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Standardization of Assays That Detect Anti-Rubella Virus IgG Antibodies

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SUMMARY

Rubella virus usually causes a mild infection in humans but can cause congenital rubella syndrome (CRS). Vaccination programs have significantly decreased primary rubella virus infection and CRS; however, vaccinated individuals usually have lower levels of rubella virus IgG than those with natural infections. Rubella virus IgG is quantified with enzyme immunoassays that have been calibrated against the World Health Organization (WHO) international standard and report results in international units per milliliter. It is recognized that the results reported by these assays are not standardized. This investigation into the reasons for the lack of standardization found that the current WHO international standard (RUB-1-94) fails by three key metrological principles. The standard is not a pure analyte but is composed of pooled human immunoglobulin. It was not calibrated by certified reference methods; rather, superseded tests were used. Finally, no measurement uncertainty estimations have been provided. There is an analytical and clinical consequence to the lack of standardization of rubella virus IgG assays, which leads to misinterpretation of results. The current approach to standardization of rubella virus IgG assays has not achieved the desired results. A new approach is required.

INTRODUCTION

Rubella virus usually causes a mild childhood infection with classical postviral symptoms of low-grade fever, maculopapular rash, lethargy, arthralgia, and myalgia. However, infection of pregnant women, especially those in the first trimester, may lead to severe congenital infection of the child, causing significant morbidity. Since the early 1970s, vaccination against rubella virus has been available, reducing the incidence of infection in countries that have well-developed vaccination programs. In most countries, clinicians are encouraged to screen all pregnant women for rubella virus IgG antibodies to confirm immunity and to offer vaccination to nonimmune individuals after delivery (1).

In 1970, the second international standard for rubella virus IgG was established. This and subsequent standards have been used by manufacturers to standardize quantitative results reported for rubella virus IgG assays. Since the 1980s, all commercial rubella virus IgG assays have reported results in international units per milliliter. However, it is apparent that standardization of rubella virus IgG assays has not been effective, with results for the same sample obtained by different assays being reported as different numbers of international units per milliliter. This situation leads to the misinterpretation of results, sometimes causing adverse clinical outcomes.

This review describes the virology of rubella virus infection and examines the history of testing for rubella virus IgG. Several factors, such as the introduction of large-scale vaccination programs and the development of new technology, have complicated the approach to the standardization of rubella virus IgG assays. By better understanding these factors and the methodology used to establish the international standards, we can propose some rea-

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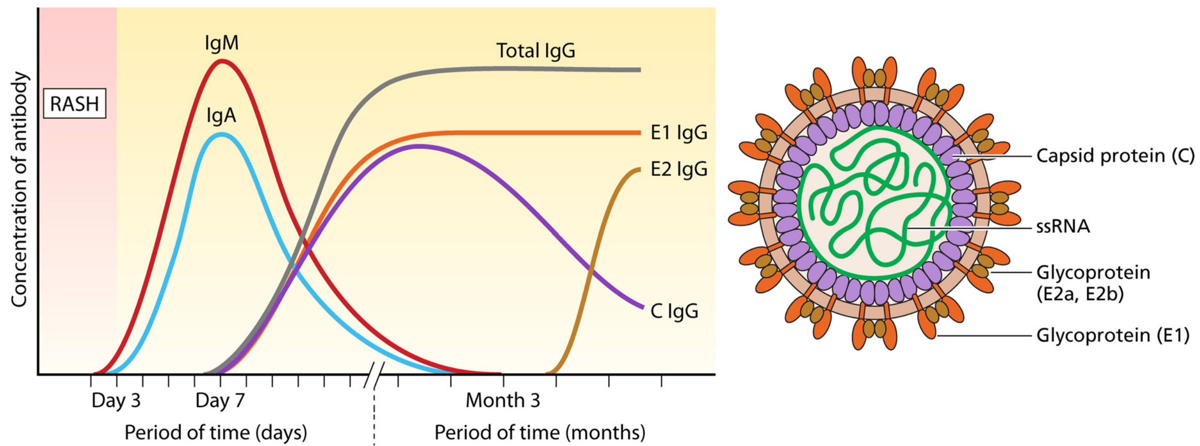

FIG 1 At the right is a schematic diagram detailing the structure of the rubella virus, including the three immunogenic antigens, i.e., two envelope (E1 and E2) antigens and a capsid (C) antigen, and single-stranded RNA (ssRNA). At the left is a plot of a normal immune response to rubella virus infections over time.

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sons why a lack of standardization of rubella virus IgG assays persists more than 40 years after the creation of the standard.

RUBELLA VIRUS

Rubella virus is a spherical, enveloped, 40- to 80-nm, 9.6-kb, positive-sense, single-stranded RNA virus of the family *Togaviridae*, the sole member of the genus *Rubivirus* (2, 3). The genome is enclosed in a capsid composed of multiple copies of a capsid protein, C (3, 4). This nucleocapsid is surrounded by a lipid bilayer embedding two viral envelope glycoproteins, E1 and E2. The outer surface of the virus has hemagglutinin-containing spike-like projections (Fig. 1). The molecular weights of the virus's four structural polypeptides are as follows: E1, 58,000; E2a, 47,000; E2b, 42,000; C polypeptide chain, 33,000 (2, 5). E1, E2a, and E2b are glycosylated and associated with the viral membrane. Two nonstructural proteins, p90 and p150, are involved in viral replication (3) but are not immunogenic. The E1 polypeptide, the largest of the two glycoproteins, is associated with the hemagglutinin function and has the predominant immunogenic reactivity in individuals exposed to the virus through natural infection, congenital infection, and vaccination. The capsid protein, C, is nonglycosylated and associated with the 40S genomic RNA. Two genotypes (6) have been identified, but only one serotype that demonstrates no cross-reactivity with other viruses has been reported.

RUBELLA VIRUS INFECTION

Unlike other togaviruses, rubella virus infects only humans. Transmission of rubella virus from person to person usually occurs via respiratory aerosols. Following inhalation of infected droplets, the virus replicates in the mucosal membranes of the upper respiratory tract, later spreading to regional lymph nodes. The period of contagiosity is approximately 5 to 7 days before and 3 to 5 days after the appearance of clinical symptoms (7).

In children, rubella virus infection causes a mild disease with symptoms including fine, distinct macules of a "rubelliform" erythematous rash detected about 16 to 20 days postinfection. The rash usually starts on the face and spreads to the trunk. It is self-limiting, fading within 48 h. Other nonspecific viral infection-like symptoms are common, including low-grade fever, malaise, lymphadenopathy, and mild transient polyarthralgia. Lymphadenopathy involving the posterior cervical and occipital nodes is common in rubella virus infections. Subclinical infections occur. Rarely, more severe symptoms such as thrombocytopenia, purpura, and encephalitis are seen (1).

Of major concern, rubella virus infection in pregnant women can lead to congenital rubella syndrome (CRS) of the infant (7–9). Rubella virus infection of women in their first trimester results in approximately 90% of the fetuses being infected and 100% of those infected having congenital deformities, often resulting in miscarriage. The risk of CRS declines as pregnancy proceeds, with CRS rarely being associated with primary infection after 16 weeks of gestation (1). CRS causes morbidity involving most major organs but particularly causes ophthalmic (cataracts, microphthalmia, glaucoma, and chorioretinitis), auditory (sensorineural deafness), cardiac, and craniofacial (microcephaly) complications (10). Hepatosplenomegaly, hepatitis, and thrombocytopenia result from liver damage. Many CRS-affected infants have severe mental impairment and delayed development. Chronic diseases such as type 1 diabetes and thyroiditis can be lifelong sequelae of CRS (11).

The economic cost of CRS in the 1980s was estimated to be about U.S. $300,000 for the lifetime support of an affected individual. The outbreak that occurred in the United States from 1962 to 1965, where approximately 12.5 million cases of rubella virus infection and 20,000 cases of CRS occurred, was estimated at the time to have an economic cost about U.S. $1.5 billion (12). In 1997, in Barbados and Guyana, the estimated costs of the lifetime treatment of individuals with CRS were U.S. $50,000 and $64,000, respectively (13).

IMMUNE RESPONSE

The humoral immune response to rubella virus infection is typical of most viral infections, with a rise in IgM followed by a slightly delayed response in specific IgG (14). Class-switching recombination allows the selection of antibody isotopes best suited to eliminating the virus. Anti-rubella virus IgM is usually detectable within 2 to 5 days after the appearance of a rash and persists for 1 to 3 months (depending on the assay used) (15). Persistence of anti-rubella virus IgM has been reported. Anti-rubella virus IgM may also be detected in reinfection and following polyclonal stimulation of the immune system (14). Even so, the detection of anti-rubella virus IgM is the main method of diagnosis of acute rubella

virus infection. This is the case especially in regions where the incidence of rubella virus infection is high, where it has a good positive predictive value and can be performed with a single sample. However, in countries where rubella virus infections are sporadic (mainly developed countries that have well-established vaccination programs), suspicion of rubella virus primary infection following a positive IgM test result must be confirmed by rubella virus IgG avidity testing, as the number of false-positive IgM results is usually greater than the number of true-positive results in a low-prevalence setting. An IgA response to acute infection has also been described.

Rubella virus IgG becomes detectable shortly after the appearance of IgM and usually remains detectable lifelong (14, 16, 17). Antibodies of different classes against E1, E2a, E2b, and C are formed and are present during an immune response following wild-type or vaccination exposure. E1 is the only antigen that is recognized by all of the antibody isotypes studied (18). Hemagglutination and neutralizing epitopes are located on the E1 polypeptide (19). It was determined that the development of E1 IgG was the dominant immunogenic response in individuals infected with wild-type virus, in CRS, and in vaccination, as most of the rubella virus-neutralizing epitopes are located on the E1 glycoprotein (18). However, the immune responses to E2 and C varied among natural infection, CRS, and vaccination. IgG antibodies to E2, which has only neutralizing antibody activity and lacks hemagglutination activity (19, 20), have been found to develop later than antibodies to E1 and may not be detectable for several months postinfection. E2 IgG antibodies were found to be more abundant in individuals with CRS than in those with natural, non-CRS infections. E2 IgG antibodies appear to persist longer than anti-C antibodies (19). The IgG, IgM, and IgA responses to C are strong during the acute phase of infection, but the levels decline over time, becoming undetectable.

Postvaccination studies indicate that although the immune response to vaccination mimics that of natural infection, the level of specific antibodies is lower than after natural infection. IgG antibodies against E1, E2, and C were detectable after vaccination of individuals who lacked immunity prior to vaccination. However, differences in the responses to the E2 and C proteins in wild-type and vaccination-induced immune responses indicate that major antigenic differences could be detected within these proteins (20).

The responses of IgG subclasses differ throughout the course of infection (14, 21–23). During the acute phase of infection, there is an initial and transient IgG3 response, along with IgA. IgG1 levels develop more slowly, increasing in both titer and affinity until IgG1 dominates the immune response (14). IgG2 and IgG4 rubella virus-specific antibodies are occasionally detected. The avidity of the IgG subclasses changes during seroconversion, starting as low-affinity antibodies and developing into high-affinity antibodies. Previous reports have indicated that the levels of antibodies detected by enzyme immunoassays (EIAs) do not always correspond to the intensities of specific bands in an immunoblot assay (18). This may be due to variability in the purity of the antigens used or variation in the substrate or conjugates employed by the assays.

In summary, the immune response to rubella virus infection is complex, involving different classes and subclasses of antibodies, maturation of avidity over time, and variable development of antibodies to up to four specific antigens. Combining the facts that different individuals can exhibit various levels of immune responses to one or more of the rubella virus antigens (24, 25) and that there is no standard antigen preparation used in the manufacture of rubella virus IgG assays, the challenge to standardize assays is significant, as it is unlikely that an individual patient's antibodies will react at the same level in assays constructed with different antigens and employing different detection chemistries.

RUBELLA VACCINATION PROGRAMS

The World Health Organization (WHO) position paper on rubella vaccines reports that most licensed vaccines are based on the live attenuated RA 27/3 strain propagated in human diploid cells (1). Each dose contains a defined number of infectious units (>1,000 PFU or 50% cell culture infective doses). The seroconversion rate after vaccination is expected to be greater than 95%. In December 2009, 130 of the 193 WHO member states had implemented a national immunization schedule. In 2015, 141 countries (72.7%) had established programs and a further 7 (3.6%) planned to implement immunization programs. Still, over 100,000 cases of CRS are recognized globally each year (1).

In 1969, a live attenuated vaccine was licensed in the United States and children between 1 and 14 years of age were given a single dose. New populations were targeted in the early 1970s, including women of child-bearing age, college students, military personnel, and some individuals in the health care setting (12). In 1978, the measles-mumps-rubella (MMR) vaccine was introduced, and in 1979, the RA 27/3 rubella vaccine replaced the HPV-77 and Cendehill vaccines. In 1989, a policy of two-dose MMR vaccination was introduced, primarily to counter sporadic outbreaks of measles (12, 26).

Rubella vaccine was licensed for use in vaccination programs in Australia and France in 1970. At that time, only adolescent girls and nonpregnant women were recommended to be vaccinated. In Australia, this protocol was replaced in 1989 with an MMR vaccination program aimed at infants 12 months of age irrespective of gender. Universal vaccination of adolescent boys and girls was introduced in 1993 (27). The current vaccination program targets boys and girls at both 12 months and 4 years of age. In France, measles-rubella vaccination of children was introduced in 1983 and MMR vaccine was introduced in 1986. Since 2005, it has been recommended that all children receive two doses of MMR vaccine, at 12 and 24 months of age. It should be noted that a reservoir of rubella virus remains, with countries such as Vietnam, China, Poland, South Africa, Indonesia, and Romania reporting more than 2,000 infections in a single year since 2011. This situation is due to inadequate vaccination coverage or "conscientious objectors" to vaccination for religious or other reasons, as seen in the "bible belt" of the Netherlands or in certain regions of the United States. The most common source of infection in countries with good vaccination coverage is through infected individuals traveling to and from regions where rubella is endemic for vacation, business, or immigration (28–31).

IMPACT OF VACCINATION

The introduction of rubella vaccination has resulted in a significant decrease in the incidence of both primary rubella virus infection and CRS in many countries. In Australia, the national notification rate for rubella virus infection fell from 23.4 per 100,000 in 1992 to 7.2 per 100,000 in 1997 and has remained at about 0.3 per 100,000 from 2003 to 2014 (11). A review of congenital rubella in Great Britain from 1971 to 1996 reported that births of CRS-

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affected babies and terminations of CRS-affected babies fell from 48 births and 742 terminations from 1971 to 1975 to 4 and 9, respectively, in 1991 to 1995 (32). In the United States, from 1962 to 1965, an estimated 12.5 million cases of rubella virus infection occurred. During the first 8 years of the vaccination program in that country, from 1969 to 1976, the numbers of rubella virus infections fell from 57,686 to 12,491 and the number of CRS cases fell from 68 to 23 (12, 33). Similar decreases have been reported in other countries where a comprehensive vaccination program has been implemented (26, 34).

One consequence of the rubella vaccination program is an overall reduction in the levels of rubella virus IgG in vaccinated individuals compared with those acquiring immunity through natural infection. It was demonstrated that, after a single dose of MMR vaccine, 9% of 307 kindergarten age children remained seronegative and a further 60% had the lowest level detectable by a neutralization assay. One month after a second dose, only 1% were seronegative and 6% had the lowest detectable level of antibodies. However, 12 years after the second vaccination, the immune response of the cohort had decreased to levels that were similar to those recorded after the initial vaccination (35). Longitudinal studies demonstrated that the percentage of women with low rubella virus IgG levels (15 to 34 IU/ml or a hemagglutination inhibition [HAI] titer of 16) increased from 2% in 1976 to 5.7% in 2000, whereas the percentage of women with an HAI titer of ≤8 or <15 IU/ml decreased from 9.4% to 2.5% over the same time period (36, 37). Revaccination of women with low antibody levels was successful in only 26% of the cases (27).

As the amount of naturally circulating virus declines because of increased herd immunity, the percentage of individuals who are naturally immune or have experienced an immunity boost because of postvaccination exposure to wild-type virus has declined as well. In Sweden, it was estimated that in 1982 up to 60% of 12-year-old school children were naturally infected, falling to 43% in 1998 and 24% in 1995 (26). The consequence of this change is that the population generally has lower levels of rubella virus IgG than before vaccination programs were introduced. This increase in low-level rubella virus IgG is one of the complicating factors when the lack of standardization of rubella virus testing is examined.

RUBELLA VIRUS IgG TESTS

The serological diagnosis of rubella virus infection and immunity began in the early 1960s after the isolation of rubella virus. Initially, viral neutralization testing (NT) was introduced but this method was cumbersome, varied in sensitivity, and generally obtained low titers (38). NT was able to detect antibodies to both E1 and E2, and reactivity in this assay is still regarded as the most reliable indicator of protective immunity. NT was replaced by the HAI test (38). This test relies on the principle that the hemagglutinin on the virus agglutinates erythrocytes of a number of animal species, including pigeons and trypsinized human group O cells. HAI detects both IgG and IgM and may be falsely negative if rubella virus-specific antibodies other than those against hemagglutinin are present, e.g., anti-C protein antibodies. Nonspecific inhibitors of HAI such as low-density beta lipoproteins need to be removed from the serum sample prior to testing; this is usually achieved through incubation with kaolin (39). However, there are many reports that highlight the variability of HAI testing within and between laboratories. The sources of variation include variability in the selection of reagents (i.e., different species and qualities of erythrocytes); potency of viral antigen; and methodologies, including temperature and time of incubation, pH of reagents, and different buffering systems; and interreader variability (40–42).

An alternative test system used in early rubella virus antibody testing was the complement fixation test (CFT), a test that relies on the detection of complement usage during an antibody-antigen reaction (38). Like HAI, CFT was labor intensive and imprecise and detected both IgG and IgM. Radial hemolysis (RH), which detects the ability of the antibody-antigen reaction to lyse erythrocytes immobilized in agar, was used to detect and semi-quantify rubella virus antibodies (43–47). In contrast to HAI and CFT, RH detects only IgG, in particular, subclasses 1 and 3. By testing a serial dilution of a standard in parallel with patient samples and constructing a standard curve based on the measurement of the annular radius of the zone of hemolysis created, IgG levels could be quantified.

The early 1980s saw the introduction of EIA for the detection of specific antibodies (42, 48–50). EIAs, which can be formatted to detect subclasses of antibodies or be directed toward specific antigens, quickly replaced HAI, CFT, and other assays to such an extent that today very few reference laboratories in the world still retain the capacity to perform these reference tests. Early commercial rubella virus IgG EIAs were usually in a microtiter plate format employing an enzymatic color-based detection system. The assays varied vastly in the choice of conjugate, substrate, and antigen, as well as incubation times and temperatures (51, 52). These assays usually demonstrated good linearity and had equivalent sensitivities and specificities. Many authors have compared the performance of EIAs over the years (52–58).

One of the first automated benchtop analyzers developed for the detection of rubella virus IgG was the Abbott IMx (59). Since then, microtiter plate technology has gradually been replaced by automated analyzers using a range of detection technologies such as immunofluorescence (bioMérieux VIDAS, Abbott AxSYM), electrochemiluminescence (Roche Elecsys), and chemiluminescence (Abbott Architect, DiaSorin Liaison, Siemens ADVIA Centaur). These changes have allowed rubella virus serology to be incorporated within general medical laboratory testing alongside general chemistry and endocrinology. Apart from the different technologies used in modern test systems, an array of different conjugates, substrates, and antigens are still used (54). A prime example is the Roche Elecsys rubella virus IgG assay, which uses several recombinant proteins as the antigen source, unlike most other assays, which use native rubella virus-derived antigens obtained as whole, purified, or partly purified virus or as a viral lysate. Irrespective of the format of the assay, all of the commercial rubella virus IgG assays currently available are calibrated against the same WHO international standard and report results quantitatively in international units per milliliter (54). Not all regulatory jurisdictions require rubella virus IgG assays to be calibrated solely by using the WHO international standard. In the United States, for example, the Code of Federal Regulations refers to a panel of 100 well-characterized rubella patient serum samples obtained from the Centers for Disease Control and Prevention to assess new assays (60).

RUBELLA STANDARDS

Tracing the history of rubella standards is difficult. Some reports, such as that of Hansen (G. A. Hansen, unpublished data, 1996), have never been released publically and require permission from

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the WHO. The first international reference preparation of anti-rubella serum was prepared in 1966. It consisted of a pool of convalescent-phase human sera. This preparation lost its potency more quickly than expected, and it was replaced in 1968. In 1970, the second international reference preparation of anti-rubella serum was established and designated BS/96.1833, also known as RUBS (Hansen, unpublished; 61). Despite its name, it was prepared from normal human immunoglobulin. Stocks of RUBS diminished over time, and in 1995, it was recognized that a replacement was needed. The candidate chosen was BS/94.1762, also prepared from pooled human immunoglobulin rather than human serum. It was designated RUB-1-94 (Hansen, unpublished; 61).

In parallel, separate standards were produced in the United Kingdom. The second British standard for anti-rubella serum, human (67/182), was established by the National Biological Standards Board in 1986 (62). It was initially and indirectly quantitated in 1974, being one of a number preparations (serum B 67/182) considered during an international collaborative study establishing the first British standard for anti-rubella serum, human (69/60), against the second WHO international reference preparation of anti-rubella serum, human (RUBS), which was established in 1970.

In reality, RUB-1-94 is not a serum standard but freeze-dried residue of human immunoglobulin diluted in equal volumes of saline. A proposed third international standard preparation was described in 1994 at the 44th meeting of the WHO Expert Committee for Biological Standards (63). The third international standard, designated RUBI-1-94, was developed by the Statens Serum Institut in Denmark and was based on British standard BS/94.1762 (61, 63). The WHO committee was informed that the proposed new standard would replace the second WHO international standard (RUBS) and "a limited collaborative assay would be arranged." RUBI-1-94 was also a normal immunoglobulin preparation derived from healthy Danish plasma donors, diluted in equal volumes of saline, and freeze-dried in 2-ml aliquots (63). In the 47th report of the WHO Expert Committee, it was noted that the nomenclature of the third international standard was confusing, as it was, in fact, an immunoglobulin preparation, and therefore, the committee voted that the RUBI-1-94 standard become the first international standard for anti-rubella virus immunoglobulin (64).

Postproduction testing of RUBI-1-94 had demonstrated no reduction in the potency of the freeze-dried samples. RUBI-1-94 stored at +50°C for 6 months in parallel with aliquots stored at −20°C showed no decline in reactivity when tested in a rubella virus IgG EIA and therefore demonstrated satisfactory stability (Hansen, unpublished). To demonstrate comparability between RUBI-1-94 and RUBS, participating laboratories were requested to test, a minimum of three times, freshly reconstituted aliquots of both standards in parallel in two different assays that they frequently used. The raw data were analyzed by comparing the results obtained with RUBS, which had an assigned potency of 1,000 IU, with the results obtained by testing RUBI-1-94 by parallel-line analysis.

The international collaborative study to assign a concentration to RUBI-1-94 was performed by 11 laboratories in seven countries (61) using one or more EIA, HAI, or RH test systems. Eight laboratories tested the standards in commercial or in-house EIAs, submitting 61 results. The commercial assays used by participating laboratories were not identified. However, the first benchtop analyzer for rubella virus IgG, the Abbott IMx, was introduced after testing took place (59), so it is assumed that microtiter plate EIAs were used. A further four laboratories submitted seven HAI results, and one laboratory submitted two RH test results. Estimates of the potency of RUBI-1-94 were 1,656 IU for the weighted mean EIA result, 1,411 IU for HAI testing, and 1,330 IU for RH testing. In combination, the geometric mean of all 70 valid test results, irrespective of the test system, was 1,592 IU. The assigned potency of RUBI-1-94 was therefore 1,600 IU.

The current instructions for use (IFU) of RUBI-1-94 provided with the standard state that this study "has almost been completed," even though the testing was finalized in 1996 and the report was submitted to the WHO in October of that year. The final results of the study were never published. The potency of RUBI-1-94 was deemed to be 1,600 IU on reconstitution. The IFU state that the ampoule can be reconstituted "in physiological saline or any other suitable fluid."

EVIDENCE OF LACK OF STANDARDIZATION

Since the release of RUBI-1-94, all commercial assays have been calibrated against this standard (54). It would be expected, therefore, that the results obtained with the same sample in different assays would be equivalent. However, that is not the case. Several peer-reviewed articles have highlighted the lack of standardization between rubella virus IgG assays reporting results in international units per milliliter (52, 54–56, 58). We first reported this discrepancy in 1992, when the assays were calibrated against the second international standard, RUBS, and were predominantly microtiter plate or bead EIAs. Fourteen years later, a similar study was conducted. The DiaSorin E.T.I. Rubek-G kit was the only microtiter plate assay still in routine use by Australian laboratories. All other microtiter plate assays had been replaced with automated, random-access platforms, and all of the new platforms had been calibrated with RUBI-1-94. More recently, a comprehensive study comparing quantitative rubella virus IgG results reported by different assays has supported the original findings (56). External quality assessment scheme (EQAS) results reported by participating laboratories have regularly demonstrated that testing the same sample in different assays produces a wide range of quantitative results. Table 1 presents results from a single EQAS sample tested in multiple laboratories with different assays. For the same sample, the mean results reported for different assays ranged from a negative value of 0.96 IU/ml to a strongly positive value of 67.0 IU/ml. Even within assays from the same manufacturer, results varied considerably.

All commercial assays use 10 IU/ml as the cutoff between immune and nonimmune status as defined by various authorities. When the immune-nonimmune cutoff was first postulated, the studies used HAI and NT. In 1978, Bradstreet et al. concluded that the minimum titer to indicate immunity should be equivalent to 24 to 48 IU (65). However, these studies used the first British standard for anti-rubella virus serum, human (69/60). The rubella subcommittee of the NCCLS (now CLSI) originally set the immune cutoff at an HAI titer of 1:8, which equated to 15 IU/ml (53). Studies conducted by Abbott Laboratories (Chicago, IL) on the Abbott IMx concluded that the cutoff for that technology was 10 IU/ml (59). In 1992, the NCCLS committee revised its recommendations to adopt the use of 10 IU/ml as the cutoff, where it has remained ever since (66).

It should be noted that most assays have an equivocal range or "gray zone." For assays lacking a gray zone, such as the Roche Elecsys rubella virus IgG assay, a patient with a rubella virus IgG

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TABLE 1 Summary of the mean of results obtained from a single EQAS sample tested in multiple commercial assays

Assay[a]	No. of results	Mean result (IU/ml)
Abbott ARCHITECT Rubella IgG CMIA	31	16.70
Abbott AxSYM Rubella IgG MEIA	5	17.50
ACON Foresight Rubella IgG EIA kit	1	53.80
Beckman Coulter Access RUBELLA IgG ChLIA	2	26.50
Bio-Rad BioPlex 2200 ToRC IgG MFIA	1	17.00
Bio-Rad Platelia Rubella IgG ELISA	1	0.96
bioMérieux VIDAS RUB IgG II ELFA	67	25.80
DiaSorin ETI-RUBEK-G PLUS EIA	2	15.00
DiaSorin LIAISON Rubella IgG CLIA	32	13.20
DIESSE CHORUS Rubella IgG ELISA	8	22.50
IMMUNOLAB Rubella IgG ELISA (quantitative)	2	34.00
Ortho VITROS Rubella IgG assay	3	43.10
Roche Elecsys Rubella IgG ECLIA	18	61.70
Siemens ADVIA Centaur Rubella G ChLIA	8	67.00
Siemens Enzygnost Anti-Rubella-Virus IgG EIA	2	45.30
Siemens IMMULITE 2000 Rubella Quantitative IgG CLEIA	4	17.30

[a] MEIA, microparticle enzyme immunoassay; ChLIA, chemiluminescent immunoassay; MFIA, multiplexed fluorometric immunoassay; ELISA, enzyme-linked immunosorbent assay; CLIA, chemiluminescence immunoassay; CLEIA, chemiluminescent enzyme immunoassay.

result of 9.9 IU/ml is interpreted as being nonreactive and a patient with a result of 10.1 IU/ml is interpreted as positive, even though the coefficient of variation of the assay is often more than 10%. These results indicate the presence or absence of antibodies. Reinfection has been described in individuals who had rubella virus IgG levels of >15 IU/ml (22, 67, 68). Therefore, the assumption that results of >10 IU/ml always confer protection against infection or reinfection and results of <10 IU/ml indicate susceptibility to infection is not correct.

METROLOGICAL APPROACH TO STANDARDIZATION AND TRACEABILITY

In order to understand better the reasons why a lack of standardization of rubella virus IgG assays continues, a review of metrological principles is necessary. These principles are detailed elsewhere (69), but briefly, they involved the creation of a certified reference material (CRM; otherwise called the primary reference standard) (70–72) and quantifying the level of the measurand (the quantity intended to be measured) by using a measurement system (73–75) or reference method(s). The traceability of secondary and working measurement standards is established through calibration against the CRM by using a calibration hierarchy (76–78). Well-established conventions for standardization of measurements, controlled by organizations such as the International Centre for Metrology (ICM), the National Institute of Standards and Technology, the International Federation of Clinical Chemistry and Laboratory Medicine, the International Organization for Standardization (ISO), and the WHO are in place (73, 79, 80). These conventions are underpinned by quality standards and terminology as specified in the international vocabulary of basic and general terms in metrology (81).

Nominated facilities act as reference laboratories. These are analytical centers of competence and are considered experts in quantifying well-defined analytes by using the best, internationally agreed measurement procedures. CRMs are pure or purified analytes having a known quantity of the measurand in question and are required to have a statement of proof of stability measured by the highest-order reference methods (82). Ideally, a CRM should have a measurement expressed in SI units (class A analytes). A good example is the 1-kg mass standard (69, 70, 82). However, only about 30 types of quantities are traceable to SI units (73). In many biological measurements, it is not possible to create such a standard and therefore "biological standards" (class B analytes) such as those obtained from the WHO are used (77, 82). Class B analytes are generally highly complex and heterogeneous mixtures found within a biological matrix (e.g., urine or serum) (77). There are three elements required to specify a measurement of class B analytes—the system (e.g., serum), the component (e.g., anti-rubella virus IgG), and the kind of quantity (essentially the biological response or biological activity)—together making up the measurand (75). Defining the kind of quantity for rubella virus IgG assays remains a challenge because of the variability of the immune response over time and from person to person.

Standards for class B analytes are supposed to be prepared with state-of-the-art purification and identification techniques, and their function is supposed to be tested in a biological system (75). Class B analytes suffer from matrix-dependent effects and often have insufficient definition of the measurand in question (77). In clinical chemistry, several working parties have addressed these deficiencies for a range of measurands such as hemoglobin A1c and human chorionic gonadotropin. These working parties began by defining the measurand in question and the method used to measure these quantities (75, 77, 78, 82). In the case of quantifying antibodies, it is recognized that there are many variables involved, including, but not limited to, the class and subclass of antibodies; polyclonal, oligoclonal, or monoclonal states; allotype, idiotype, and isoforms; avidity and affinity maturation; fragmentation, denaturation, and mutation; and the antibodies being free or complexed (83).

Reference methods are considered to be "definitive methods." According to the ICM, for class A analytes, isotype dilution with mass spectrometry, colorimetry, gravimetry, titrimetry, and the determination of freezing point depression are primary methods yielding results in SI units (74). Determination of the levels of class B analytes relies on the measurement of their bioactivity or biological "kinds of quantity," which are highly dependent on the measurement system used (69, 74, 77, 78, 82, 83). It is important, therefore, that the reference method used to assign a unitage to the CRM is well defined, robust, and reproducible in nature and the measurand being measured is clearly defined (73, 74, 79, 84). EIAs used to detect and quantify protein can be disturbed by interfering substances; this is also known as a "matrix effect" (78, 83). As stated by Müller, the "Use of poorly characterized methods with unknown trueness or of calibrators of lesser standardization is not acceptable for value assignment. When such approaches are used for analytes measured by immunoassays, ...they are not accurate, not traceable and not in agreement with the principles of metrology" (74).

The WHO standard RUBI-1-94 has an assigned value of 1,600 IU and is considered a class B CRM. Once the value of a CRM is defined, all subsequent CRMs must be compared with the original CRM, not the immediate predecessor, or else the value becomes a "moving target" (75). A hierarchy of secondary and working standards is used to demonstrate traceability and commutability from the CRM ultimately to the patient test result through a traceability chain, which has been well described elsewhere (69, 76). Traceability is defined as a "Property of a measurement result whereby the result can be related

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to a reference result through a documented, unbroken chain of calibration, each contributing to the measurement uncertainty" (69). The hierarchy of traceability is primarily the responsibility of the assay manufacturer (73, 85), who should provide, along with calibration standards, evidence of traceability, including an estimate of the uncertainty budget. This information is rarely, if ever, provided in the IFU of rubella virus IgG assays.

When a primary reference material is intended to be used to directly assign values to manufacturers' calibrators, extensive testing for commutability is required. Commutability is defined by ISO 15194 as the "ability of a material to yield the same numerical relationships between the results of measurements by a given set of measurement procedures, proportion to measure the same quantity, as those between the expectation of the relationship obtained when the same procedures are applied to other relevant types of material" (79). Simply put, the RUB-1-94 standard should achieve the same result with any assay testing for anti-rubella virus IgG. Plainly, this is not the case.

PROPOSED REASONS FOR LACK OF STANDARDIZATION

The processes used to establish RUBI-1-94 fail by almost all of the principles of metrology. RUBI-1-94 is not a pure or purified analyte and is not a well-defined measurand. The material used to develop the standard is an immunoglobulin preparation derived from pooled human plasma. The processes used to concentrate antibodies are not described, and no studies investigating changes in biological function due to concentration methods or lyophilization are reported. Indeed, the IFU state that "the use of an immunoglobulin preparation as a reference material for diagnostic assays of human sera in clinical laboratories is not an ideal solution." The lyophilized sample is reconstituted in physiological saline or "any other suitable diluent," ignoring potential matrix effects. It should be noted that this immunoglobulin preparation consists of polyclonal antibodies from multiple but undisclosed numbers of individuals and is provided to the user at a concentration of 1,600 IU/ml, which requires a dilution to achieve a concentration within the range of medically significant levels. Only a brief statement on stability is provided with the standard. Limited postproduction accelerated stability testing results have been documented, but these details were not released publicly.

The reference methods used to assign a value to RUBI-1-94 could not be considered reference procedures. The value was assigned by testing with HAI, RH, and EIAs that have since been superseded by arguably superior technology. HAI and RH tests detect all antibody subclasses and do not measure IgG antibodies specifically. Both methods are qualitative or, at best, semiquantitative. The EIAs used by the eight laboratories participating in the value assignment processes were unidentified. It is stated that they had both commercial and in-house origins. It is assumed that each EIA measured rubella virus IgG-specific antibodies. No detail is given describing the antigen source or type or the substrate or conjugates used. No assay performance characteristics, such as linearity, which is critical to value assignment, were provided. The assays used to assign the IU value cannot be described as "highest-order reference methods," and each family of assays (HAI, RH, and EIA) measures different functional biology. All of the assays used to assign the value to the standard are now obsolete.

No commutability studies of the international standard have been conducted, although incidental observations arising from method comparisons would cause commutability to be questioned. All manufacturers of commercial rubella virus IgG assays indicate in their IFU that they calibrate their assays against WHO standard RUBI-1-94. However, the principles of traceability are not described; that is, there are no details regarding the creation of secondary or working standards or how the calibrators are developed to correspond to these standards. Of note, no value of measurement uncertainty associated with the traceability chain is reported in these IFU or elsewhere.

It is obvious that some commercial assays are measuring different biological functionalities, as observed from the difference in the values obtained when patient samples are tested in different assays. Several papers have described (i) significant differences in patient test results reported for different assays, (ii) a spread of results reported in international units per milliliter for patient samples having the same HAI titer, or (iii) a range of results when a dilution series of the international standard or a secondary standard was tested. Therefore, it could be deduced that commutability between methods does not exist.

ANALYTICAL IMPACT OF POOR STANDARDIZATION

As stated above, a level of 10 IU/ml has been nominated as the "cutoff" between immunity and nonimmunity (53). By any analytical standards, this strict cutoff is arbitrary, especially considering the imprecision of the assays. Studies have indicated that the dose-response relationship of most rubella virus IgG assays is linear and that the sensitivities and specificities of the assays are comparable (52, 54). Therefore, the main analytical, and therefore clinical, consequence of poor standardization of rubella virus IgG assays (discussed below) is around the cutoff. When a patient with low levels of rubella virus IgG is tested in different assays, lack of standardization causes some samples to be reported as greater than 10 IU/ml and some to be reported as less than that cutoff.

Two comprehensive studies of this situation have been published. In 2013, we sought to determine the true presence or absence of anti-rubella virus IgG in 500 samples that were reported to have levels of 40 IU/ml or less. A total of 100 samples with results from each of five commercial instrument-based immunoassays (IAs) (a total of 500 samples) were collected and tested with HAI and an immunoblot assay. The qualitative result of the IA was then compared with a status derived from the HAI and immunoblot test results. Concordance of the IA qualitative result with the status ranged from 50.0 to 97.8%, indicating significant variation in qualitative results at low levels (86). A separate study tested 325 samples that were reported as having results of less than 10 IU/ml by one of nine commonly used commercial IAs. Each sample was tested in all of the nine IAs and also by HAI, immunoblotting, and NT. Only 129 of the 325 samples were negative in all nine IAs. A total of 59% of the samples reported as negative by an IA were found to have antibodies detectable by the immunoblot assay (56).

NRL, Australia, conducts an international quality control (QC) program for infectious disease testing called QConnect. Samples are manufactured and, where possible, are traceable to an international standard. For the rubella virus IgG QC program, aliquots of QConnect RubellaG QC samples (formerly AcroMetrix, Benicia, CA, and now Thermo Fisher Scientific, Fremont, CA.) were provided to participating laboratories. The QConnect RubellaG QC sample is calibrated against RUBI-1-94 to 10 IU/ml by procedures having an ISO 17511 traceability claim (87). Participating laboratories tested the QConnect sample periodically over long periods of time (up to 12 months) and reported the

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results into an internet-based QC monitoring software called EDCNet. The results of the QC testing for the calendar year 2014 indicated that there was a large variation between the results reported by the assays with the mean results expressed in international units per milliliter (number of results in parenthesis) for the Abbott ARCHITECT Rubella IgG chemiluminescence microparticle immunoassay (CMIA), bioMérieux VIDAS RUB IgG II enzyme-linked fluorescence assay (ELFA), Roche Elecsys Rubella IgG electrochemiluminescence immunoassay (ECLIA), and the Siemens ADVIA Centaur Rubella G assay as being 12.86 (3,175), 21.11 (38), 26.48 (453), and 48.30 (389), respectively (previously unpublished data).

CLINICAL IMPACT OF POOR STANDARDIZATION

It is fair to state that quantification of results from patients having high levels of anti-rubella virus IgG has little clinical impact, as the patient will be immune irrespective of the quantitative value at the upper end of medical importance. However, because of vaccination programs, the percentage of individuals having low levels of rubella virus has increased, compounding the difficulties of clinical interpretation of immune or nonimmune status (37).

The use of IU implies that, in general, the scientific community and health professionals consider serologic assays to be standardized and assume that results obtained by different assays are comparable. Actually, the antigen(s) used in the assays (total virus, recombinant antigens), the conjugate, and the assay format (indirect, sandwich, competition, capture) differ from one assay to another. Under these conditions, rubella virus IgG results or interpretations may be very different and even discordant, depending on the assay used (56). Discrepancies between assays have been confirmed by several studies (52, 54–56, 86) and have an impact on diagnosis (confusing results resulting in confusing clinical management of pregnant women). Indeed, pregnant women tested in different laboratories with different assays may receive contradictory result interpretations, i.e., negative by one method and positive by another (false seroconversion). More significantly, false diagnosis of a rubella virus primary infection may be induced by an artificial rise in the rubella virus IgG titer. A recent paper may highlight this phenomenon (88). Pregnant women had antenatal screening conducted by different laboratories using unspecified assays. Once admitted, the women were tested with the Siemens Advia Centaur rubella virus IgG assay, which reports rubella virus IgG levels higher than those reported by many other assays. It was concluded that of the 298 women who had screening rubella virus IgG levels reported as $<$10 IU/ml, 19 "seroconverted" because the subsequent Centaur results were reported as $>$40 IU/ml. It is our opinion that these "seroconversions" are due to differences in the quantitative results of the assays and that, had the early and later samples been tested together in the same assay (i.e., in parallel), as is the recommended practice, no difference in titer would have been detected, even though some of the Centaur levels were $>$500 IU/ml. This situation has been observed previously (56) and may lead to unnecessary anxiety for a pregnant woman and her family, termination of pregnancy, and/or medicolegal complications. Moreover, depending on the sensitivity of the assay used to determine the immune status, a proportion of the population tested will be considered susceptible and unnecessarily (re)vaccinated (58).

Lack of standardization has also impacted seroprevalence studies, the results of which depend on the assays used. These studies are particularly important in supporting the goal of rubella elimination. Indeed, rubella seroprevalence data for 2013 collected from England, Germany, Australia, and France show that the rate of "negative" or equivocal results of rubella virus IgG testing is higher in vaccinated populations (11, 29, 33, 36, 89). More recently, the evaluation of incidence of rubella virus infection during pregnancy in Texas appears to have been distorted by the poorly standardized assays in a country that eliminated rubella years ago (88).

FUTURE APPROACHES

It is not possible to identify an approach to further standardize rubella virus IgG assays by using the existing RUB1-1-94 international standard, given the deficiencies of the material and the fact that rubella virus IgG testing essentially seeks a qualitative response of immune/nonimmune, or more correctly, the presence or absence of specific rubella virus IgG. An informal Rubella Working Party is developing a panel of samples deemed to have no detectable rubella virus IgG. It is hoped that this panel will be available to all manufacturers of rubella virus IgG assays and it will be used to optimize the assay's specificity, signaling a potential move away from reporting results in international units per milliliter. If necessary, an assay-specific unit could be applied, as recommended by the IUPAC-International Federation of Clinical Chemistry consensus paper (84) until such time as an appropriate international standard can be developed by using metrological principles. Recommendation 4.3 states that "For quantities having no recognized kind-of-quantity name with a definable dimension, the term 'arbitrary' should precede the usual kind-of-quantity name and a reference to the 'procedure' and to the calibrator should follow the kind of quantity in the systematic name."

For metrological principles to be followed, the measurand and the biological kind of quantity must be clearly defined. A single, defined, and pure state that relates to the functional activity of the antibody needs to be described. This is difficult because rubella virus IgG is a complex molecule that exists in a mixture of various states. As rubella virus antigen E1 is the immunodominant antigen (18), monoclonal anti-rubella virus E1 IgG may be an example of a measurand that could be used to fulfill metrological principles, as it can be clearly defined, i.e., molecular weight, structure, etc., and its activity can be measured. However, assays would require E1 as the antigen source as a purified or recombinant protein. To measure the reactivity of the monoclonal antibody, a reference method would be required. The Biacore assay (Biacore AB, Uppsala, Sweden), which uses kinetic analysis on a surface plasmon resonance-based biosensor (14), is an example of a potential reference method, although a high level of reproducibility within and between reference laboratories would need to be confirmed. This approach would address the metrological requirements for the production of a standard but would be still be insufficient for clinical use.

The assays used in laboratory medicine to determine immunity to rubella virus detect and measure a much broader variety of antibodies than just anti-E1 antibodies. The immune response is to a complex set of antigens, which vary from individual to individual as the disease progresses and in response to biological factors such as immune status. The immunodominant responses of different individuals may not be to the same antigen. Some individuals fail to produce a response to some antigens. Therefore, an international standard based on the quantification of antibodies specific to only E1 would be of little use clinically. It may, however,

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be of use in pharmacology to quantify the immune potency of sera following vaccinations. Indeed, the WHO technical committee in 1994 concluded that "there were cases in which a single international standard might not be appropriate in use in both therapeutic and diagnostic areas" (63).

There have been reports on the immune reactions to natural infection, CRS, and vaccination (14, 24, 25, 49, 68, 90, 91). Much of this has been performed with research-only assays developed to measure the responses to individual antigens. There have also been studies investigating the level of protection elicited by detectable antibodies. However, to our knowledge, there have not been any studies by the manufacturers or the users of commercial assays to determine if, and how much, detected antibodies protect an individual. Therefore, a review of assay IFU would reveal that the intended use of the assay is not to determine a level of protective immunity but to detect circulating antibodies. It remains to be determined what level of antibodies detected by an assay is protective.

One outstanding issue remains to be discussed. European *In Vitro* Directive 98/79/EC states that "The traceability of values assigned to calibrators and/or control materials must be assured through available reference measurement procedures and/or available reference materials of a higher order" (85). This may be interpreted to indicate that manufacturers need to ensure that the calibrators provided are traceable by metrological principles to the reference material, in this case, RUBI-1-94. If metrological principles were to be adhered to, manufacturers should at least provide evidence of the traceability of their calibrators to the international standard along with an uncertainty budget (85). A common understanding and agreement on how these requirements affect the standardization of rubella virus IgG assays, as well as other quantitative serological assays, would be useful, as the lack of standardization of rubella virus IgG assays also exists among hepatitis B surface antibody assays. As is the case with HBa1c, the development of a working party composed of a national metrological institute(s), manufacturers of assays, clinicians, and experts in the field of infectious disease serology is an appropriate next step.

The alternative approach is to stop the use of the standard for calibrating rubella virus IgG (and arguably anti-hepatitis B surface antigen) assays and return to qualitative assays. Quantitative tests require the results to be linear, forcing the reactivity of high negative sample values (e.g., 8 or 9 IU/ml) to be close to those of low positive samples (e.g., 11 or 12 IU/ml). The move to qualitative assays would allow manufacturers to better separate the negative and positive populations, leading to assays similar to anti-HIV antibody assays, which have much greater clinical sensitivity and specificity than rubella virus IgG assays.

The international standard could be used to calibrate functional assays such as NT or HAI tests for use in seroprevalence studies, where the level of protective immunity and standardization are necessary for the comparison of data.

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