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Comparison of two commercial ELISA kits for detection of rubella specific IgM in suspected congenital rubella syndrome cases and rubella IgG antibodies in a serosurvey of pregnant women

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ABSTRACT

Enzyme linked immunosorbent assay (ELISA) for antibody identification, is important for laboratory confirmation of rubella infection in different settings. The Enzygnost rubella ELISA, widely used in the World Health Organization (WHO) Global Measles and Rubella Laboratory Network, is expensive and often unavailable. Qualitative and quantitative performance of the Euroimmun ELISA was compared with the Enzygnost ELISA, for detection of rubella specific IgM, using 283 sera collected from suspected congenital rubella syndrome (CRS) patients and IgG antibodies using 435 sera from a serosurvey among pregnant women.

Good qualitative agreement was observed for detection of both rubella specific IgM (94.7% agreement and κ of 0.86) and IgG (96.3% agreement and κ of 0.84). Bland–Altman analysis for IgG yielded a mean difference of 0.781 IU/ml with 97.1% values within ±2 SD of the mean difference.

Our study findings suggest that Euroimmun ELISA may be considered for detection of rubella specific IgM in suspected CRS cases and rubella specific IgG in surveillance studies.

1. Introduction

Rubella is an acute, contagious vaccine preventable viral infection, which usually causes a mild exanthematous febrile illness in children or young adults. Infection with rubella virus during pregnancy, particularly in the first trimester, can severely affect the fetus and may result in miscarriage, stillbirth or birth of infant with congenital malformations known as congenital rubella syndrome (CRS) (Murhekar et al., 2018).

In September 2013, the World Health Organization (WHO) Regional Committee for South-East Asia decided to adopt the goal of measles elimination and rubella/CRS control in the South-East Asia Region by 2020 (Thapa et al., 2015). The Government of India is committed towards this goal. India introduced measles-rubella (MR) vaccine in 2017 and launched mass vaccination campaign targeting children aged 9 months to <15 years with plans to expand the campaigns in a phased manner (Murhekar et al., 2018). Laboratory based surveillance for measles and rubella is well established in India. Surveillance for CRS was initiated in six sentinel sites in 2016 (Murhekar et al., 2018).

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The first serosurvey to estimate rubella sero-prevalence among pregnant women, was conducted between March–June 2017, at these sentinel sites (Muliyil et al., 2018).

Enzyme linked immunosorbent assay (ELISA) for antibody detection plays an important role in laboratory confirmation of congenital rubella syndrome and postnatal rubella. Laboratory tests recommended for confirmation of CRS include all of the following: (a) detection of rubella specific IgM antibodies (b) sustained rise of rubella specific IgG antibodies and (c) rubella virus detection by reverse transcription polymerase chain reaction (RT-PCR) (WHO, 2007). In addition, detection of rubella specific IgG antibodies is important to estimate the seroprevalence in different populations, including pregnant women (Gilbert et al., 2017; Shih et al., 2016).

In the facility based surveillance for CRS established in India, a commercial ELISA kit (Euroimmun, Luebeck, Germany) is used for laboratory diagnosis, after considering factors like ease of procurement, timeliness of supply, experience of laboratory experts and cost. In the present investigation, performance of Euroimmun ELISA was evaluated against another reference ELISA kit – the Enzygnost Rubella ELISA kit (Siemens Healthcare GmbH, Henkestr. Erlangen, Germany). This kit is widely used across the WHO Global Measles and Rubella Laboratory Network (GMRLN), including in India. Comparability of results was studied, for detection of rubella specific IgM and IgG antibodies, among suspected cases of CRS and pregnant women respectively.

2. Methods

2.1. Ethics statement

The study was approved by the Institutional Ethics committees of all sentinel surveillance sites. Participants were enrolled in the study after obtaining written informed consent from pregnant women and parents / guardians of infants.

2.2. Study population and case definition

Suspected cases were recruited as per WHO-recommended standards for CRS surveillance (Murhekar et al., 2018). As per the surveillance protocol, any infant, less than 1 year of age having any of the following criteria was considered as suspected CRS: (a) structural heart defect (b) hearing impairment (c) any of the following eye signs – cataract, glaucoma, pigmentary retinopathy (d) maternal history of fever with rash during pregnancy (e) strong clinical suspicion (including features of growth retardation, microcephaly, hepatomegaly, mental retardation etc.). Suspected CRS cases were identified among infants (0–11 months) attending pediatrics, ear nose and throat (ENT), ophthalmology and cardiology Outpatient Departments (OPDs) of six sentinel hospitals. Suspected CRS patients meeting any of the following criteria were considered as laboratory confirmed CRS: (a) detection of rubella specific IgM antibodies (b) sustained rise of rubella specific IgG antibodies, as determined on at least two occasions between 6 and 12 months. In addition, oropharyngeal (OP) swabs were collected from infants aged ≤5 months and rubella virus was detected by reverse transcription polymerase chain reaction (RT-PCR) assay and positive samples were genotyped (WHO, 2007). Pregnant women attending antenatal clinics at the sentinel hospitals between March–June 2017 were eligible to participate in the serosurvey.

2.3. Laboratory investigations

2.3.1. CRS surveillance

Sera from all suspected CRS patients were aliquoted, with one aliquot used for testing at sentinel surveillance site, one stored as back up at site and one stored at −80 °C until transported to the Indian Council of Medical Research (ICMR)- National Institute of Virology (NIV), Pune, on dry ice. Sera samples from suspected CRS patients collected between December 2016 and October 2017 were tested once a week, at the sentinel sites for detection of rubella specific IgM and IgG antibodies using Euroimmun ELISA (Euroimmun, Luebeck, Germany). As per the laboratory algorithm (Murhekar et al., 2018), results interpreted as positive, negative and equivocal were conveyed to the treating physician. Equivocal samples were re tested in duplicate and best of three results reported. For any patient whose result still remained equivocal (IgM and /or IgG), a follow up sample at an interval of four weeks was collected and tested.

OP swabs were processed, aliquoted, stored at −80 °C and dispatched on monthly basis, on dry ice to NIV, Pune for testing.

2.3.2. Rubella serosurvey

Serum samples ($n = 1800$) collected from the pregnant women were tested for rubella specific IgG antibodies (Euroimmun) at the sentinel sites between March–June 2017 (Muliyil et al., 2018). Results were interpreted as positive, negative and equivocal. No follow up samples were collected in case of equivocal results. As a part of quality control, 25% positive and 20% of negative samples from each site selected randomly were sent to NIV, Pune for retesting. (Samples with equivocal results were not included for quality control testing as this could confound the final analysis including calculation of sensitivity, specificity Cohen's kappa etc.)

Procurement of ELISA kits was centralized and all the sentinel sites were provided kits transported in cold chain.

Laboratory investigations at NIV, Pune.

All samples from suspected CRS patients were retested for IgM antibodies using both Euroimmun and Enzygnost Rubella ELISA. Similarly, samples from serosurvey were repeat tested for IgG antibodies using both Euroimmun and Enzygnost Rubella ELISA. Rubella specific IgG titres were extrapolated from a four-point standard curve comprising calibrators with different concentrations of antibodies, as per manufacturer's instructions. Titers above the upper limit of detection of 200 IU/ml were denoted as 201 IU/ml and values below lower limit of detection (1 IU/ml), were taken as 0.5 IU/ml (Euroimmun). Titres for IgG (Enzygnost) were obtained using the α and β constants provided by the manufacturer. The interval between ELISA testing at sentinel sites and NIV, Pune was 4–6 weeks.

Samples, whose results were discordant between the two kits, were retested in duplicate and 2 of 3 results were considered final. Chemiluminiscence assay (CLIA) was performed at two of the network laboratories for samples which remained discordant after testing in triplicate. The VITROS 3600 Immunodiagnostic system (Orthoclinical Diagnostics, Raritan, NJ) and the Liaison (DiaSorin, Saluggia, Italy) systems were used for testing IgM and IgG respectively. A sample was assigned as positive or negative when concordance of at least two of the three methods was obtained (Butchko and Jordan, 2004).

All OP swabs were tested only at NIV, Pune. Diagnostic RT PCR assay for all OP swabs and genotyping for representative positive samples was carried out as per WHO guidelines (WHO, 2007). Briefly, the viral RNA was extracted using QIAamp Viral RNA Kit (Qiagen Hilden, Germany Catalog no: 52906) according to manufacturer's protocol. RT PCR was performed using Superscript III one step RT PCR kit (Invitrogen, Carlsbad, California, USA Catalog no: 12574–026) to amplify a 185-nucleotide region in the E1 coding region (nucleotides 8807 to 8991) using forward primer (5′-CAA CAC GCC GCA CGG ACA AC-3′) and two reverse primers (5′-CCA CAA GCC GCG AGC AGT CA-3′) and (5′-CCA CGA GCC GCG AAC AGT CG-3′). After the RT step for 30 min at 50 °C and denaturation for 5 min at 95 °C, the reaction mixture was incubated for 35 cycles of 95 °C for 30 s, 60 °C for 30 s, and 72 °C for 1 min followed by extension at 72 °C for 10 min. The positive control was RNA from rubella Virus Vaccine Strain RA 27/3. Negative Controls were included with each set of clinical specimens. The PCR products were resolved on 2% agarose gel, stained with SYBR gold nucleic acid gel stain (Life technologies corporation, Eugene, Oregon, USA, Catalog no: S11494) and visualized.

2.4. Measurement of intra assay and inter assay precision

The intra assay precision was calculated by testing duplicates of randomly selected samples within a single run, by the two ELISA systems for both rubella specific IgM and IgG. For determination of inter assay precision, positive kit controls of same lot provided by the manufacturer, were run repeatedly on different days. The coefficient of variation (CV) was calculated for the OD values.

The mean absorbance of positive control wells was compared between the two kits for rubella specific IgM and IgG. The characteristics of the kits used in the study are provided (Supplementary Table 1).

2.5. Statistical analysis

Qualitative agreement between the two kits was assessed for both rubella specific IgM and IgG by calculating percent agreement and Cohen's kappa coefficient (k) statistic to assess the degree of (inter-rater) agreement for serum antibody status. Standard formulae were used to calculate percent agreement and the relative sensitivity and specificity of results by Euroimmun kit considering Enzygnost as the reference standard. As titers estimated by both IgG kits were not normally distributed (Kolmogorov–Smirnov Z = 3.15 for Enzygnost and 4.81 for Euroimmun kit), a logarithmic transformation of original data was performed. Paired quantitative measurements of rubella specific IgG ($n = 422$) were then compared by Bland - Altman analysis and plots (Bland and Altman, 1986). The results ($n = 13$) which had the value zero by either/both kits were not included in quantitative comparison by Bland Altmann plot. Statistical analysis was performed using STATA software (Version 13, StataCorp, Texas, USA) and Open Epi version 3 software.

3. Results

A total of 283 sera from 253 suspected CRS patients and 435 sera from pregnant women were available for testing. The median age of 253 suspected CRS patients was 2 (IQR: 0–7) months; 70.4% of them were below the age of 5 months while 29.6% were aged ≥6 months. Diagnostic RT-PCR for rubella was positive in 31 (21.1%) of 147 OP swabs from infants aged ≤5 months and genotype 2B was confirmed in seven representative samples from the different sites. The cases whose OP swabs confirmed PCR positive were all positive for rubella specific IgM.

3.1. Comparative performance of Euroimmun and Enzygnost kits for detection of rubella specific IgM

Around 28% (80/283) of the sera from suspected CRS patients were positive for IgM antibodies by Euroimmun ELISA and 22.9% (65/283) by Enzygnost. A concordance of 94.7% (268/283) was observed for rubella IgM results tested by Euroimmun and Enzygnost assays. Cohen's kappa (κ) was 0.86 (95% CI: 0.75–0.98), with a sensitivity of 100% (95% CI: 94.4–100) and specificity of 93.1% (95% CI: 88.96–95.79) on comparing Euroimmun with Enzygnost results (Table 1). Of fifteen discordant samples, nine were of sufficient quantity to be tested by CLIA for anti-rubella IgM (Table 2). All except one of the samples could be classified as positive or negative. If the borderline result of this sample is considered positive, (as the value is close to upper cut off of 1.1), a final positive result may be reported.

The mean absorbance of positive control (PC) wells for 14 assays, by Euroimmun (1.38) was significantly higher ($p = 0.0009$) than Enzygnost PC, for 16 assays (1.02). Good reproducibility was observed for both ELISA systems within the same assay with an average CV of 5.27 for Euroimmun and 6.50 for Enzygnost assays (Table 3). Comparison of the inter assay performance showed that Enzygnost assay had a higher coefficient of variation (CV) than Euroimmun for both IgM and IgG (Table 3).

3.2. Evaluation of the performance of Euroimmun and Enzygnost kits for detection of rubella specific IgG

Test results of 435 samples were considered for estimation of percent agreement of rubella specific IgG by Euroimmun and Enzygnost kits. Three hundred and seventy one (85.3%) samples were positive by Euroimmun and 387 (89%) by Enzygnost assays respectively. Concordance of 96.3% (419/435) for Euroimmun vs. Enzygnost results was noted. Cohen's kappa of 0.84 (95% CI: 0.75–0.93) was observed, with sensitivity of 95.9% (95% CI: 93.4–97.4) and specificity of 100% (95% CI: 92.6–100) on comparing Euroimmun with Enzygnost results (Table 1). Of 16 discordant samples, five sera were available in sufficient quantity and tested by CLIA for anti-rubella IgG. All samples were negative by Euroimmun and CLIA and positive by Enzygnost, with final classification as negative.

The mean absorbance of positive control (1.36) of 14 ELISA assays by Euroimmun, was significantly higher ($p = 0.0025$) than Enzygnost positive control absorbance over 16 assays (1.045). Good reproducibility was observed for both ELISA systems within same assay with average CV of 5.76 for Euroimmun and 6.99 for Enzygnost assays (Table 3). Comparatively lower inter assay precision was noted with Enzygnost assay, with CV similar to that provided by the manufacturer - upto16.2% for IgM and14.8% for IgG.

Bland - Altman analysis (Fig. 1) for paired quantitative results of rubella specific IgG yielded a mean difference of 0.781 IU/ml (95% limits of

Table 1
Qualitative comparison of Euroimmun vs. Enzygnost IgM and IgG.

Euroimmun IgM ELISA	Enzygnost IgM ELISA		
	Positive	Negative	Total
Positive	65	15	80
Negative	0	203	203
Total	65	218	283
Euroimmun IgG ELISA	Enzygnost IgG ELISA		
	Positive	Negative	Total
Positive	371	0	371
Negative	16	48	64
Total	387	48	435

Table 2
Comparative analysis of nine samples with discordant results of rubella specific IgM.

Number of samples	Pattern of results	Final classification
5	Euroimmun: *Positive* Enzygnost: *Negative* CLIA: *Non-reactive*	Negative
3	Euroimmun: *Positive* Enzygnost: *Negative* CLIA: *Positive*	Positive
1	Euroimmun: *Positive* Enzygnost: *Negative* CLIA: *Borderline (1.04)*[*]	Indeterminate

CLIA = Chemiluminescence Immunoassays (CLIA).
Out of 15 discordant samples, only 9 were of sufficient quantity to be tested.
[*] *If borderline result is considered positive as the value is close to upper cut off of 1.1, final result may be reported as positive.*

Table 3
Intra assay and Inter assay precision.

Test	ELISA Kit	Intra assay precision		Inter assay precision		
		Number Tested	Average CV%	Number Tested	Mean(±SD) of Positive Control	Average CV%
IgM	Euroimmun	22	5.27	6	1.53 ± 0.12	8.46
	Enzygnost	18	6.50	6	0.49 ± 0.08	16.23
IgG	Euroimmun	32	5.76	4	1.44 ± 0.18	12.45
	Enzygnost	23	6.99	4	1.35 ± 0.24	17.5

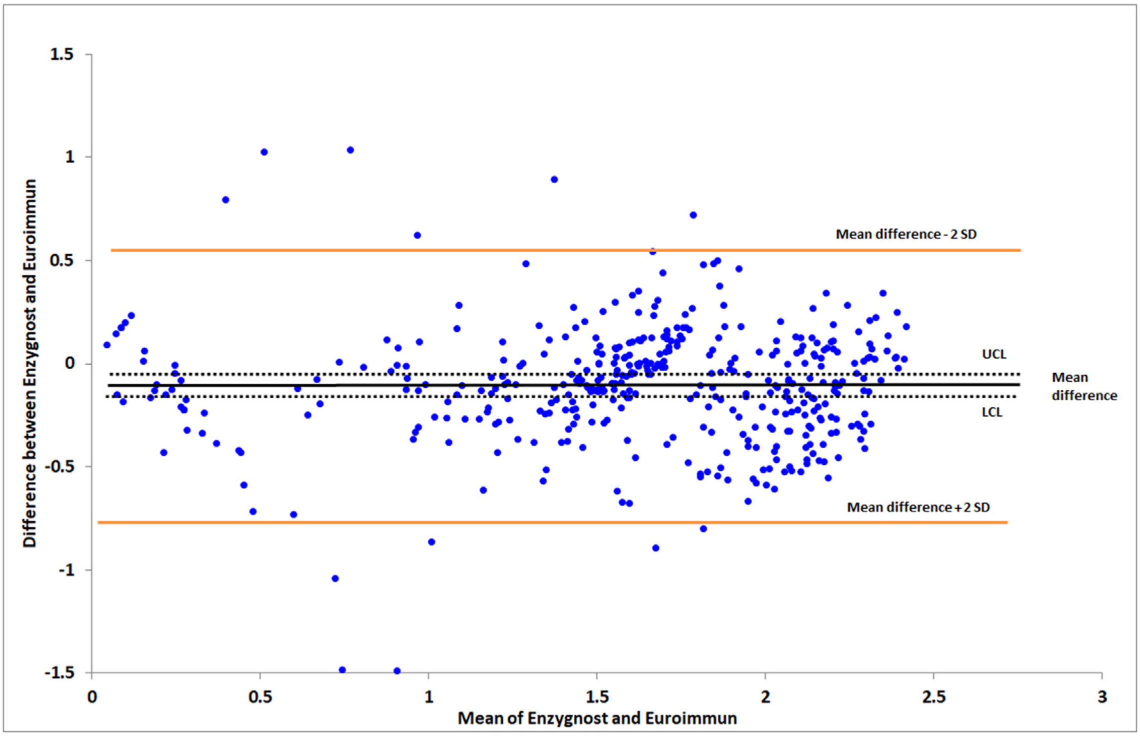

Fig. 1. Title Bland Altmann plot showing quantification of agreement between two methods with IgG titres transformed to logarithmic scale. Each dot represents paired reading of a single sample with the average of the two measurements (by Euroimmun and Enzygnost) plotted along the horizontal axis and the difference between the two methods plotted along the vertical axis.

agreement, 0.725 and 0.840 IU/ml). The majority of values (97.1%) were within ±2 SD of the mean difference. Euroimmun ELISA provided on an average, higher titres as compared to Enzygnost (Fig. 2).

4. Discussion

Serological studies of rubella are gaining importance in the light of the ongoing global control program for CRS. Detection of rubella specific IgM in suspected CRS cases remains the frontline diagnostic tool for laboratory confirmation and ELISA is one of the most common methods in use. Detection of rubella specific IgG by ELISA, is of value in population based studies, particularly among pregnant women, to understand their susceptibility to infection. The Enzygnost ELISA kit, which is widely used in the WHO network, is also the kit of choice for case based surveillance of measles-rubella in India. Although a well-established and validated kit, it is expensive (Supplementary Table 1) and often unavailable in many laboratories in India, which are not directly linked to the network or which are working on population based studies. This therefore requires the use of other commercial kits, causing variations in reporting of results.

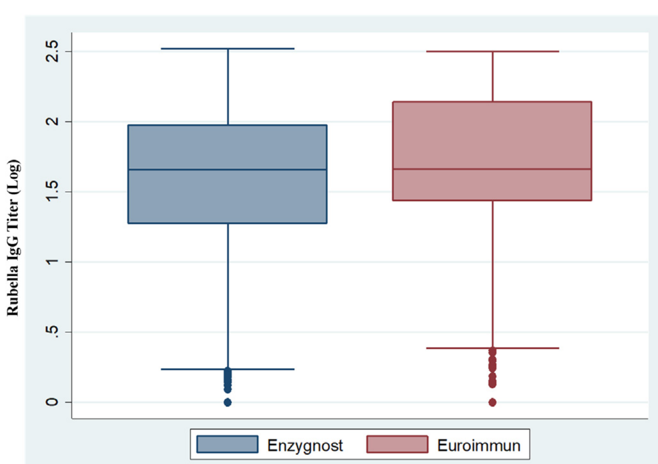

Fig. 2. Title Box plot distribution of IgG titers (log values – y axis) from two methods (x axis) Box plot showing distribution of rubella IgG titre values obtained by testing with two kits. The boxes demonstrate the quartiles of the titre values determined by both assays. The whiskers are extended to show the distribution of rest of the values. The line inside the box is the median and the dots below the whiskers indicate outliers.

Through the present study, a uniform strategy of laboratory testing for CRS (Murhekar et al., 2018) and population survey of pregnant women (Muliyil et al., 2018) was established for the first time in India. This study reports on evaluation of the comparability between Euroimmun and Enzygnost ELISA kits for rubella specific IgM in suspected cases of congenital rubella syndrome and rubella specific IgG in serosurvey of pregnant women. Previous reports from India on CRS in different "at risk groups" (Dewan and Gupta, 2012), have used different testing strategies for laboratory confirmation of CRS.

Our results demonstrate satisfactory performance of both kits. Good individual reproducibility was observed, as evidenced by intra assay precision for both kits, with slightly higher CV for inter assay precision (Table 3). Mean absorbance and extinction ratio of positive control wells for both IgM and IgG were significantly higher in Euroimmun assays. Both the Euroimmun and Enzygnost kits are based on the principle of indirect ELISA, with purified antigen obtained from inactivated cell lysate of rubella virus, propagated in different cell lines. Contamination to some extent with cellular by-products, leading to nonspecific binding and reactions is therefore expected, particularly for IgG (Thomas and Morgan-Capner, 1988), although it may be lower for IgM detection in young infants. The Enzygnost assay attempts to minimize nonspecific results, through use of a control antigen well, in addition to a viral antigen well for each sample (Pedneault et al., 1994). In addition a correction factor is applied, which is calculated by dividing the nominal value of anti-rubella reference Positive/Negative (P/N) with the mean test result of reference P/N. This would have contributed to lower extinction ratios for the Enzygnost assays.

Our data shows a good qualitative agreement between the two ELISA kits for detection of both anti rubella IgM and IgG (Table 1). Sensitivity was observed to be 100% for Euroimmun IgM testing as compared to Enzygnost results, with specificity of 93.1%. This indicates that while no false negative results were identified by Euroimmun, there is a risk of false positivity, which was addressed in the present study by including a third testing strategy- CLIA. Considering that inter rater agreement, in this case inter kit agreement, may be due to chance, we have applied Cohen's kappa statistics to understand the true agreement between the two kits. Our data ($\kappa = 0.86$) shows “almost perfect agreement” as per Jacob Cohen's original interpretation (Cohen, 1960). Recent interpretation (McHugh, 2012) identifies the κ to indicate strong agreement. In cases of rubella specific IgG testing by Euroimmun, specificity was 100%, indicating that no false positive results were obtained. The risk of detection of false negative results remained, with a sensitivity of 95.9%. Cohen's kappa was 0.84, indicating almost perfect (Cohen, 1960) or strong agreement (McHugh, 2012) between the two tests. Availability of seronegative samples was limited, due to the overall high seropositivity observed in the study, which could contribute to the high relative specificity observed.

Discordance was observed in 15 (5.3%) of IgM and 16 (3.7%) of IgG results. While it was not possible to test all these samples by an alternative method (CLIA), those tested could be classified as true positive or negative based on best of three test results. Some of the discordant results observed in our study might be the result of variations in the types and source of antigen used in the different assays.

Quantitative comparison of IgG results by Bland Altman analysis was also satisfactory (Fig. 1). Some variation in rubella specific IgG results using different ELISA kits is expected and widely recognized (Dimech, 2016). Since the 1980s, rubella virus IgG assays have been calibrated against the same WHO international standard rubella virus serum-RUBI-1-94 (Dimech, 2016). RUBI-1-94 is not a pure or purified analyte and the reference method used to assign a value to RUBI-1-94 namely hemagglutination inhibition (HAI)/ radial hemolysis (RH) have been superseded by superior technology (Bouthry et al., 2016).

Data from our study (Table 1), shows that a slight overestimation of IgM positivity (~5%) is possible with Euroimmun ELISA. The issue could be resolved by correlation of reports of individual suspected CRS cases with clinical presentation and other investigational findings.

Resolution of IgG testing and interpretation is usually required for serosurvey in an adult population, where high population prevalence is expected. There is a possibility of reporting false negative IgG results using the Euroimmun kit, as noted in our study (~3%). An earlier evaluation of eight commercial rubella IgG assays also had a similar observation (Dimech et al., 2008). Reporting a false negative rubella specific IgG result is considered more acceptable than a false-positive result. Such reports can possibly lead to vaccination or anxiety for a pregnant woman (Dimech et al., 2008). However, a false positive report could result in missing a candidate for vaccination, who could get infected if exposed to the virus. The outcome of testing in CRS cases where sustained positivity of two sequential IgG test is required for confirming the diagnosis, is beyond the scope of this study.

Our study did not include equivocal samples in the comparative analysis. This was primarily because the comparisons were a quality control exercise; therefore, equivocal results were excluded as they could confound final analysis in terms of sensitivity, specificity and Cohen's kappa statistics. This limitation can be addressed through further studies including samples classified as equivocal by different kits. The variability of kits calibrated using the WHO second reference standard have been widely recognized and the role of “gray zone” reporting highlighted (Dimech et al., 2016).

In conclusion our results show good agreement of Euroimmun and Enzygnost kits for rubella specific IgM (qualitative) and IgG (qualitative and quantitative). Euroimmun ELISA may be considered an acceptable substitute to Enzygnost ELISA for detection of rubella specific IgM in suspected CRS cases and rubella specific IgG in surveillance studies.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.diagmicrobio.2019.01.009.

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Author contribution

Planned the study: *GNS, MVM, NG, RV, AMA*; Centre wise testing and interpretation: *AMA, MPS, VN, CR, AM, SN and MG*; CLIA analysis: *AMA, MPS*; Data compilation and analysis: *RV, SG, VJ;* QC*: VJ, SG and OK*; Statistical support: *SMJ;* First draft: *RV*; Critical review and final inputs: *GNS*. All authors read and approved the final draft of the manuscript.

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