Clinical and Vaccine Immunology

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***Clin. Vaccine Immunol.* 2013, 20(2):255. DOI: 10.1128/CVI.00603-12.**
Published Ahead of Print 19 December 2012.

CVI
Journals.ASM.org

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Since the 1980s, commercial anti-rubella virus IgG assays have been calibrated against a WHO International Standard and results have been reported in international units per milliliter (IU/ml). Laboratories testing routine patients' samples collected 100 samples that gave anti-rubella virus IgG results of 40 IU/ml or less from each of five different commercial immunoassays (CIA). The total of 500 quantitative results obtained from 100 samples from each CIA were compared with results obtained from an in-house enzyme immunoassay (IH-EIA) calibrated using the WHO standard. All 500 samples were screened using a hemagglutination inhibition assay (HAI). Any sample having an HAI titer of 1:8 or less was assigned a negative anti-rubella virus antibody status. If the HAI titer was greater than 1:8, the sample was tested in an immunoblot (IB) assay. If the IB result was negative, the sample was assigned a negative anti-rubella virus IgG status; otherwise, the sample was assigned a positive status. Concordance between the CIA qualitative results and the assigned negative status ranged from 50.0 to 93.8% and 74.5 to 97.8% for the assigned positive status. Using a receiver operating characteristic analysis with the cutoff set at 10 IU/ml, the estimated sensitivity and specificity ranged from 70.2 to 91.2% and 65.9 to 100%, respectively. There was poor correlation between the quantitative CIA results and those obtained by the IH-EIA, with the coefficient of determination (R^2) ranging from 0.002 to 0.413. Although CIAs have been calibrated with the same international standard for more than 2 decades, the level of standardization continues to be poor. It may be time for the scientific community to reevaluate the relevance of quantification of anti-rubella virus IgG.

Infection with the rubella virus usually results in a mild childhood illness. However, infection during the first trimester of pregnancy can result in the neonate developing congenital rubella syndrome (1). For this reason, rubella vaccination programs have been established (2–5). In Australia, most diagnostic testing for rubella immunity is performed as part of an antenatal screen to ensure that the mother has protective levels of antibody. A hemagglutination inhibition titer greater than or equal to 1:16 and/or an antibody concentration greater than 10 or 15 IU/ml, depending upon the assay, is considered protective (6, 7). Some laboratories choose to report a "gray zone" to indicate uncertainty in the degree of protection conferred by low anti-rubella virus IgG levels. In Australia, the most frequently used gray-zone range is 10 to 30 IU/ml.

Since the 1980s, commercial assays used for the quantification of anti-rubella virus IgG have been calibrated against the World Health Organization (WHO) international standard rubella virus serum and test results have been reported in international units per milliliter (IU/ml) (8, 9). In theory, the calibration of assays should lead to standardization of quantitative results (10). However, several reports have indicated that quantitative anti-rubella virus IgG results reported by different assays are not always comparable (8, 9, 11). Consequently, individuals presenting to laboratories using different assays may be given different clinical interpretations, especially if their anti-rubella virus IgG levels are low.

Recently, several new commercial anti-rubella virus IgG assays have become available. New assays require validation prior to introduction into routine use (12). A common approach to validation used by laboratories is the comparison of results obtained from the new assay with those obtained from the assay routinely used by the laboratory (11, 13, 14). However, if a difference in qualitative or quantitative results obtained with the two assays occurs, it is difficult to elucidate which assay's result is correct (11).

In this study, the results of a hemagglutination inhibition assay (HAI) (15, 16) and a rubella immunoblot (IB) assay (17–20) were used to assign an anti-rubella virus IgG status. Samples with test results less than or equal to 40 IU/ml were collected from collaborating laboratories using each of five different commercial anti-rubella virus IgG immunoassays (CIAs). The 500 samples were tested by HAI and IB assays, and the qualitative results from the CIAs were compared with the assigned anti-rubella virus IgG status. To investigate the accuracy of quantification of anti-rubella virus IgG levels by the CIAs, all samples were tested in an in-house enzyme immunoassay (IH-EIA) developed to detect and quantify low levels of anti-rubella virus IgG.

MATERIALS AND METHODS

Samples. For each of five CIAs used routinely in collaborating laboratories, 100 routine clinical samples giving an anti-rubella virus IgG test result of 40 IU/ml or less, totaling 500 individual samples, were collected. After initial CIA testing in the collaborating laboratories, the samples were transported at ambient temperature to a central laboratory where they were stored at −20°C until further testing was performed. Prior to testing, the samples were thawed and tested in a testing strategy as described below. All testing, including repeat testing, was performed within 1 week of thawing. After testing, the remaining volume was divided into aliquots

Received 21 October 2012 Returned for modification 13 November 2012
Accepted 2 December 2012
Published ahead of print 19 December 2012
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doi:10.1128/CVI.00603-12

TABLE 1 Summary of the assay characteristics of five commercial immunoassays

Assay characteristic	Description or value for indicated commercial immunoassay				
	Abbott Architect	Abbott AxSYM	Roche Elecsys	bioMérieux Vidas	Ortho Vitros
Solid phase	Microparticles	Microparticles	Magnetic beads	Solid-phase receptacles	Plastic wells
Antigen	Partially purified rubella virus	Partially purified rubella virus (strain HPV77)	Rubella-like particles and recombinant E1 antigen	Rubella antigen (strain MR 383)	UV-treated rubella antigen from cell culture
Detection system	Chemiluminescence	MUB[a]	Chemiluminescence	MUB	Luminescence
No. of calibrators	6	6	2	1	4-parameter logistic curve
Calibration range (IU/ml[b])	0–500	0–500	0.17–500	0–250	0–350
Standard	RUB-1-94[c]	WHO standard (not specified)	RUB-1-94	RUB-1-94	RUB-1-94
Negative range (IU/ml)	<4.9	<5.0	<10.0	<5.0	<9.99
Equivocal range (IU/ml)	5.0–9.9	5.0–9.9	NA[d]	5.0–10.0	NA
Low positive (IU/ml)	NA	NA	NA	NA	10.0–14.9
Positive range (IU/ml)	≥10.0	>10.0	≥10.0	≥10.0	≥15.0

[a] MUB, methylumbelliferyl.
[b] IU/ml, international units per milliliter.
[c] RUB-1-94, WHO standard (1st International Standard).
[d] NA, not applicable.

in 1-ml vials for future use. No sample underwent more than three freeze-thaw cycles.

Tests. Samples were tested using the manufacturer's instructions in one of five CIAs: Architect rubella IgG (Abbott Diagnostics, Abbott Park, IL), AxSYM rubella IgG assay (Abbott Diagnostics, Abbott Park, IL), Vidas Rub IgG II (bioMérieux, Durham, NC), Vitros rubella IgG (Ortho Clinical Diagnostics, Buckinghamshire, United Kingdom), and Elecsys rubella IgG (Roche Diagnostics, Mannheim, Germany). A description of the characteristics of each CIA is presented in Table 1. To determine the anti-rubella virus IgG status, all 500 samples were tested in a commercial HAI and IB assay. To assess the accuracy of quantification, the samples were tested in the IH-EIA.

(i) HAI assay. All 500 samples were tested in the Siemens RubeHIT (Siemens Health Care, Marburg, Germany). Briefly, nonspecific agglutinins were removed by incubating samples and controls in a kaolin suspension (250 g per liter) for 20 min at room temperature. The samples were centrifuged at 3,000 × *g* for 10 min, and the supernatant was retained. Doubling dilutions of the supernatant were made in a $CaCl_2$-bovine albumin-NaCl-$MgSO_4$ (CANM) saline solution, provided with the kit. A standard concentration of rubella antigen, at 4 to 8 hemagglutinating units per 25 μl, was added to each dilution, and the reaction mixture was incubated for 1 h at room temperature. A 4% (vol/vol) suspension of human erythrocytes was added to each well, and the reaction mixture was incubated overnight at room temperature. The highest dilution in which the hemagglutination was inhibited was considered the endpoint titer. All test results were scored by each of two different individuals without reference to the results determined by the other. Any discrepant results were reviewed by both readers, and a consensus result was obtained. Samples with HAI results of 1:16 or greater were deemed to be HAI positive. To ensure that any lack of reactivity was not due to technical error, tests of samples with a HAI titer of 1:8 or less were repeated in duplicate. Samples with HAI results repeatedly less than or equal to 1:8 were considered HAI negative.

With each HAI test run, negative, low-positive, and high-positive controls, provided by the manufacturer, were tested. Negative, low-positive, and high-positive external controls with known reactivity were also tested in each test run. Each control was required to produce an HAI titer within 1 doubling dilution of its target result for the run to be considered valid. No runs were invalid.

(ii) Immunoblot analysis. All HAI-positive samples were tested in the recombBlot rubella IgG (Mikrogen Diagnostik, Neuried, Germany) according to the manufacturer's instructions. The nitrocellulose strips provided by the manufacturer contained recombinant rubella antigens which were separated by SDS-PAGE and transferred to the nitrocellulose membrane. Briefly, 20 μl of sample or control was diluted in 2 ml of Tris buffer and incubated with the nitrocellulose strip containing rubella antigen overnight at room temperature with gentle shaking. After incubation, the strips were washed and a rabbit anti-human IgG-horseradish peroxidase conjugate was added. The strips were incubated for 1 h at room temperature and washed. Tetramethylbenzidine substrate was then added, forming a color reaction where anti-rubella virus IgG present in the sample was bound to the rubella antigens (E1, E2, c, and an E1/E2 complex) on the strip. An E2 weak-positive control, provided by the manufacturer, was tested with each set of 20 samples. The intensity of the color reaction of the E2 control acted as an assay cutoff, with any band being considered positive if its intensity was greater than that of the intensity of E2 control band. All results were scored independently by two different individuals, and any discrepant readings were resolved by consensus. Any sample having one or more reactive bands was deemed IB positive. To ensure that any lack of reactivity was not due to technical error, all samples with a negative IB result were retested in a single assay.

(iii) In-house EIA. An IH-EIA was developed by NRL. Microtiter flat-bottom plates (Nunc, Roskilde, Denmark) were coated with 50 μl of rubella virus antigen (HPV-77 strain) (MyBioSource, San Diego, CA) at a concentration of 0.5 μg/well in a carbonate buffer (pH 9.6) and incubated overnight at 37°C. After washing in PBS-T (phosphate-buffered saline [pH 7.4] containing 0.01% [vol/vol] Tween 20), 150 μl of blotto (50 mM Tris-HCl [pH 8.0], containing 5% skim milk powder, 2 mM $CaCl_2$, 150 mM NaCl, and 0.2% Nonidet P-40) was added, and the reaction mixture was incubated for 1.5 h at 37°C to block nonspecific binding. Plates were washed three times with PBS-T, and then 10 μl of control or sample was diluted in 90 μl of blotto and added to each well. A plate shaker was used to ensure adequate mixing before incubation at 37°C for 1 h. After being washed with PBS-T, 100 μl of mouse anti-human IgG conjugated to horseradish peroxidase (Southern Biotechnology Associates, Birmingham, AL) diluted 1:1,000 in blotto was added to each well, and the reaction mixture was incubated for 1 h at 37°C. After washing, 100 μl of substrate, 2 mM ABTS (2′-azinobis [3-ethylbenzthiazoline-6-sulfonic acid]), in 25 mM sodium citrate buffer (pH 4.5) containing 0.3% hydrogen peroxide was added to each well, and the reaction mixture was incubated in the dark at room temperature for 20 min. The reaction was stopped with the ad-

TABLE 2 Qualitative test results obtained from five commercial immunoassays compared with an anti-rubella virus IgG status[a]

Assay	Samples with negative rubella IgG status						Samples with positive rubella IgG status					
	Total no. of samples	No. of samples with indicated assay result			Concordance with status (%) (95% CI)		Total no. of samples	No. of samples with indicated assay result			Concordance with status (%) (95% CI)	
		Negative	Equivocal	Positive	Equivocal result considered negative	Equivocal result considered positive		Negative	Equivocal	Positive	Equivocal result considered negative	Equivocal result considered positive
Abbott Architect	23	13	5	5	69.2 (48.1–84.9)	50.0 (30.3–69.6)	77	1	7	69	89.6 (80.0–95.1)	90.9 (81.6–96.0)
Abbott AxSYM	9	6	2	1	NA[b]	NA	91	2	5	84	92.3 (84.3–96.6)	97.8 (81.5–99.6)
bioMérieux Vidas	6	6			NA	NA	94	6	18	70	74.5 (64.2–82.7)	93.6 (86.1–97.4)
Ortho Vitros	32	29	1	2	93.8 (77.8–98.9)	90.6 (73.8–97.5)	68	6	7[c]	55	80.9 (69.2–89.0)	91.1 (81.1–96.4)
Roche Elecsys	44	29		15	65.9 (50.0–79.1)	NA	56	5		51	91.1 (79.6–96.7)	NA

[a] Concordance of commercial immunoassay results with status, including 95% confidence limits (95% CI), were estimated by considering equivocal results to be both negative and positive.
[b] NA, not applicable.
[c] Seven Ortho Vitros results between 10 and 15 IU/ml were considered low positive rather than equivocal.

dition of 50 μl of 5% oxalic acid. The optical density of each control and sample was read at 405 nm.

Each microtiter plate contained an eight-point standard curve. The standard curve was constructed using duplicate, doubling dilutions of the WHO international anti-rubella immunoglobulin standard (RUBI-1-94) starting at a concentration of 200 IU/ml. An anti-rubella virus IgG external quality control (QC) sample (AcroMetrix, Benicia, CA), calibrated at 20 IU/ml, was tested at least once in each plate. The results of the QC sample were used to determine the assay's repeatability and reproducibility. To determine the linearity of the IH-EIA, a secondary standard, independently calibrated against RUBI-1-94 (2°STD; AcroMetrix, Alkmaar, The Netherlands), and consisting of seven doubling dilutions from 68 to 1.0625 IU/ml was tested and quantified using the standard curve. The IH-EIA was validated using a panel of known anti-rubella virus antibody-positive ($n = 95$) and -negative ($n = 25$) samples.

Testing strategy. All 500 samples were screened using the HAI. All samples having an HAI titer of 1:8 or less were assigned a negative anti-rubella virus antibody status. As HAI detects both rubella virus IgG and IgM, any sample that had an HAI titer of greater than 1:8 was tested in the IB assay. If the IB result was negative, the sample was assigned a negative anti-rubella virus IgG status. If the IB result was positive, that sample was assigned a positive anti-rubella virus IgG status. All 500 samples were then tested on the IH-EIA.

Analysis. The qualitative results of the CIAs were compared with the assigned anti-rubella virus IgG status derived from the HAI and IB testing; where more than 20 results were available for analysis, the percentage of concordance with the assigned status and 95% confidence intervals (95% CI) were determined for each CIA. The quantitative results reported by the CIAs were analyzed using a receiver operating characteristic (ROC) analysis (Analyze-it for Excel; Analyze-it Software, Leeds, United Kingdom) in order to predict the CIAs ' sensitivities and specificities (12, 21). The quantitative results obtained from the CIAs were also compared with those obtained from the IH-EIA using the coefficient of determination (R^2) and Bland-Altman analyses (Analyze-it for Excel; Analyze-it Software, Leeds, United Kingdom) (22–24).

RESULTS

All samples that were initially negative in the HAI and IB were negative on repeat testing. The qualitative results reported by each of the five CIAs were compared with the assigned anti-rubella virus IgG status. The percentage and 95% CI of CIA qualitative results that were concordant with the assigned status were estimated by assuming equivocal results to be either negative or positive (Table 2). As each CIA had a different set of 100 samples analyzed, different proportions of the 100 samples were assigned a positive or negative status. The range of quantitative test results reported by each CIA for samples assigned a negative or positive status is represented graphically in Fig. 1. Only the bioMérieux Vidas reported all samples with a negative status as negative ($n = 6$) but reported 6 and 18 samples assigned a positive status ($n = 94$) as negative and equivocal, respectively. The Roche Elecsys reported 5 of 56 samples with a positive status as negative and 15 of 44 samples with a negative status as positive.

A total of 23 samples assigned a negative status, ranging from 0 to 15 samples per assay, were reported as positive by a CIA (Table 3). Although assigned a negative status by the testing strategy, 19 of the 23 samples had a HAI titer of 16 or greater. All 19 had a negative IB results. HAI detects antibody reactivity against E1 antigens but not E2 or core. Three of the remaining four samples had an HAI titer of 8 and a positive IB result, with evidence of antibody reactivity to E2 antigen. Only 1 of the 23 samples had negative HAI and IB results. The highest positive CIA test result obtained on a sample with a negative status was 36 IU/ml, reported by the Abbott AxSYM. The 15 Roche Elecsys-positive results obtained from samples with a negative status ranged from 10 to 35 IU/ml. A total of 20 samples assigned a positive status, ranging from 1 to 6 samples per assay, were reported as negative by a CIA. All 20 samples were positive for both the HAI and IB tests.

The CIA results were used to perform ROC analysis. Using a cutoff of 10 IU/ml, the ROC analysis was used to determine the predicted sensitivity and specificity, including the 95% CI, of the CIAs for this population of samples (Table 4). The predicted sensitivity for the CIAs ranged from 70.2% for the bioMérieux Vidas to 91.2% for both the Ortho Vitros and the Abbott AxSYM. The predicted specificity ranged from 65.9% for the Roche Elecsys to 100% for the bioMérieux Vidas. However, it is noted that the confidence limits, especially for the specificity calculations, were large due to the relatively low number of samples assigned a negative status. Further, the sensitivity and specificity are not reflective of the assays' performances when testing a normal population, as the samples in this study were selected as having low positive reactivity.

The 23 QC test results obtained from 10 test runs gave a mean of 25.1 IU/ml and a coefficient of variation (CV), expressed as a percentage, of 19.6%. A further 39 QC test results, obtained from a single run, gave a mean of 26.5 IU/ml and a CV of 9.4%.

The 2°STD was tested in two test runs, once in duplicate and once in a single assay, for a total of three test results for each of the seven panel members. When the results of the 2°STD test were

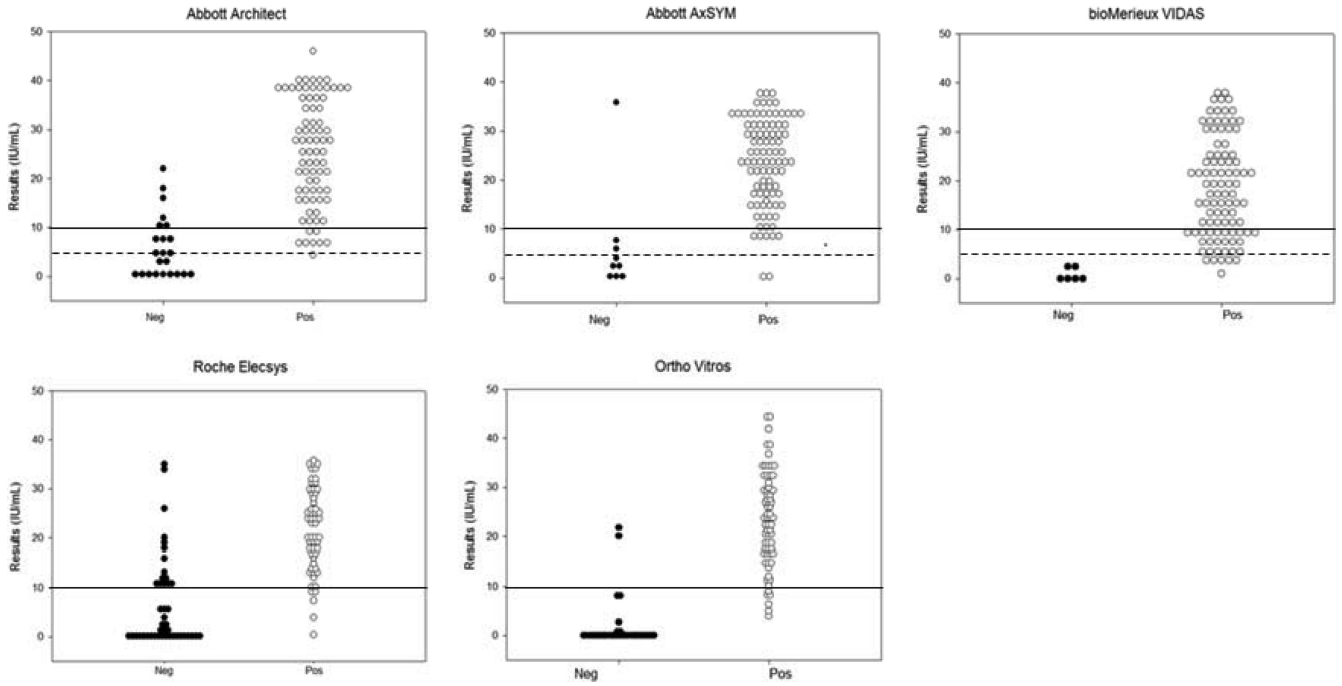

FIG 1 Dot histograms of the quantitative test result, expressed in international units per milliliter (IU/ml), of 100 low-positive (Pos) and negative (Neg) results obtained from each of five commercial immunoassays, for a total of 500 results, plotted against an assigned negative or positive status. The assay's cutoff is represented with a horizontal line, and equivocal ranges are represented with a hashed line.

plotted against the expected value, the R^2 was 0.99 and the equation describing the correlation was $y = 0.90x + 6.26$.

Of the 500 samples, 497 were tested in the IH-EIA. Three samples had insufficient volume to complete the testing. Of the 497 samples tested, 115 were assigned a negative status and 382 a positive status when tested in the HAI and the IB assay. The quantitative results reported by the CIAs were compared with those reported by the IH-EIA. Using Bland-Altman analysis, the IH-EIA quantitative results were within the 95% confidence limits of agreement for all CIA quantitative results up to approximately 60 IU/ml. At concentrations higher than this, the IH-EIA consistently reported levels of anti-rubella virus IgG that were higher than those reported by the CIAs. The R^2, slope, and bias of the comparison of IH-EIA quantitative results with the corresponding CIA result were estimated with and without the IH-EIA results greater than 60 IU/ml (Table 5). When the samples having an IH-EIA result of greater than 60 IU/ml were removed, the R^2 ranged from 0.210 to 0.421, indicating a lack of correlation between the results of the IH-EIA and each of the CIAs. When all test results were used to calculate the R^2, the R^2 values were even lower. Using Bland-Altman analysis, the IH-EIA had a positive bias compared with each of the CIAs, ranging from 3.71 to 9.11 IU/ml.

TABLE 3 The number and range of quantitative test results reported by five commercial anti-rubella virus IgG immunoassays that were discordant compared with an assigned anti-rubella virus IgG status[a]

	CIA-positive results for samples assigned a negative status		CIA-negative results for samples assigned a positive status	
Assay	No.	Range (IU/ml)	No.	Range (IU/ml)
Abbott Architect	5	11–22	1	4.3
Abbott AxSYM	1	36	2	0.0–0.5
bioMérieux Vidas	0		6	1.0–4.0
Ortho Vitros	2	20–22	6	3.9–9.1
Roche Elecsys	15	10–35	5	0.4–9.5

[a] CIA, commercial anti-rubella virus IgG immunoassay.

DISCUSSION

The instructions for use (IFU) of the international rubella standard state that "RUB-1-94 consists of freeze-dried residuals of 2.0 ml of a mixture of normal human immunoglobulin and an equal volume of sterile distilled water." It was calibrated against the second International Standard for Rubella Serum and was estimated to contain 1,600 IU per ampoule. This standard has been used to calibrate commercial anti-rubella virus IgG assays since 1995 (8–10). The European *in vitro* diagnostics directive 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" (25). However, others have questioned the practicality of standardizing biologicals that are traceable not to Système International d'Unités (SI) units but to arbitrary units such as WHO international standards, stating that "immunogenic proteins such as viral proteins are generally highly complex and heterogeneous mixtures in biological fluids" (26). In these cases, the reference materials and the biological fluids are therefore "nonidentical," which consequently invalidates the basic principle of traceability: to compare like with like (27). Indeed, the WHO standard is polyclonal in nature and it is generally not possible to create a secondary standard or certified reference material that would behave in the same manner when tested in the same immunoassay. The shape of the curve generated by the secondary standards would always differ from that generated by the WHO stan-

TABLE 4 Estimation of the sensitivity and specificity, using receiver operating characteristic analysis with a cutoff set at 10 international units per milliliter, of five commercial immunoassays using samples having low-level and negative results to anti-rubella virus IgG[a]

ROC analysis parameter	Abbott Architect	Abbott AxSYM	bioMérieux Vidas	Roche Elecsys	Ortho Vitros
Curve area	0.93	0.88	1.00	0.88	0.97
Predicted specificity (%)	78.3	88.9	100	65.9	93.8
95% confidence interval	56.3–92.5	51.8–99.7	54.1–100.0	50.1–79.5	79.2–99.2
Predicted sensitivity (%)	89.6	91.2	70.2	89.3	91.2
95% confidence interval	80.6–95.4	83.4–96.1	59.9–79.2	80.4–97.0	81.8–96.7

[a] ROC, receiver operating characteristic.

dard, and this would be most apparent at the lower region of the curve (28). Considering the differences in the formulations of the CIAs (Table 1), in particular, in the antigen(s) bound to the solid phase, a lack of standardization between CIAs is not surprising.

Past studies have demonstrated a lack of standardization between some assays used to quantify anti-rubella virus IgG in serum (8, 9). In conjunction with a lack of standardization, vaccination programs have resulted in lower levels of anti-rubella virus IgG being detected in the population (2, 3). This lack of standardization and the number of individuals having vaccine-induced low-level anti-rubella virus IgG can cause difficulties in the interpretation of the results, especially when the result is close to the cutoff of the assay (7, 29). Studies indicate that the sensitivities and specificities of many commercial EIAs are similar (9, 14, 30, 31). However, as most CIAs use 10 IU/ml as a cutoff for immunity, different qualitative results for the same sample are reported by different assays. Therefore, results generated from an individual's sample that is tested in one assay cannot be compared with results obtained in other assays. So when results are obtained from acute and convalescent samples and each sample is tested in different assays, the results may resemble a seroconversion to anti-rubella virus IgG, with the early sample testing negative in one assay and later samples testing positive in another. Potentially, these results may be interpreted as evidence of a recent rubella virus infection. This situation may lead to anxiety for the patient or even a recommendation for termination of pregnancy. Therefore, consecutive samples from the same individual should be tested together with the same assay (11).

As new commercial immunoassays are introduced to the market, scientists comparing the results obtained from the new assay with those obtained from their routine assay experience difficulty in elucidating discordant test results. Testing samples with discordant test results on a third assay is not recommended (32, 33). Comparison of qualitative results with those obtained from a gold standard reference test is preferred (12, 28). For anti-rubella virus IgG testing, HAI, viral neutralization, and Western blot analyses have been considered appropriate reference tests, although very few laboratories worldwide retain the expertise for neutralization testing (6, 15, 16, 19). Further, these tests are manual and complex and also subject to variation (15, 16, 34). Of the 23 samples assigned a negative status but having a positive CIA result, 22 had a positive result in either HAI or IB testing but a negative result in the other test. The negative result may have been due to a lack of sensitivity of the assay. Also, HAI detects only antibodies to E1 antigen. This phenomenon caused three samples to be assigned a negative status in the testing strategy, whereas the CIA reported a positive result and the IB assay had evidence of E2 antibody reactivity. The HAI can detect anti-rubella IgM, whereas the IB test and the CIA detect only IgG-specific antibodies. The presence of anti-rubella virus IgM may explain why some samples were HAI positive but IB negative. These results underline the difficulty of selecting a reference testing strategy to confirm qualitative anti-rubella virus IgG results.

The qualitative test results of the CIAs were compared with a status determined by HAI and IB testing. If the equivocal results in the CIAs were assumed to be positive for anti-rubella virus IgG, the qualitative results of all CIAs gave greater than 90% concordance with the assigned positive status. If the equivocal results in the CIAs were assumed to be negative, the percentages of concordance of the qualitative test results on the Abbott Architect and Roche Elecsys with the assigned negative status were poor, at 69.2 and 65.9%, respectively. However, the concordance of the qualitative results on the Ortho Vitros with assigned negative status was higher at 83.8%. These findings are not indicative of the sensitiv-

TABLE 5 Summary of the analysis of correlation between quantitative results, reported as international units per milliliter obtained from five commercial anti-rubella virus IgG immunoassays and an in-house immunoassay calibrated with the World Health Organization international standard rubella virus serum RUB-1-94[a]

	Commercial EIA				
Analysis category and parameter	Abbott Architect	Abbott AxSYM	bioMérieux Vidas	Roche Elecsys	Ortho Vitros
Samples with IH-EIA results less than 60 IU/ml					
Coefficient of determination (R^2)	0.421	0.210	0.276	0.227	0.420
Slope	0.58	0.52	0.42	0.41	0.49
Intercept (IU/ml)	2.48	14.04	5.78	6.41	4.42
All samples					
Coefficient of determination (R^2)	0.354	0.106	0.169	0.063	0.110
Slope	1.35	0.90	1.12	0.60	0.94
Intercept (IU/ml)	10.50	15.12	15.12	20.57	23.41

[a] IH-EIA, in-house immunoassay; IU/ml, international units per milliliter.

ities and specificities of the assays for testing an unselected population, as the samples in the study were preselected for low positive reactivity. Generally, CIAs have sensitivity greater than 98% and specificity of greater than 85% (9, 31).

The IFU for the two Abbott assays and bioMérieux Vidas specify equivocal ranges from 5.0 to 9.9 IU/ml, whereas the Ortho Vitros IFU specify an equivocal range of 10.0 to 14.9 IU/ml. The Roche Elecsys IFU do not state an equivocal range. Only the Abbott AxSYM and the Roche Elecsys reported test results greater than 30 IU/ml. No CIA reported a positive result greater than 36 IU/ml for a sample that was assigned a negative status. The use of a strict cutoff of 10 IU/ml invariably results in the reporting of false-positive and -negative test results. Many Australian laboratories testing for anti-rubella virus IgG choose to report results between 10 and 30 IU/ml as positive but add a clinical interpretation stating that the clinical significance of the result is in doubt. The sources for this uncertainty are the imprecision of the assay and biological false reactivity of some samples in the assay. Establishing and reporting a gray zone allows laboratories to follow the manufacturer's instructions while acknowledging the uncertainty of the clinical interpretation at low levels of antibodies.

The qualitative nature of the reference test results does not aid in determining the accuracy of quantification of antibodies. The present study evaluated quantitative CIA results by testing sets of samples having negative or low-level rubella virus IgG in an IH-EIA, which was directly calibrated using RUBI-1-94 and designed to be linear at low levels of anti-rubella virus IgG. Results obtained from the IH-EIA were shown to be precise, with repeatability (within-run precision) of less than 10% and reproducibility (between-run precision) of 20%. Excellent correlation with results obtained from the independent secondary standard, 2°STD, indicated that the IH-EIA was accurate to a level of about 60 IU/ml. When the quantitative results of CIAs were compared with those obtained from the IH-EIA, the level of correlation was very poor for all CIAs and the IH-EIA had a positive bias ranging from 3.7 to 9.1 IU/ml. This would support the theory that CIAs lack standardization, particularly at low levels of rubella virus IgG, even though the calibrators of all but one CIA were traceable to RUBI-1-94.

All assays experience imprecision (35). In our experience conducting QC programs for infectious disease testing for more than 10 years, results of testing the same QC sample on manual microtiter plate assays generally show variation of less than 20% whereas those from instrument-based serology assays show variation of less than 15%. The results of a peer-comparison QC program using a QC sample calibrated at 20 IU/ml against RUBI-1-94 demonstrated that the imprecision of CIAs ranged between 5 and 20%, with a measurement uncertainty (MU) of about 2 to 5 IU/ml (unpublished data). That is, when the MU is 5 IU/ml, a result of 10 IU/ml has a 95% confidence of being between 5 and 15 IU/ml. Therefore, the interpretation of low-positive test results is difficult when both the imprecision and lack of standardization (bias) of the assays are considered.

This study has demonstrated the difficulty in both standardizing assays designed to detect and quantify antibodies and, consequently, using quantitative results to set immune/nonimmune cutoffs. Both imprecision and bias contribute to these difficulties (35). However, the main contributing factor to the lack of standardization is the poor implementation of traceability protocols. When the WHO standard was developed, there was insufficient definition of the analyte as required in traceability. The approach taken did not consider factors such as biological variation and the complexity of the proteins being detected, the reactivity of these antigens with antibodies of differing levels of avidity and affinity, the characteristics of the assay being used, and the stage of disease of the patient being tested (26, 27). Anti-rubella virus IgG testing has no quantitative reference method with known and defined uncertainty. Indeed, the results of testing of RUBI-1-94 were never published, no details of the methods for calibration are available, and the manufacturer's IFU indicates that "This study has almost been completed."

Although an international reference standard has been available since the 1980s and has been used to calibrate assays for the detection and quantification of anti-rubella virus IgG, it has not led to greater levels of standardization between commercial assays and issues reported in 1992 remain. It may be time for the scientific community to question the relevance of quantification of anti-rubella virus IgG. It may be possible for manufacturers of commercial assays to assign a cutoff for their assays by maximizing sensitivity and specificity using well-validated panels of samples with a known status, disregarding the WHO standard, and no longer reporting the anti-rubella virus IgG results in IU/ml but as a qualitative result.

ACKNOWLEDGMENTS

We acknowledge all laboratories providing samples for this study: Douglass Hanly Moir Pathology, San Pathology, Eastern Health Pathology, Pathology One, and Adelaide Pathology in Australia and Hackensack University Medical Center in the United States.

Equal financial sponsorship was provided by each manufacturer.

REFERENCES

1. **Gregg NM.** 1991. Congenital cataract following German measles in the mother. 1941. Aust. N. Z. J. Ophthalmol. **19**:267–276.
2. **Francis BH, Hatherley LI, Walstab JE, Taft LI.** 1982. Rubella screening and vaccination programme at a Melbourne maternity hospital. A five-year review. Med. J. Aust. **1**:502–504.
3. **Francis BH, Thomas AK, McCarty CA.** 2003. The impact of rubella immunization on the serological status of women of childbearing age: a retrospective longitudinal study in Melbourne, Australia. Am. J. Public Health **93**:1274–1276.
4. **Reef SE, Cochi SL.** 2006. The evidence for the elimination of rubella and congenital rubella syndrome in the United States: a public health achievement. Clin. Infect. Dis. **43**(Suppl 3):S123–S125.
5. **Robertson SE, Cutts FT, Samuel R, Diaz-Ortega JL.** 1997. Control of rubella and congenital rubella syndrome (CRS) in developing countries, part 2: vaccination against rubella. Bull. World Health Organ. **75**:69–80.
6. **NCCLS.** 1997. Detection and quantification of rubella IgG antibody: evaluation and performance criteria for multiple component products, specimen handling, and use of test products in the clinical laboratory; approved guideline. NCCLS, Wayne, PA.
7. **Skendzel LP.** 1996. Rubella immunity. Defining the level of protective antibody. Am. J. Clin. Pathol. **106**:170–174.
8. **Dimech W, Bettoli A, Eckert D, Francis B, Hamblin J, Kerr T, Ryan C, Skurrie I.** 1992. Multicenter evaluation of five commercial rubella virus immunoglobulin G kits which report in international units per milliliter. J. Clin. Microbiol. **30**:633–641.
9. **Dimech W, Panagiotopoulos L, Francis B, Laven N, Marler J, Dickeson D, Panayotou T, Wilson K, Wootten R, Dax EM.** 2008. Evaluation of eight anti-rubella virus immunoglobulin G immunoassays that report results in international units per milliliter. J. Clin. Microbiol. **46**:1955–1960.
10. **Forsgren M.** 1985. Standardization of techniques and reagents for the study of rubella antibody. Rev. Infect. Dis. **7**(Suppl 1):S129–S132.
11. **Guillet G, Grangeot-Keros K, Vauloup-Fellous C.** 2012. Discrepancies between rubella IgG results: which assay is right, which assay is wrong? Abstr. 22nd European Congress of Clinical Microbiology and Infectious Diseases (ECCMID), poster P963. John Wiley and Sons, Inc., Hoboken, NJ.

12. **CLSI.** 2008. Clinical evaluation of immunoassays; approved guidelines—2nd ed. CLSI document I/LA21-A2 28. CLSI, Wayne, PA.
13. **Diepersloot RJ, Dunnewold-Hoekstra H, Kruit-Den Hollander J, Vlaspolder F.** 2001. Antenatal screening for hepatitis B and antibodies to *Toxoplasma gondii* and rubella virus: evaluation of two commercial immunoassay systems. Clin. Diagn. Lab. Immunol. **8**:785–787.
14. **Vlaspolder F, Singer P, Smit A, Diepersloot RJ.** 2001. Comparison of Iimmulite with Vidas for detection of infection in a low-prevalence population of pregnant women in The Netherlands. Clin. Diagn. Lab. Immunol. **8**:552–555.
15. **Public Health Laboratory Services Standing Advisory Committee on Viral Reagents.** 1978. Haemagglutination-inhibition test for the detection of rubella antibody. J. Hyg. (Lond) **81**:373–382.
16. **Cradock-Watson JE.** 1991. Laboratory diagnosis of rubella: past, present and future. Epidemiol. Infect. **107**:1–15.
17. **Cusi MG, Metelli R, Valensin PE.** 1989. Immune responses to wild and vaccine rubella viruses after rubella vaccination. Arch. Virol. **106**:63–72.
18. **Cusi MG, Rossolini GM, Cellesi C, Valensin PE.** 1988. Antibody response to wild rubella virus structural proteins following immunization with RA 27/3 live attenuated vaccine. Arch. Virol. **101**:25–33.
19. **Nedeljkovic J, Jovanovic T, Mladjenovic S, Hedman K, Peitsaro N, Oker-Blom C.** 1999. Immunoblot analysis of natural and vaccine-induced IgG responses to rubella virus proteins expressed in insect cells. J. Clin. Virol. **14**:119–131.
20. **Wilson KM, Di Camillo C, Doughty L, Dax EM.** 2006. Humoral immune response to primary rubella virus infection. Clin. Vaccine Immunol. **13**:380–386.
21. **Buttner J.** 1993. Evaluation of diagnostic and prognostic measures, p 25–30. *In* Haeckel R (ed), Evaluation methods in laboratory medicine. HCH, Weinhiem, Germany.
22. **Bland JM, Altman DG.** 1986. Statistical methods for assessing agreement between two methods of clinical measurement. Lancet **i**:307–310.
23. **Bland JM, Altman DG.** 1995. Comparing methods of measurement: why plotting difference against standard method is misleading. Lancet **346**: 1085–1087.
24. **Bland JM, Altman DG.** 1999. Measuring agreement in method comparison studies. Stat. Methods Med. Res. **8**:135–160.
25. **European Commission.** 1998. Directive 98/79/EC of the European Parliament and the Council on in vitro diagnostics (IVD) medical devices 1998. Official Journal of the European Communities. European Commission, Enterprise and Industry DG, Brussels, Belgium.
26. **Lequin RM.** 2002. Standardization: comparability and traceability of laboratory results. Clin. Chem. **48**:391–393.
27. **American Association for Laboratory Accreditation.** 2012. P102-A2LA policy on measurement traceability. The American Association for Laboratory Accreditation, Frederick, MD.
28. **Davies C.** 1994. Principles, p 23–46. *In* Wild D (ed), The immunoassay handbook. Stockton Press, New York, NY.
29. **O'Shea S, Dunn H, Palmer S, Banatvala JE, Best JM.** 1999. Automated rubella antibody screening: a cautionary tale. J. Med. Microbiol. **48**:1047.
30. **Greenwood NP, Ovsyannikova IG, Vierkant RA, O'Byrne MM, Poland GA.** 2010. A qualitative and quantitative comparison of two rubella virus-specific IgG antibody immunoassays. Viral Immunol. **23**:353–357.
31. **Medici MC, Martinelli M, Albonetti V, Chezzi C, Dettori G.** 2008. Evaluation of rubella virus immunoglobulin G (IgG) and IgM assays with the new Vidia instrument. J. Clin. Microbiol. **46**:1847–1849.
32. **Hadgu A.** 1996. The discrepancy in discrepant analysis. Lancet **348**:592–593.
33. **Miller WC.** 1998. Bias in discrepant analysis: when two wrongs don't make a right. J. Clin. Epidemiol. **51**:219–231.
34. **Schmidt NJ, Lennette EH.** 1970. Variables of the rubella hemagglutination-inhibition test system and their effect on antigen and antibody titers. Appl. Microbiol. **19**:491–504.
35. **Dimech W, Francis B, Kox J, Roberts G.** 2006. Calculating uncertainty of measurement for serology assays by use of precision and bias. Clin. Chem. **52**:526–529.