB results.

The results of these 11 cases were all negative for Liaison[®] Rubella IgG test, although with titers between 0.68 and 9.74IU/mL. With VIDAS[®] RUB IgG II test, 8 of them were negative, although with titers between 2 and 9UI/mL, 1 was positive with a titer of 16UI/mL and the other 2 were equivocal with titers of 10 and 13UI/mL. Of the 8 cases that say they were not vaccinated and whose results were negative by IB, all presented titers of <0.20 IU/mL and 0 for Liaison[®] Rubella IgG test and VIDAS[®] RUB IgG II test, respectively. In the only case in which the participant said she had not been vaccinated and the BI was doubtful, the titers were <0.20IU/mL and 0 for Liaison[®] Rubella IgG test and VIDAS[®] RUB IgG II test, respectively.

There were 48 (33 %) cases in which participants did not know whether they were vaccinated or did not respond, but had a positive IB. Of these, 47 were negative with Liaison[®] Rubella IgG test, with titers between 0.27 and 9.84UI/mL, and one was positive with a titer of 12.2UI/mL. With VIDAS[®] RUB IgG II test, 34 were negative with titers

Table 1

Overall results of the different tests: IB (criterion 1), Liaison and VIDAS.

IB results	Liaison results		VIDAS results		
	Low-positive	Negative	Positive	Negative	Equivocal
Positive	3	115	8	76	34
Negative	0	24	0	24	0
Equivocal	0	4	0	4	0
Total	3	143	8	104	34

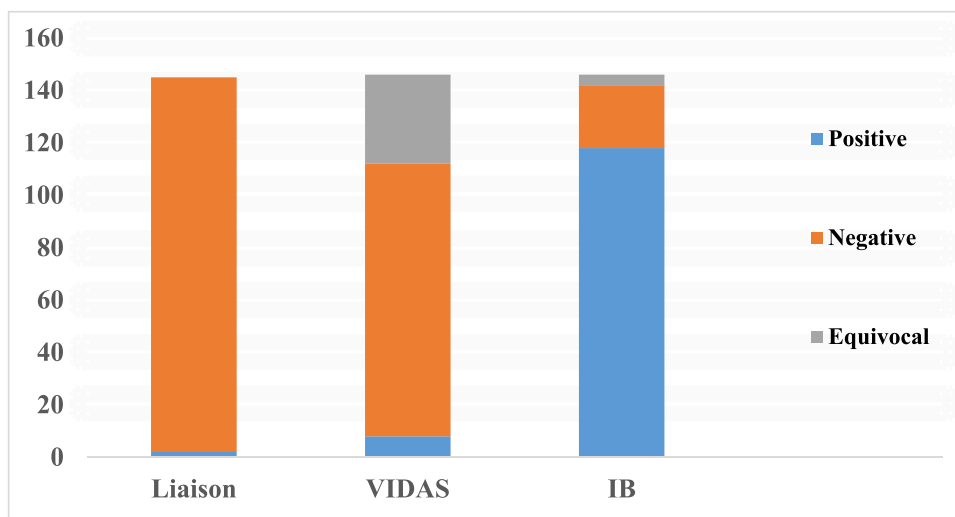


Fig. 1. Overall results of the different tests: IB (criterion 1), Liaison and VIDAS.

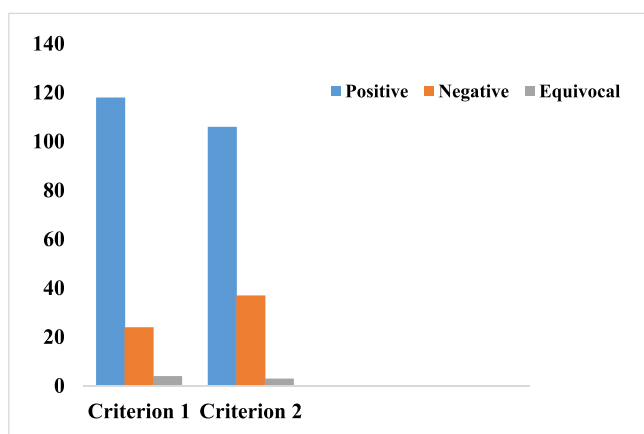


Fig. 2. Comparison of results obtained with IB, according to criteria 1 and 2.

Table 2

IB results according to vaccination status.

IB results	Vaccinated	Unvaccinated	Do not know/Do not answer
Positive	59	11	48
Negative	1	8	15
Equivocal	0	1	3

between 2 and 9UI/mL, 10 were equivocal with titers between 10 and 14UI/mL and 4 were positive with titers between 15 and 23UI/mL. In the 15 cases who did not know if they had been vaccinated and whose IB result was negative, the titers varied between <0.20 and 1.32IU/mL, in Liaison® Rubella IgG, and were 0IU/mL, in VIDAS® RUB IgG II test. In the other three who did not know their vaccination status and whose IB was incorrect, the titers varied between <0.20 and 3.94IU/mL in Liaison® Rubella IgG test and between 0 and 2IU/mL in VIDAS® RUB IgG II test.

4. Discussion

Serological tests for rubella are part of the routine follow-up of pregnant women in most countries. However, despite false negative IgG results being often found [10], this issue is usually not addressed in the recommendations of public health authorities [11].

IB has been described as more reliable for identifying immune status

due to its greater sensitivity than commercial immunoassays [8,9]. The commercial IB used in our study was previously compared with a neutralization technique, and most of the samples (82.9 %) gave concordant results, which led the authors to state that the IB technique should be considered a reference method [7]. In another study, women who tested IgG negative by commercial immunoassay had positive anti-E1 RV-IgG and raised a secondary immune response to vaccination. The same study reports that 96.8 % of the women who had a negative immunoblot before immunization raised a primary immune response to vaccination, thus reinforcing the concept that IB is a good marker of past infection [10]. Therefore, based on these studies, the IB was chosen as the reference method for our research.

The interpretation of the IB is not consensual, with at least two different interpretation criteria, as previously described in “Material and methods” [7,9]. These criteria were used in our study, which resulted in 81 % and 73 % positive results with the immunoblot, depending on the criteria followed. Despite this difference, it is unequivocally demonstrated that most sera classified as negative by immunoenzymatic methods have antibodies recognized by the immunoblot, that may provide the necessary protection [10].

Both criteria also agree that the equivocal zone of commercial immunoassays should be removed, and these results should be considered positive. For example, if equivocal results with the commercial VIDAS® RUB IgG II immunoassay (between 10 and 15UL/mL) had been considered positive, our study’s percentage of positives would increase from 5 % to 29 %. Although the rate of false negatives with the immunoassay continued to be significant, with this change there would be greater agreement between the results obtained. Additionally, the establishment of new cut-offs for commercial immunoassays may also contribute to improving the correlation between assays. In the present study, despite the low sample size, it was possible to verify that when the BI was negative, the Liaison® Rubella IgG varied between <0.20 and 1.32 IU/mL, and with VIDAS® RUB IgG II the titer was always 0 IU/mL, both well below the established cut-offs.

Regarding vaccination status, of the 60 women who said they were vaccinated, 59 had a positive BI. Only one woman who claimed to be vaccinated had a negative IB result, and a negative immunoassays result (both with a total absence of antibodies detected). It was impossible to verify whether this negative result was due to a non-response to vaccination, which is rare but possible [12].

Of the 20 women who said they were not vaccinated, 11 had positive IB. Although it cannot be determined with certainty why these women had immunity, it must have been for one of the following two reasons: i) contact with the wild virus, since over twenty years ago the virus was

still endemic (less likely hypothesis), or ii) they were vaccinated, but do not have records of this vaccination. As expected, of the 8 cases who stated that they had not been vaccinated and whose results were negative by the IB, all presented negative results in both commercial immunoassays.

In one comparative study, the specificity of the commercial immunoassays was described as 100 %, but the sensitivity in samples with low-level hemagglutination inhibition assay titers was 82.4 % and 90.8 % for Liaison[®] Rubella IgG and VIDAS RUB IgG II, respectively, if considering the 10UI/mL cut-off [13].

A limitation of our study is that only these two commercial tests were evaluated, meaning these results cannot be extrapolated to all other tests. However, in one study [9], 14 different rubella IgG immunoassays were compared using 150 clinical samples and the IB as the reference, and a high number of false-negative IgG immunoassays was also observed. Furthermore, in a 2013 study, using hemagglutination inhibition assay and IB as reference tests in 100 samples, the estimated sensitivity and specificity of five commercial immunoassays ranged from 70.2 to 91.2 % and 65.9 to 100 %, respectively [14]. Therefore, we can assume that other commercial tests, not evaluated in our study, would probably point in the same direction, that is, most sera with negative results would be positive with IB.

The main conclusion of this study is that most of the negative results obtained with commercial immunoassays are false negative results, which is in line with previous studies and confirming that this situation has not changed [8–10]. These false negatives convey the mistaken idea that these women are not protected against the rubella virus, promoting the inappropriate recommendation that they should be vaccinated [10].

Our main recommendation to reduce these false negatives is to eliminate equivocal areas of immunoassays so that results obtained in this area are considered positive. Establishing new (lower) thresholds for these tests would also help to improve this situation.

Another recommendation is to create an algorithm that states when IB should be performed, in IgG-negative cases, which would resolve most situations. Algorithms for carrying out complementary tests are already routinely used in many laboratories for prenatal diagnosis, such as IgG avidity in the case of positive IgM for CMV [13]. This would avoid unnecessary revaccinations and restrict the impact of false negative results on seroprevalence determinations, which are crucial for rubella elimination campaigns.

CRedit authorship contribution statement

Maria Chasqueira: Writing – review & editing, Supervision, Data curation. **Sara Costa:** Investigation, Formal analysis, Data curation. **Margarida Paixão:** Writing – review & editing, Formal analysis. **Margarida Albuquerque:** Resources, Conceptualization. **Maria José Sousa Rego:** Resources, Conceptualization. **Paulo Paixão:** Writing – review & editing, Writing – original draft, Methodology, Conceptualization.

Declaration of competing interest

Nothing to declare.

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Data availability

The data supporting this study's findings are available on request from the corresponding author.

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