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COMMENTARY

Where to Now for Standardization of Anti-Rubella Virus IgG Testing

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The lack of standardization of rubella IgG testing continues to be a problem 20 years since the standard was introduced. The situation is complex and poorly understood. As demonstrated by an article in this issue (E. Bouthry, M. Furione, D. Huzly, A. Ogee-Nwankwo, L. Hao, A. Adebayo, J. Icenogle, A. Sarasini, M. Grazia Revello, L. Grangeot-Keros, and C. Vauloup-Fellous, J Clin Microbiol 54:1720–1725, 2016, http://dx.doi.org/10.1128/JCM.00383-16), the problem remains. The situation is far from being resolved, but at least the process for change has started.

The lack of standardization of tests that report anti-rubella IgG results in international units per milliliter has long been recognized (1). Only recently has a concerted effort been undertaken to understand, document, and address the issue. Recently, a comprehensive review of standardization of rubella IgG tests was published (2) in an attempt to better understand the reasons for this situation, to highlight the consequences of poor standardization, and to suggest future activities that may alleviate the problem. Indeed, the lack of standardization is not confined to rubella IgG tests but is also experienced in anti-hepatitis B virus surface (anti-HBs) antigen and other serological test kits calibrated against an international standard.

Testing for anti-rubella IgG is mainly confined to women of childbearing age. A primary rubella infection during the first trimester of pregnancy has a high likelihood of causing congenital infection resulting in severe morbidity, including ophthalmic, auditory, cardiac and craniofacial complications, or neonatal death (3). Preferably testing should occur prior to pregnancy, but usually it is performed at the first antenatal visit. A negative or low-positive result may indicate the need for vaccination, whereas a positive result indicates that the individual has detectable antibodies and therefore is protected against infection (2).

Although rubella IgG tests are calibrated using the World Health Organization (WHO) 1st International Standard for Anti-Rubella Immunoglobulin (RUBI-1-94) and report results in international units per milliliter, there is a lack of standardization of the results reported by different test kits (2, 10). The consequence of this poor standardization is that a patient's results are dependent upon what test kit is used. A person may obtain a negative result from one test kit and a positive result from another (4, 5, 10). A false-positive result may provide an incorrect sense of protection, whereas a false-negative result may cause an unnecessary vaccination. In extreme situations, a woman obtaining a false-negative result may choose to terminate a pregnancy if she has contact with rubella while pregnant.

So why has this situation occurred? The answer is complex and based on historical issues that have collectively contributed to the problem. Regulations have built up barriers to its resolution. Several factors have further contributed to the situation: the use of a quantitative result to determine immunity, immunization programs having changed the characteristics of the immune response from being wild type to vaccine induced, and poor adherence to metrological principles when initially designing the international standard.

Although there are a number of WHO international standards for antibodies to infectious diseases—for example, anti-toxoplasma IgG, anti-parvovirus B19 IgG, and syphilitic plasma IgG and IgM—only two infectious disease antibodies are routinely reported in international units per milliliter: anti-rubella IgG and anti-HBs antigen. Both analytes have immune "cutoffs" based on these quantitative results; generally 10 IU/ml for anti-rubella IgG and 10 mIU/ml for anti-HBs antigen. Therefore, in the case of some anti-rubella IgG tests, a result of 9.9 IU/ml is deemed negative and a result of 10.1 IU/ml is deemed positive (2). Other test kit manufacturers provide a "gray zone" or equivocal range of about 10% around the cutoff.

Rubella vaccination generally elicits a lower level of antibody response than wild-type infection (6). Therefore, countries with a comprehensive immunization program have populations with low-level antibodies that approach the cutoff of test kits, thereby increasing the likelihood that different test kits report divergent qualitative results. Another factor that contributes to divergent results is that different test kits measure different antibodies (2). The rubella virus has three main immunogenic antigens: two envelope antigens and one capsid antigen. The immune response to these antigens varies over time and is dependent on the exposure: i.e., vaccination or wild-type or congenital infection (7). The immune response to rubella virus is similar to most other viral infections, with the development of different subclasses of IgG and the increase in avidity of IgG over time (8). Test kits vary markedly in their manufacture, using different conjugate antibodies, antigens bound to the solid phase, and detection chemistries. This matrix of variables creates a testing environment that is difficult to standardize and that calibration with an international standard cannot overcome.

The RUB-1-94 WHO international standard is a freeze-dried residue of human immunoglobulin diluted in equal volumes of saline. The level assigned to this standard (1,600 IU) was initially

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measured using test systems that are no longer commonly used: hemagglutination inhibition (HAI), radial hemolysis (RH), and in-house enzyme immunoassays (EIA) (2). HAI and RH tests detect both IgG and IgM, are labor intensive, and are subject to high levels of variability. However, the rubella IgG tests used today are predominantly performed with high-throughput laboratory automation using chemiluminescence, fluorescence, or electrochemiluminescence detection chemistries, with antigens sourced from either purified native virus or recombinant proteins and having polyclonal and/or monoclonal conjugates (4). It is not surprising that calibration of these test kits using RUB-194 has not overcome this range of variables.

The principles of standardization of test systems require (i) a reference method, (ii) a certified reference material (CRM), and (iii) the development and measurement of secondary and working standards calibrated against the CRM using a calibration hierarchy and having an estimated uncertainty of measurement of the results (9). RUB-1-94 is defined as the CRM. However, the "measurand" (the substance being measured) in the standard is ill-defined, being a polyclonal mixture of antibodies, and is not representative of the measurand in current test systems. The test systems used to establish the level of RUB-1-94 were not quantitative, detected a mixture of measurands, and are subject to variability. Therefore, the approach used to establish the rubella standard was suboptimal.

Future approaches to resolve the situation are currently under discussion. Remaining with the current paradigm is not acceptable. One potential solution is to return to reporting anti-rubella IgG results qualitatively. This approach would allow test kit manufacturers to develop assays that separate the negative and positive populations in a manner that is employed in tests such as anti-HIV or anti-hepatitis C virus (anti-HCV). In the latter test kits, the means of the negative and positive populations are usually more than 10 standard deviations from the cutoff (the delta value), something that cannot be achieved in quantitative tests. Recently a panel of 100 rubella IgG-negative samples were collected and made commercially available (Biomex, Heidelberg, Germany) to aid manufacturers to optimize their test kit's specificity. However, there are two major regulatory barriers to this approach. These test kits are regulated as *in vitro* diagnostic devices (IVDs). Many regulatory bodies require IVDs to be calibrated to a standard if one exists. Additionally, even if a qualitative test kit is allowed, the process of reformulating and resubmitting the IVD is costly and requires many years of work.

An alternative would be to embrace metrological principles and use a reference method with known analytical characteristics to detect and measure a well-defined measurand—for example, anti-rubella E1 IgG. However, this standard would have limited utility unless all manufacturers' tests only measured this measurand.

There are some circumstances in which an anti-rubella IgG international standard might be useful. One example is in standardizing plaque neutralization assays for use in seroprevalence studies. However, it is clear that the use of the standard in calibrating immunoassays is not working and a new approach is required. The debate will continue and requires input and consensus from scientists, clinicians, test kit manufacturers, regulators, and bodies such as the WHO and CDC before resolution can be achieved.

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