

Manual for the Laboratory-based Surveillance of Measles, Rubella, and Congenital Rubella Syndrome

Chapter 9. Laboratory testing for determination of population immune status

Overview

Other than testing that is performed for case confirmation, the laboratories in the GMRLN are often responsible for providing laboratory support for serological surveillance conducted on a selected population or community. These seroprevalence studies are performed to assess the impact of vaccination programmes and aid in the identification of susceptible groups.

Seroprevalence studies involve aspects of study planning and design as well as the serologic testing and analysis of results of specimens collected from a defined population over a specified period of time. The publication, *Guidance on conducting serosurveys in support of measles and rubella elimination in the WHO European Region*, provides additional information including sampling design [1].

While EIA testing may be performed to confirm the presence of measles- or rubella-specific IgG antibody to assess the immunity of individuals (e.g., health-care personnel), detection of IgG by EIA is also the most common method utilized for the estimation of population immunity to measles and rubella. Population-based seroprevalence studies can help characterize the immune profile of target populations, however, there are serious limitations and challenges. Careful study design is required to generate quality data that are useful for identification of susceptible populations and at-risk groups. Adequate resources and support are needed to ensure results are available in a timely fashion.

Depending on the scope of the study and the resources available, residual serum specimens submitted for diagnostic testing (convenience or opportunistic sample) are often used, or serum specimens may be collected according to the sampling strategy. While other sources of antibody have been used in seroprevalence studies such as oral fluid, breast milk, or whole blood, these fluids have a reduced concentration of antibody compared to serum and the sample volume used in an assay to measure IgG or a test that measures neutralizing antibody is difficult to adjust to accommodate that variation [2].

This chapter includes a discussion of the relationship between the measurement of virus-specific IgG and the determination of measles or rubella immunity. Guidelines are provided for the evaluation and interpretation of equivocal results in the calculation of seroprevalence. The last section provides an overview of the critical elements to consider in the planning, preparation, and coordination of seroprevalence studies.

Note: In this manual, commercial measles and rubella IgG assays are referred to as EIAs for simplicity but may include assays with alternative detection systems.

In this chapter:

- 9.1 Detection of virus-specific IgG and protective immunity
- 9.2 Determination of measles immunity
- 9.3 Determination of rubella immunity
- 9.4 Guidelines for re-testing and interpretation of discordant and equivocal results
 - 9.4.1 Re-testing specimens and selecting outcomes to use in prevalence calculations
 - 9.4.2 Handling discordant results
 - 9.4.3 Repeatedly equivocal results
- 9.5 Planning and coordination for seroprevalence studies
- Bibliography to chapter 9

9.1 Detection of virus-specific IgG and protective immunity

The cut-offs for commercial measles and rubella IgG EIAs were established for maximizing specificity as the assays were designed to evaluate the immune response for individual patient management and not for population antibody prevalence calculations [3]. A positive result in a well-validated measles or rubella IgG EIA demonstrates a serologic response to antigenic stimulation elicited by either vaccination or natural infection. Although susceptibility to disease is inferred by a negative or equivocal result for virus-specific IgG, not all individuals with such a result may actually lack immunity to measles or rubella [4,5]. It is understood that immunity is a complex process and that IgG antibody detection provides only a marker or surrogate for immunity. A useful review of the principles of protection elicited by vaccination and

interpretation of immune markers is provided in the 2013 WHO publication, *Correlates of vaccine-induced protection: methods and implications* [6].

In countries and regions with high vaccination coverage that has been sustained over several decades, there are diminishing numbers of individuals with naturally-acquired immunity to measles or rubella. Lower levels of antibody induced by vaccination compared to those from disease are reflected in results obtained from population-based serologic studies [3, 7-8]. Consequently, negative or equivocal IgG results can be expected with commercial IgG EIAs when assessing immunity in populations with strong immunisation programmes. The selection of an EIA for the evaluation of immune status in vaccinated populations requires careful consideration of the assay specifications and validation methodology and may require additional testing of specimens with results in the equivocal range.

When testing a panel of serum specimens for measles- or rubella- specific IgG using different commercial EIAs, the results that are obtained can be discordant. This has been a particularly challenging issue for rubella IgG assays [10]. The variation in results from different assays may be due to one or more factors including the antigen preparation, assay format, assay platform, and the detection system. Commercial IgG kits that offer a quantitative result for IgG by transformation of optical density values obtained in the EIA to a titre or concentration in international units per millilitre (IU/ml) may be designed to allow an evaluation of a rise in titre (e.g., Enzygnost® Anti-Measles Virus/IgG) or are intended to correlate with a protective level of antibodies (e.g., Enzygnost® Anti-Rubella Virus/IgG). Regardless of calibration to a reference standard by the manufacturers of rubella IgG EIAs, the assays are not standardized against each other [10-12].

Analyses of serum specimens with equivocal results for either measles or rubella IgG by respective virus-specific kits have demonstrated that equivocal samples by EIA are usually above the cut-off for protection when tested in virus neutralization assays [11,13,14]. If equivocal results can be included as positive results, the sensitivity is greatly increased as well as the concordance observed between results obtained from different assays. The development, validation and characteristics of widely used commercial IgG EIAs and the specific

recommendations for their use are discussed separately for measles and rubella in the sections that follow.

9.2 Determination of measles immunity

The measles plaque reduction neutralization (PRN) assay detects functional neutralizing antibodies which offer the best correlate to protection *in vivo* [15]. Prior to development of the PRN assay, the hemagglutination inhibition (HAI) assay had been in widespread use, but has been replaced by the PRN assay, which has superior sensitivity and consistency than the HAI assay. The PRN assay is regarded as the gold standard for evaluation of the humoral immunity to measles.

The PRN test requires enumeration of plaques formed by the cytopathic effect induced by measles virus growth in Vero cells. Assays are generally performed in 12- or 24-well plates using serial dilutions of test serum alongside reference or standard serum samples. Results can be quantified and compared to the protective level of neutralizing antibody in mIU/ml. The serum titre used to determine measles immunity is based on the evaluation of PRN titres that appeared to provide protection from clinical disease during a measles outbreak [16]. In that study, an outbreak of measles occurred at a Boston college that included students who had participated in a blood drive prior to exposure. The PRN titre that corresponded to the protective titre was ≥ 120 mIU/ml when standardized against the WHO measles antibody international standard (currently the WHO 3rd international standard; NIBSC 97/648).

The PRN titre is calculated from the dilution that reduces the number of plaques by 50%, transformed into mIU/ml using a conversion based on the concentration of the measles international standard and the end point titre of the standard in the assay run. Standardization and use of appropriate controls to ensure test validity of PRN assays is critical. PRN assays are difficult to standardize, are technically demanding, and take up to seven days to complete. In practice, there is significant inherent variation in the assay, which is addressed by testing the serum in triplicate [17]. Therefore, the PRN assay is impractical for large-scale surveys and is used primarily for vaccine immunogenicity trials and for studies leading to vaccine licensure and can be used to validate IgG EIA data.

In many countries, there are several commercially available measles IgG EIAs. The commercial EIAs vary according to the antigen source (e.g., purified viral lysate or recombinant viral proteins) and the test format. Many assays utilize a recombinant measles nucleoprotein for the antigen as antibodies directed to this protein are the most abundant following infection or vaccination [18]. While absence of an immune response to the nucleoprotein suggests susceptibility to infection, it is the neutralizing antibodies that are directed against the measles hemagglutinin (and to a lesser extent, the fusion protein) that confer protective immunity [19]. The performance of different kits is variable, particularly for serum with antibody measured in the low positive range. However, positive results using well-validated EIAs show a good correlation with results obtained by the measles PRN assay [7,8,12]. The EIAs that are described as semi-quantitative include a formula for converting optical density values into titre units, but the titres are intended to evaluate whether a significant rise in titre has occurred and are not equivalent to a PRN titre.

Suboptimal sensitivity for detection of measles IgG by EIA is a particular concern in cohorts with vaccine-induced immunity. Studies have demonstrated that serum specimens that fall into the equivocal range for IgG using several of the commercial EIA kits often have PRN titres ≥ 120 mIU/ml [7,8,12]. One approach is to screen the serum specimens by EIA and those specimens with results in the equivocal range (and a percentage of negatives) can be evaluated by PRN [7,8].

There has been over 10 years of experience with an in-house multiplex immunoassay (MIA) based on Luminex Technology [8,20]. It is basically a bead-based ELISA. The method is easy to multiplex in one reaction and requires less serum than required for testing in an EIA. Beads can be engineered in the laboratory and the antigen is linked to color-coded beads. The assays have correlated well with other methods such as PRN and has both anti-F and anti-H IgG functionality.

9.3 Determination of rubella immunity

There has not been an opportunity to evaluate the level of neutralizing antibodies that are protective against rubella in a similar manner as was done for measles. Instead, expert

committees established a minimum level of antibodies that are considered protective against rubella disease based on studies of persons exposed to wild rubella virus [5]. At the time of those studies, the HAI assay was the gold standard technique for detection of anti-rubella antibodies. However, the HAI could be affected by nonspecific inhibitors of agglutination, producing false-positive results. Serial dilutions $\geq 1:8$ were not affected by this and thus an HAI titre of 8 (1:8) was set as the threshold titre to define immunity to rubella. The corresponding concentration in international units was 15 IU/ml [5].

Improvements in methodology led to the replacement of the HAI by other methods including passive latex agglutination tests, immunofluorescent assays, radial haemolysis tests and virus neutralization. However, most laboratories have adopted the EIA for detection of rubella IgG antibodies. Most of the rubella IgG EIAs have been calibrated against an international rubella standard available at the time of assay development and provide quantitative results in IU/ml. Many of the first generation of rubella IgG EIAs have been replaced by automated systems and some of these utilize different detection systems including chemiluminescence [11].

A reduction of the cut-off or threshold for protection from 15 to 10 IU/ml has been accepted by most programmatic users of rubella assays [11]. However, the absence of detectable rubella-specific IgG does not necessarily correspond to susceptibility, nor does the presence of antibody measured at >15 IU/ml guarantee protection, as some rubella reinfections have occurred despite demonstration of titres >15 IU/ml [5]. Rubella IgG EIAs are calibrated using either of the two contemporary rubella standards, RUB-1-94 or RUBI-1-94.

The origin and description of the rubella standards is complex and beyond the scope of this manual and is available elsewhere [10,14]. However, the international standards for rubella were not developed according to metrological principles. This compromises the suitability of the standard for use as an analyte for calibration of rubella IgG EIAs. Investigators have recognized the need for standardization among rubella IgG assays and have raised questions regarding the accuracy and appropriateness of the 10 IU/ml cut-off in assessing immunity to rubella. Many have also voiced concerns regarding the validity of quantitative reporting of rubella IgG results [10-14].

Similar to tests that measure measles neutralizing antibody, the assays for measuring rubella neutralizing antibody titre have not been suitable for use in population studies. However, a rubella virus-specific immunocolorimetric neutralization assay, adapted to a high-throughput system for measuring neutralizing antibody titres with high sensitivity and good reproducibility has been described [21]. The neutralization titres (Nt) provided by this assay were shown to have good concordance with results of serum specimens tested by a commercial immunoblot (IB) test [11]. In a study comparing the sensitivity and specificity of eight commercial rubella IgG assays, serum specimens with Nt/IB concordant results were used for the evaluation. The results supported evidence in previous reports that showed no loss in specificity when equivocal results obtained in the rubella IgG EIAs are interpreted as positives. The protocol for the rubella neutralization assay (developed at CDC) is provided in Annex 9.1.

9.4 Guidelines for re-testing and interpretation of discordant and equivocal results

The following three sub-sections were included in a draft (30 August 2015) of a seroprevalence guidance document (a WHO-sponsored publication).

9.4.1 Re-testing specimens and selecting outcomes to use in prevalence calculations

A randomly-selected stratified subset of specimens should be re-tested immediately for purposes of quality assurance. The study protocol should specify whether the second test should use the same EIA kit as the first, or a different kit. In addition, the study protocol may direct that the second testing should be performed in a different laboratory. The specimens selected for re-testing should include:

- All equivocal results from the first run
- A subset of negative results from the first run
- A subset of positive results from the first run

After the second test, a 3x3 concordance table (sometimes called a *confusion matrix*) should be constructed to assess the concordance of the results. Table 9.1 shows an example of this type of

table. Note that in this example, most specimens that were negative in the first run were also negative in the second run. Although some positives had an equivocal result in the second run, most specimens that were positive in the first run were positive in the second test. There were no positive specimens that re-tested as negative, or *vice versa*. These results are highly concordant and do not give any indication that a quality control problem existed in the first run.

Table 9.1. High concordance between first and second test results

	First run negative	First run equivocal	First run positive
Second run negative	180		
Second run equivocal	20	2	30
Second run positive		8	170

In contrast, the data in Table 9.2 show discordant results. There were 18 specimens with results that changed categories, from negative to positive (n=10) or positive to negative (n=8). This casts doubt on the quality of the first run, and indicates that the entire set of specimens from the first run (not just the subset) should be re-analyzed.

Table 9.2. Low concordance between first and second test results

	First run negative	First run equivocal	First run positive
Second run negative	101		84
Second run equivocal		2	
Second run positive	108	8	126

For seroprevalence calculations, each specimen must ultimately be assigned an outcome value: *positive*, *negative*, or *equivocal*. There are two issues to be determined regarding the outcome values:

1. What value should be assigned to specimens with discordant results?
2. What value should be assigned to specimens with repeatedly equivocal results?

9.4.2 Handling discordant results

As described above, some specimens should be tested at least twice to confirm that there was not an important quality problem in the first run. If the valid run indicators show a problem, or the concordance analysis between the first and second runs shows a problem, then all the specimens in the first run will need to be re-analyzed, and that process should be repeated until there is no longer a concern about quality.

Even when most results are concordant upon re-testing, a small number of specimens may have discordant results. For different runs, different kits, or different laboratories, the study protocol should stipulate a hierarchy of outcomes that makes it clear which result to use for each specimen in the prevalence calculation. An example of the decision tree, or hierarchy, may be:

- Use the negative and positive outcomes from the first run, first kit, first laboratory, if the re-test validation for the positive and negative results in the first run meets expected quality.
- If there are specimens with equivocal results in the first run and a number of those same specimens yield either negative or positive results in a subsequent, validated run, then substitute the earlier equivocal result may be replaced. Record and later summarize the number of specimens whose equivocal results were replaced.

In this hierarchy, earlier positive and negative results are not replaced, but initial equivocal results can be superseded if positive or negative results are obtained by subsequent re-testing. An important principle is that the policy for designation of the outcome value for each specimen should be stated clearly, adhered to consistently, and documented. It is important to document the number of values that were used from the first run and the number of values that were overturned by testing performed with alternative kits or tested in another laboratory.

9.4.3 Repeatedly equivocal results

If the results for a specimen remain equivocal after subsequent testing, then the protocol will need to provide clear guidance about how to categorize the outcome in prevalence calculations.

Three options may be adopted for treatment of equivocal results and the calculation of prevalence.

(1) Include equivocal specimens as seropositive. For some commercial EIAs, it will be biologically probable that specimens with equivocal results are in fact seropositive because the cutoff for a positive result is set at a level of IgG that is higher than the minimum level required for protection. The cut-offs for the kit may be evaluated by testing well-characterized serum panels by an assay that measures neutralizing antibody. For example, results by EIA and plaque neutralization suggested that equivocal results/titres obtained by EIA could be regarded as positive in a study that measured measles susceptibility using the same assay across several countries [12]. The study protocol should indicate that these specimens will be counted as positive. The number of specimens that repeatedly test equivocal should be documented and then reclassified as positive before proceeding with the prevalence calculation.

(2) Include equivocal specimens as seronegative. Alternatively, in some situations the conservative choice will be to consider equivocal results as negative and thereby err on the side of slightly underestimating the population prevalence of immunity. If the study organizers prefer this approach, then document the preference in the study protocol and be sure to summarize the number of specimens that test repeatedly as equivocal. The specimens are then reclassified as negatives and proceed with the prevalence calculation.

(3) Retain a separate category for equivocal results. A third option is to count equivocal results separately and maintain the proportion of equivocal results in the population in a distinct category. This approach allows the reader to conduct a sensitivity analysis by combining the equivocal results with either the positive or negatives. Calculations performed by adding equivocal results to either positives or negatives allows the reader to evaluate the extent to which the equivocal results affect prevalence estimates.

9.5 Planning and coordination for seroprevalence studies

Population serologic studies together with mathematical modelling techniques have been widely used to investigate transmission patterns of infections and to inform and target vaccination activities. In addition, data from seroprevalence studies is an importance component in the

verification process for disease elimination. These activities rely on laboratory support and appropriate planning and coordination are vital to ensure that usable information is generated with a judicious use of time and resources. Therefore, the laboratory should be involved and consulted during the early stages of planning.

The source of the serum specimens for the serosurvey may consist of convenience samples available from previous studies or available from hospitals. The volume of serum must be adequate for repeat testing. A clear understanding of how the data generated from the study will be used is necessary to help guide the protocols that the laboratory will follow. The items to consider include the following:

1. Clearly identify the public health concern or question, tailored to the specific country context, that the study is expected to address
2. Provide the reasons for the laboratory assay selected, details for IQC, EQA
3. The coordination required between the field team and laboratory to oversee specimen transport logistics, specimen storage, responsibility for specimen labelling and recording of data
4. Appropriate statistical analysis and database management including data entry and cleaning

It is recommended that guidelines for conducting population seroprevalence studies are carefully followed when planning a study. One such resource is *Guidance on conducting serosurveys in support of measles and rubella elimination in the WHO European Region* [2].

Bibliography to Chapter 9

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