



Calibration and Evaluation of Quantitative Antibody Titers for Measles Virus by Using the BioPlex 2200

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ABSTRACT The BioPlex 2200 (Bio-Rad Laboratories, Hercules, CA) is a rapid, automated platform, which can screen large numbers of specimens for antibodies to measles, mumps, rubella, and varicella. Although approved for producing qualitative results, in this study we validated the test (off-label) to allow reporting of quantitative results. To do this, we used the third anti-measles World Health Organization standard to generate a calibration curve that allowed relative fluorescence intensity to be translated into quantitative antibody titer (antibody units [AU]/ml). The results from the BioPlex 2200 and the reference plaque reduction neutralization test (PRNT) exhibited a reasonable correlation following an exponential function, but correlation was poor in low-titer samples. Using a receiver operating characteristics analysis, an equivocal zone for the BioPlex 2200 was established between ≥ 0.13 and < 1.10 AU/ml to achieve 100% specificity (95% confidence interval [CI] = 83.2 to 100%) and 100% sensitivity (95% CI = 93.5 to 100%) versus PRNT. By determining an equivocal range requiring confirmation by PRNT, we can avoid underestimating the levels of immunity through false-negative results and optimize methods for seroepidemiological studies.

KEYWORDS BioPlex, immunobead, measles, quantitative, antibody titers, neutralizing antibody, immunity, seroepidemiology

The reemergence of measles and mumps over the last decade (1–3) has highlighted the need for countries to better understand the protective level of immunity within their populations. Seroepidemiology is an effective means of estimating population immunity in order to assess susceptibility to outbreaks and predict the risk of future epidemics of vaccine-preventable disease (VPD) (4). Measles, rubella, and varicella have well-defined thresholds of antibody titers which correspond to immunity, but there is currently no World Health Organization (WHO) standard or threshold defined for mumps (5–11).

One of the challenges facing epidemiologists is finding an appropriate test method for quickly assessing antibody titers on a large number of samples. Although neutralization assays are considered the gold standard for the quantitative determination of neutralizing antibody titers (12, 13), these assays are not ideal for seroepidemiology studies because they are cumbersome and labor-intensive. Most traditional enzyme immunoassays (EIA) used in diagnostic laboratories are also not ideal for seroepidemi-

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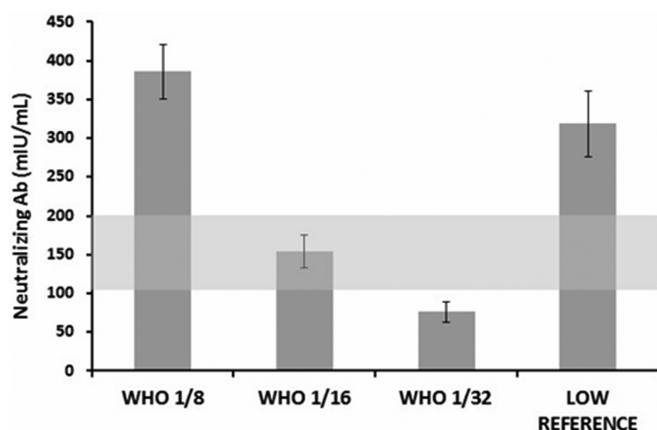


FIG 1 Calibration of PRNT to the WHO standard. Data were generated using eight replicates from different runs of 4-fold serial dilution of the third WHO international standard of measles immunity. The light gray area represents twice the 95% CI of the chosen threshold of immunity (1:16 dilution corresponding to 152 mIU/ml). The low reference serum tested significantly above the equivocal range.

ology studies. These assays often generate a qualitative result (immune or nonimmune) and are sometimes more specific than they are sensitive, especially at lower antibody titers, to ensure that susceptible individuals are not missed (14, 15). Rather than a binary positive or negative result, seroepidemiology studies require specifically validated methods that provide accurate quantitative antibody titer data describing different levels of antibodies spanning the entire spectrum of immunity, (16–18). Statistical models are used to calculate susceptibility thresholds from the antibody titer. These thresholds can be calculated for each age group and/or birth cohort. In addition, traditional EIA methods require multiple reactions to evaluate protection from multiple VPDs. Multiplex methods that measure multiple analytes in a single reaction offer the benefit of streamlined testing, which is imperative when screening the large numbers of sera needed for seroepidemiology.

The BioPlex 2200 MMRV IgG assay (Bio-Rad Laboratories, Hercules, CA) is a rapid, highly automated test that is capable of high-throughput screening of large numbers of specimens (19). It provides a qualitative interpretation of serology against measles, mumps, rubella, and varicella (MMRV). Specimens tested on the platform are reported as positive (immune), equivocal, or negative (nonimmune). Previous work has shown that the qualitative results produced by this commercially available assay correlate well with existing EIA methods (20). Because the BioPlex 2200 MMRV IgG assay generates a quantifiable value (relative fluorescence intensity [RFI]), it is possible that this assay can be used as a quantitative method, which is off-label from the commercial product. In the present study, we used the anti-measles WHO standard to generate a calibration curve that correlated RFI to quantitative antibody titer. This allowed us to compare the calculated measles antibody titer from the BioPlex 2200 MMRV IgG assay titer to the plaque reduction neutralization test (PRNT) reference method (21).

RESULTS

Calibration of PRNT using the WHO standard. The threshold of immunity was extrapolated to 180 mIU/ml based on the first international standard (4, 22) and to 120 mIU/ml based on the second international standard (21). The threshold of immunity using the third international standard is still considered to be 120 mIU/ml by PRNT based on equivalence with the second international standard (23, 24). We determined that a 1:16 dilution of the third international standard measured at 152 ± 20 mIU/ml (95% confidence interval [CI]), and it was not significantly different from 120 mIU/ml threshold since it is within the equivocal range of twice the 95% CI (25). We therefore used this dilution as the standard to set the range of equivocal PRNT result at 112 to 192 mIU, based on twice the calculated 95% CI (Fig. 1). This corresponds approximately

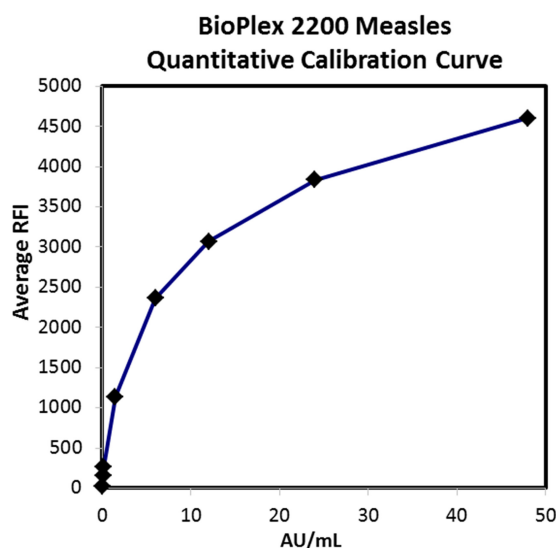


FIG 2 Calibration curve in units of relative fluorescent intensity (RFI) and AU/ml. Twofold serial dilutions of the WHO measles third international standard (3,000 to 5.5 mIU/ml) were tested on the BioPlex 2200 in triplicate. The RFIs from the BioPlex 2200 MMRV IgG assay were used to generate a calibration curve to allow the calculation of titers expressed in AU/ml. The generation of the calibration curve allows for standardization of RFI values and correction for variation between runs and reagents.

to a probability of >95% that a measurement above or below the equivocal range is a true positive or negative, respectively.

Calibration of the BioPlex 2200 using the WHO standard. Overall, the BioPlex 2200 MMRV IgG assay had good replicate precision ranging from 1.6 to 5.3% (% coefficient of variation). Using 2-fold serial dilutions of the WHO measles third international standard, the calibration curve fits well ($R^2 = 1.000$), with the calculated results in antibody units (AU)/ml close to the expected values (Fig. 2 and 3). The qualitative numeric value of 1.1 AI is equivalent to the quantitative value of 1.1 AU/ml (Fig. 4). Overall, titers generated by the BioPlex 2200 MMRV IgG assay and PRNT correlated best with an exponential function ($y = 0.1159e^{0.0061x}$, $R^2 = 0.64$) (Fig. 5). The considerable variance between the titers measured by BioPlex 2200 MMRV IgG assay and PRNT is due to low correlation between the titer of neutralizing antibodies measured by PRNT and the titer of total antibodies measured by EIA methods, especially at lower values (14, 15, 26).

Determination of threshold for best agreement between the two methods using ROC. PRNT titers in the 148 residual samples ranged from 7.2 to 1,023.7 mIU/ml (85 immune, 40 nonimmune, and 23 equivocal). Given that the results from the BioPlex 2200 MMRV IgG assay and the PRNT correlated poorly in low-titer samples, we used receiver-operating-characteristic (ROC) curve (27, 28) analysis to define an equivocal zone for the BioPlex 2200 MMRV IgG assay and determine the cutoff value for the best agreement of the BioPlex 2200 MMRV IgG assay with PRNT (Fig. 6). The ROC analysis, which assumed all equivocal results as negative, showed a negative agreement (specificity) of 100% (95% CI = 94.3 to 100%) using a positive cutoff of ≥ 1.1 AU/ml (red line; Fig. 5) for the BioPlex 2200 MMRV IgG assay. The positive agreement (sensitivity) was 64.7% (95% CI = 54.1 to 74.0%) (Table 1). All specimens that tested below 0.13 AU/ml by the BioPlex 2200 MMRV IgG assay (blue line Fig. 5) were also determined to be negative by PRNT (Table 1). Using the established BioPlex 2200 and PRNT positive cutoff plus an established equivocal range, the positive and negative agreement was 100%.

DISCUSSION

An ideal immunoassay for seroepidemiological studies would be accurate, easy to use, and capable of measuring multiple markers at once. We demonstrate here that the BioPlex 2200 MMRV IgG assay can be used to generate quantitative antibody titers to

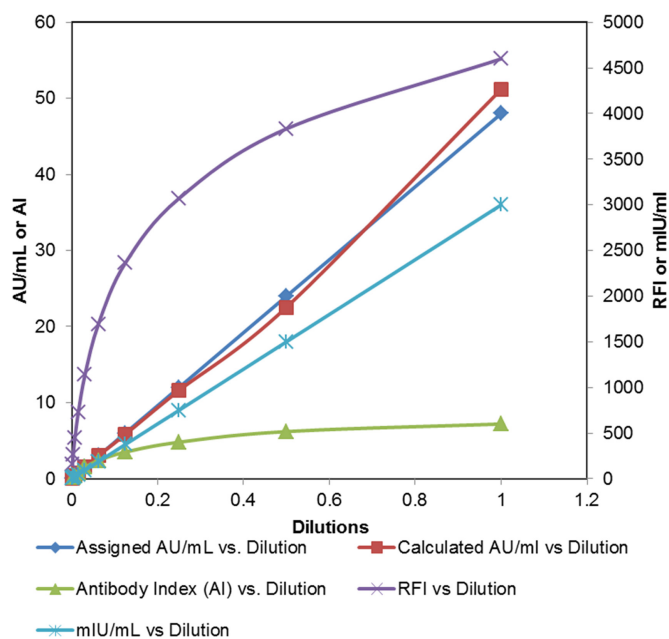


FIG 3 Evaluation of the third WHO measles international standard using the BioPlex 2200 measles IgG assay. Data were generated using three replicates from 2-fold serial dilutions of the third WHO international standard of measles immunity, which has an undiluted concentration of 3,000 mIU/mL. The assigned AU/mL are BioPlex 2200 values assigned to the WHO standard based on the dilution factor and used for calibration. The calculated AU/mL values are quantitative results generated by the BioPlex 2200 using a six-level 4PL (four-parameter logic log) calibration curve. The antibody index (AI) values are qualitative results generated by the BioPlex 2200 using a two-point calibration curve. The RFI values are raw signals generated by the BioPlex 2200.

measles. In this study, the correlation of the quantitative result of the BioPlex 2200 MMRV IgG assay and the reference method (PRNT) is higher for “nonequivocal” specimens (>192 mIU/mL) and specimens with low concentrations of neutralizing antibody do not correlate well. This observation has been previously reported for other EIA methods and is attributed to the fact that PRNT only measures neutralizing IgG, whereas EIA-based tests measure total anti-measles IgG (14, 15, 20, 26, 29). This lack of sensitivity may lead to an underestimation of the number of protected individuals in seroepidemiological studies.

Previous data looking at qualitative results using two different EIAs showed reduced sensitivity for determining immunity compared to PRNT with approximately false-negative results in 10% of sera which correlated to low levels of neutralizing antibody (30). Rabenau et al. (31) found that although EIA and neutralization methods had good

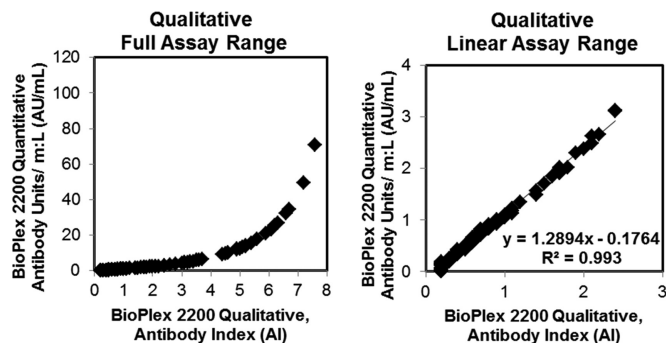


FIG 4 Qualitative versus quantitative BioPlex 2200 analyses. (Left) The qualitative full assay is calibrated with two calibrators and therefore has a reduced linear range compared to the quantitative assay, which used a six-level 4PL calibrator math model, resulting in a larger dynamic range. (Right) The linear range of the qualitative assay is ~3 AI.

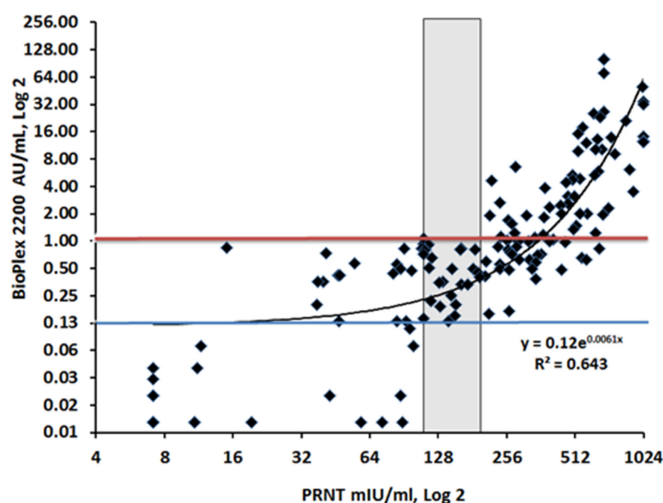


FIG 5 Quantitative results on the BioPlex correlate best with PRNT at titers above 1.10 AU/ml and below 0.13 AU/ml. The gray rectangle shows the “equivocal” range of PRNT (between 112 and 192 mIU/ml). The red line represents the cutoff for BioPlex (1.10 BioPlex 2200 AU/ml) above which BioPlex shows 100% specificity compared to PRNT for this set of data. The blue line represents a cutoff for BioPlex (0.13 BioPlex 2200 AU/ml) below which BioPlex shows 100% sensitivity compared to PRNT.

correlations (Spearman = 0.71), 1.5% of the samples had false-negative EIA results. However, the neutralization assay in that study was not calibrated to the WHO standard, and these authors considered a neutralization titer of 1/10 protective (31). Another study (15) evaluating serum taken from 9-month-old infants 4 weeks after vaccination found that automated EIA methods failed to detect 263 of 454 seroconversions detected by PRNT. In that study, the PRNT was calibrated against the WHO second international standard, and the majority of those that failed documented seroconversion by EIA had lower levels of neutralizing antibodies (<470 mIU/ml).

Our study used the current WHO third international standard to ensure the comparability of results between the two assays. The potency of this standard has been shown to be ~2-fold higher for enzyme-linked immunosorbent assay-based tests than for PRNT, unlike the first and second international standards (25), and therefore we did not attempt to convert the BioPlex MMRV IgG results into mIU/ml.

The poor performance at low antibody levels may be a result of the differences between the test methods. Neutralization is commonly accepted as the most sensitive

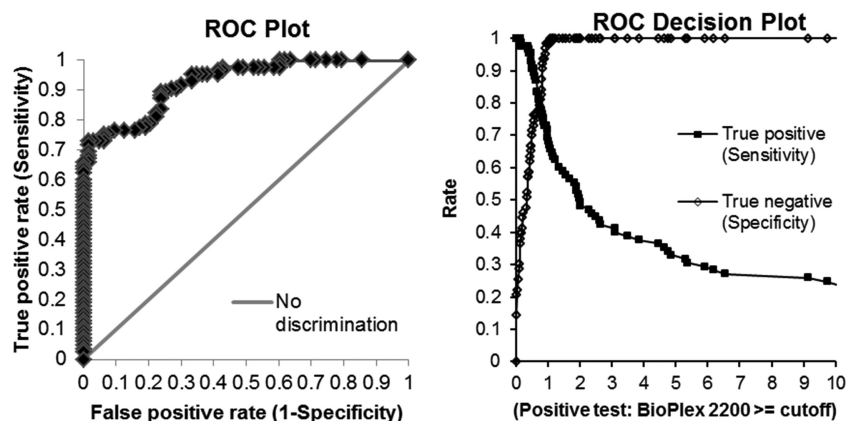


FIG 6 ROC analysis of BioPlex 2200 versus PRNT. ROC analysis was performed for the BioPlex 2200 measles IgG assay to determine the positive cutoff required to achieve 100% specificity versus PRNT immunity status, as well as the cutoff necessary to achieve 100% sensitivity. This information was used to establish the BioPlex 2200 equivocal zone for sample testing by PRNT. For the ROC analysis, 148 samples were evaluated; all equivocal samples were considered nonimmune.

TABLE 1 BioPlex 2200 measles immune status agreement versus PRNT immune status using the BioPlex quantitative cutoff derived by ROC analysis^a

PRNT result	Measles antibody titer (AU/ml) ^b		
	Positive	Negative	Total
Positive	55	30	85
Negative	0	63	63
Total	55	93	148

^aThe cutoff was determined to be all equivocal samples considered negative (nonimmune). Percent agreement: positive, 64.7 (54.1 to 74.0); negative, 100 (94.2 to 100).

^bConsidered positive if >1.1 .

assay for detecting protective antibodies. The BioPlex 2200 MMRV IgG assay captures IgG antibodies directed at all measles antigens, including those that may not contribute to immunity, such as antibodies directed against nucleoprotein. PRNT measures with greater sensitivity neutralizing antibodies, directed at specific epitopes of the surface hemagglutinin and fusion proteins (24, 30, 32). These differences make direct comparison of the two assays problematic, especially at low antibody titers (14, 15, 26). A study examining sera from health care workers in the Netherlands found that the seropositivity rates of four different qualitative EIAs compared to PRNT ranged from 89 to 97%, with the greatest variation in the cohort born in from 1975 to 1985, suggesting waning immunity in individuals with antibodies generated from a single MMR vaccine rather than natural exposure. The EIA that performed best was an in-house Luminex bead-based multiplex assay that used purified whole virus that provided an efficient display of the measles glycoproteins (29). Dorigo-Zetsma et al. found that equivocal results in the EIAs were positive by PRNT; however, in the present study, 26/74 equivocal BioPlex specimens were not immune by PRNT. Based on the validation data in this study, specimens with antibody titers determined to be between 0.13 and 1.10 AU/ml by the BioPlex 2200 MMRV IgG assay will require retesting using PRNT. Samples of ≥ 1.1 and <0.13 AU/ml for the BioPlex 2200 measles IgG assay exhibit 100% agreement with PRNT regarding immune status. This is consistent with previous data showing EIAs and PRNT titers correlated well when PRNT titers were <8 and $>1,052$ with most discrepancies occurring in the range of 8 and 120 (14).

The PRNT is cumbersome, technically demanding, and slow. It has a turnaround time of 7 days, making it a poor assay for high-throughput screening. The BioPlex 2200 MMRV IgG assay has the potential to simplify the screening of large numbers of specimens. It has a higher throughput and reduced turnaround time compared to automated conventional plate-based EIA (20). By combining both a high-throughput screening assay and the PRNT reference method, one can maximize the efficiency for screening while maintaining accuracy in specimens with low levels of antibodies.

The method of screening samples by EIA and confirming them by PRNT on select specimens is not a new concept (15, 26). In a serological study of infants at 4 weeks postimmunization, 27% of the specimens with an EIA result below a predetermined cutoff to maximize sensitivity and specificity required PRNT to ensure that all seroconversions were detected (15). By establishing an equivocal zone for the BioPlex 2200 MMRV IgG assay between 0.13 and 1.10 AU/ml, we can limit the number of specimens requiring confirmation by PRNT and ensure that we have accurate results for seroepidemiological studies. Assuming that 85% of the population has antibodies above the threshold for immunity to measles (based on laboratory seropositivity data in Nova Scotia [data not shown]), we would expect that only 15 to 20% of the specimens in a serosurvey study will require PRNT confirmatory testing. However, this may still pose a challenge in developing countries where PRNT is not readily available.

Our study does have limitations. The serum samples used had no clinical information associated with them. As such, we could not determine whether the antibodies were due to vaccination or natural infection. Both factors can influence the performance of EIA testing. As such, it is not possible to determine the true risk of acquiring

measles if exposed in an individual whose specimens fall within the BioPlex 2200 equivalent zone. The upper-limit quantitative threshold of the BioPlex is 48 AU/ml, and thus specimens with results above this level may not generate accurate titers. However, this would be well above the defined surrogate marker of immunity.

Despite its lower sensitivity similar to other EIA formats, we showed here that the BioPlex 2200 MMRV IgG assay can be used to generate quantitative data and that, by establishing an equivocal zone, we can limit the number of specimens needing confirmation by PRNT. This maximizes the throughput and accuracy needed for sero-epidemiological studies. Although the present study is limited to measles, to further streamline seroepidemiology testing, future studies will use other WHO standards to generate calibration curves for the other analytes of MMRV and allow for the quantification of antibody titers to multiple analytes in a single small-volume reaction. This will be particularly important when using sera collected in large prospective cohort studies, such as the Canadian Health Measures Study (33), that have limited specimen volumes.

MATERIALS AND METHODS

Study design. A total of 148 anonymized residual patient specimens submitted for routine testing of immune status, previously categorized as immune ($n = 50$), nonimmune ($n = 50$), or equivocal ($n = 48$) using the Enzygnost measles IgG assay (Siemens Healthcare Diagnostics, Oakville, Ontario, Canada), were tested by the BioPlex 2200 MMRV IgG assay at the Nova Scotia Health Authority's QEII Microbiology Laboratory (Halifax, Nova Scotia, Canada). Further testing using PRNT was performed at the National Microbiology Laboratory (Winnipeg, Manitoba, Canada). These sera were stored at -20°C and, because they were anonymized, no clinical information was available regarding age, sex, or vaccine status. The samples were chosen based on their estimated immunity status (to allow thorough validation using equivocal and negative specimens) and do not represent current population immunity in Canada. Both the numeric titers and the qualitative categorical results of the BioPlex 2200 MMRV IgG assay were compared to the PRNT results. The local Institutional Review Board at each study site obtained ethics approval for the use of anonymized residual sera and approved the overall study design.

BioPlex 2200 MMRV IgG. The BioPlex 2200 MMRV IgG assay is a multiplex flow immunoassay that simultaneously detects and identifies antibodies to multiple antigens in a single test reaction (19, 20). The BioPlex 2200 system combines 5 μl of patient sample, sample diluent, and a reagent containing a population of four different dyed microspheres coated with different antigens to detect the presence of IgG antibodies for measles, mumps, rubella, and varicella-zoster viruses. The dyed bead identity is determined by the fluorescence of the dyes and the quantity of antibody captured by the antigen is determined by the fluorescence of an anti-human IgG-phycoerythrin-labeled conjugate. Raw data are calculated in relative fluorescence intensity (RFI). When run on the BioPlex 2200 instrument, the RFI was normalized to an antibody index (AI), which is a qualitative numeric result, using a two-level calibration curve. The AI values are displayed to the operator. The sample AI result is compared to established negative and positive ranges, <0.9 AI (negative) and ≥ 1.1 (positive), to generate a qualitative status (positive, negative, or equivocal). The generation of the calibration curve is necessary to standardize RFI and correct for variation between runs and reagents. For the purpose of this study, we used the RFI values from the BioPlex 2200 MMRV IgG test results to generate a calibration curve using dilutions of the WHO measles third international standard, which allowed us to calculate antibody quantitative titers. BioPlex 2200 measles quantitative results are expressed as antibody units (AU)/ml in order to differentiate the quantitative result from the qualitative AI. BioPlex results cannot be directly converted into mIU/ml using the third international standard. Previous studies have established that the third international standard has different potencies for PRNTs and EIAs (25). As such, we felt it is more accurate to describe the titer in terms of AU. To generate a calibration curve, 2-fold serial dilutions of the WHO measles third international standard (3,000 to 5.5 mIU/ml) were tested on the BioPlex 2200 in triplicate. BioPlex 2200 measles AU/ml values for the WHO third international standard were derived by assigning the 1/32 dilution a value of 1.5 AU/ml, which equals 1.5 AI. The 3,000 mIU/ml standard was assigned a value of 48 AU/ml ($1.5 \text{ AU/ml} \times 32$). The calculated value of 48 AU/ml was used to determine the values for all other standard levels. The calculated value was divided by the dilution factor for all other standard levels.

Plaque reduction neutralization. Measles-specific neutralizing antibodies were measured using plaque reduction neutralization (adapted from reference 21). A dilution series of heat-inactivated sera was incubated with the Edmonston strain of measles virus for 2 h to neutralize it. The mixture was then inoculated on a confluent layer of Vero cells (American Type Culture Collection, CCL-81). The cells were overlaid with medium containing 2% carboxymethyl cellulose and, after 5 days of growth, the cells were fixed and stained to assess measles plaque formation. The plaques formed in each well of the dilution series of serum-neutralized virus, and of the nonneutralized virus control, were counted and used to determine the 50% neutralizing dose (ND_{50}) of the serum with the Karber formula [$\log_{10} \text{ND}_{50} = m - \Delta(\Sigma P - 0.05)$], where m is the \log_{10} of the highest dilution, Δ is the constant interval between dilutions expressed as \log_{10} and ΣP is the sum of all the proportions of number of plaques/average number of plaques for the virus control. The value was converted to mIU/ml using a unit

constant calculated by comparing the predicted concentration of the WHO third international standard to its ND_{50} in each assay run. The PRNT was calibrated to the WHO third international standard for anti-measles (National Institute for Biological Standards and Controls, UK, code 97/448) using eight replicates from different runs of 4-fold serial dilutions of the international standard. A low-titer serum from an individual immunized with three doses of MMR vaccine was also used as a reference for PRNT.

Statistical methods. A four-parameter logistic regression model was used for the quantification of measles IgG using the BioPlex 2200 MMRV IgG assay. BioPlex 2200 MMRV IgG AU/ml results were compared to the PRNT averages, and the 95% CIs and regressions were calculated using Microsoft Excel. The ROC was calculated using Analyze-It software (Analyze-It Software, Ltd., United Kingdom).

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H.S. is employed by Bio-Rad Laboratories. The other authors have no conflicts of interest to declare.

T.F.H., H.S., S.B., N.S.C., and A.S. were involved in the conception and design of the study. C.J. and E.M. were responsible for the creation and coordination of specimen panels, specimen testing, and collation of the data. T.F.H., H.S., A.S., S.B., and N.S.C. analyzed and interpreted the data. T.F.H., H.S., S.B., and A.S. drafted the manuscript. All authors revised the manuscript critically for important intellectual content. All authors reviewed and approved the final draft of the manuscript.

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