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Detection of Rubella Virus-Specific Immunoglobulin G in Saliva by an Amplification-Based Enzyme-Linked Immunosorbent Assay Using Monoclonal Antibody to Fluorescein Isothiocyanate

A. J. VYSE,[1]* D. W. G. BROWN,[1] B. J. COHEN,[1] R. SAMUEL,[2] AND D. J. NOKES[3]

Enteric and Respiratory Virus Laboratory, Central Public Health Laboratory, London NW9 5HT,[1] and Department of Biological Sciences, University of Warwick, Coventry CV4 7AL,[3] United Kingdom, and Christian Medical College, Vellore, India[2]

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An immunoglobulin G (IgG)–capture enzyme-linked immunosorbent assay (ELISA) for rubella virus is described. The assay uses a fluorescein isothiocyanate (FITC)–anti-FITC amplification system. The detection limit of the ELISA was approximately 7 IU of rubella virus-specific IgG per ml of serum sample. For saliva samples the performances of the capture ELISA and previously described radioimmunoassay were assessed, and the results of those two assays were compared to the rubella virus-specific IgG result obtained by a commercial ELISA (Behring Enzygnost) with a panel of paired serum and saliva samples. This comparison showed that the capture ELISA with saliva was more sensitive than the radioimmunoassay and that the results correlated better with the serum IgG result than the results of the radioimmunoassay did, with an overall sensitivity of 82% and a rank correlation of 0.68, whereas the sensitivity and rank correlation for the radioimmunoassay were 74% and 0.45, respectively. For subjects of 10 years of age or younger, the ELISA with saliva had a sensitivity of 94% and a specificity of 100% compared to the results of the ELISA (Behring Enzygnost) for rubella virus-specific IgG with corresponding serum samples. The sensitivity was much lower for subjects ages 17 years or older. The assay may have wider epidemiological use with saliva specimens, particularly those from children.

Rubella virus (RV) is an enveloped virus with a positive-sense, single-stranded RNA genome. It belongs to the *Togaviridae* family and is the only member of the genus *Rubivirus* (16). The infection caused by RV in children or adults is usually mild, and patients with RV infection present with fever and skin rash. Many cases are asymptomatic. Accurate laboratory diagnosis of past or recent rubella is essential for both clinical and epidemiological studies and for the design and monitoring of vaccination programs (3, 16). Serological techniques that detect RV-specific immunoglobulin G (IgG) are the methods most commonly used for the diagnosis of past infection (3, 8).

For large epidemiological studies the collection of blood can be difficult, particularly for those populations outside the clinical environment and those disliking the invasive nature of venipuncture (10, 11). As a noninvasive alternative, saliva provides a body fluid that contains antibodies of diagnostic significance, and the antibody content of salivary crevicular fluid reflects that of plasma but has lower concentrations. It is, however, possible to detect antibodies to a variety of viral antigens in saliva, especially by use of sensitive antibody-capture assays (5, 10, 12). Furthermore, the collection of saliva specimens has several advantages over venipuncture: it is convenient and can be done by untrained persons, e.g., parents, and is painless and less hazardous than venipuncture, thus giving better access to large populations and hard-to-reach groups such as children.

Detection of salivary RV-specific IgG by radioimmunoassay has been described previously (13). While this assay is sensitive and well characterized, enzyme-linked immunosorbent assay (ELISA) is preferable because it avoids radioactive waste and is technically less demanding than radioimmunoassay and the technology involved is more easily transferable between laboratories. We describe here the development and evaluation of an antibody-capture ELISA for the detection of RV-specific IgG in saliva. The assay will be useful for both epidemiological and diagnostic studies.

* Corresponding author. Mailing address: Enteric and Respiratory Virus Laboratory, Central Public Health Laboratory, 61 Colindale Ave., London NW9 5HT, United Kingdom. Phone: 44 181 2004400. Fax: 44 181 2001569. E-mail: avyse@phls.co.uk.

MATERIALS AND METHODS

Saliva collection. Saliva was collected and extracted from sterile foam swabs (Malvern Medical Developments, Worcester, United Kingdom) as described previously (2, 19), except where stated below.

Sera, saliva, and paired serum-saliva panels. All samples were stored at −20°C until required for testing.

Control sera. RV IgG-positive (256 IU/ml) and RV IgG-negative (<4 IU/ml) sera from healthy blood donors were identified with a commercial ELISA kit (Behring Enzygnost; Behringwerke AG, Marburg, Germany). The World Health Organization (WHO) second international RV antibody standard (National Institute of Biological Standards and Controls, Potters Bar, United Kingdom) of 80 IU/ml was used to assess assay sensitivity.

Serum-saliva pairs. Four panels comprising 197 serum-saliva pairs were used (Table 1). All sera were tested by the Behring ELISA. Panel 1 consisted of 97 pairs that were positive for serum RV-specific IgG antibody and that were obtained from children involved in a study of measles-mumps-rubella (MMR) vaccination of preschool children. These samples were provided by E. Miller and M. Ramsay, Public Health Laboratory Service Communicable Disease Surveillance Center. Thirty-six of the saliva samples were collected with foam swabs, 30 were collected with the Orasure device (Epitope Inc., Beaverton, United Kingdom), and 26 were collected with the Omni-SAL device (Saliva Diagnostics Systems Ltd., Singapore); for 5 saliva samples the collection device was not recorded. The saliva samples were also tested by RV-specific IgG capture radioimmunoassay (GACRIA) (12). Panel 2 consisted of 24 pairs of samples negative for serum RV-specific IgG antibody. These samples were from a study of congenital RV infection in southern India (6). The saliva samples were collected with the Orasure device and were tested by the RV-specific GACRIA. Panel 3 consisted of 76 pairs of samples; 14 were negative and 62 were positive for serum RV-specific IgG antibody. The samples were from the Christian Medical College, Vellore, India. The saliva samples were collected with the Omni-SAL device and were tested by the RV-specific GACRIA. This panel was categorized

TABLE 1. Comparison of the amplification-based GACELISA and GACRIA for detection of RV-specific IgG in saliva with the Behring ELISA with serum[a]

Serum-saliva panel (no. of samples)	Age range	Sensitivity (%)		Specificity (%)		PPV (%)		NPV (%)		No. of samples with the following result[b]					
		G	R	G	R	G	R	G	R	B+, G+, R+	B+, G+, R−	B+, G−, R+	B+, G−, R−	B−, G−, R+	B−, G−, R−
1 (97)	3.5–4 yr	94.8	95.8	NA	NA	100	100	NA	NA	89	3	4	1	0	0
2 (24)	4 mo–6 yr	NA	NA	100	100	NA	NA	100	100	0	0	0	0	0	24
3a (55)	17–34 yr	60.8	29.4	100	100	100	100	16.7	10	15	14	0	22	0	4
3b (21)	5 mo–10 yr	90.9	90.9	100	90	100	90.9	90.9	90	10	0	1	1	1	9
Overall (197)	4 mo–34 yr	82	74.4	100	97.3	100	99	56.4	46.8	114	17	5	24	1	37

[a] G, amplification-based GACELISA; R, GACRIA; B, Behring ELISA; NA, not applicable; CI, confidence interval; +, positive result; −, negative result.
[b] No samples fell into the categories B−,G+,R+ or B−,G+,R−.
[c] The coefficient of agreement of the kappa statistic is based on comparison of the results of the Behring ELISA with those of the amplification-based GACELISA and the results of the Behring ELISA with those of GACRIA. It can be calculated only when both samples are positive and negative by the Behring ELISA.
[d] The *P* value in effect compares samples with B+,G+,R− and B−,G−,R+ results with samples with B+,G−,R+ results, which are the totals when the results of one method agree with those of the Behring ELISA and the results of the other method do not.

by the ages of the donors. Panel 3a consisted of 55 pairs of samples from subjects ages 17 to 34 years, and panel 3b consisted of 21 serum-saliva sample pairs from subjects ages 5 months to 10 years.

Anti-RV-FITC conjugate. Monoclonal antibody (MAb) to RV hemagglutinin (18) (Laboratory of Microbiological Reagents, Central Public Health Laboratory) was purified by a modification of the caprylic acid precipitation method and was conjugated to fluorescein isothiocyanate (FITC) as described previously (15, 17).

Rubella FITC–anti-FITC GACELISA. After a series of experiments to optimize assay conditions the following procedure was used for the rubella FITC–anti-FITC IgG antibody-capture ELISA (GACELISA).

Wells of microtiter plates (Immuno Module Maxisorb U8 immunoplates; Life Technologies Ltd., Paisley, United Kingdom) were coated with 100 μl of rabbit antibody to human IgG (gamma chain specific; Dako Ltd., Ely, United Kingdom), diluted 1/1,000 in 0.05 M sodium carbonate-bicarbonate buffer (pH 9.6), and incubated at 37°C for 18 h. After washing with phosphate-buffered saline (PBS) containing 0.05% Tween 20 (T20), the wells of each plate were incubated at 37°C on a plate shaker at 500 rpm successively with 100 μl of the following: undiluted saliva or a 1-in-100 dilution of serum in PBS containing 10% fetal calf serum (FCS) and 0.2% T20 for 30 min; RV hemagglutinin (Laboratory of Microbiological Reagents) diluted 1 in 10 in PBS containing 10% FCS and 0.2% T20 for 1 h; a 1/500 dilution of the anti-RV MAb–FITC conjugate in PBS containing 10% FCS, 5% normal rabbit serum (NRS), 2% human serum negative for anti-RV-specific IgG (NHS), and 0.2% T20 for 2 h; and mouse anti-FITC horseradish peroxidase-conjugated MAb (Chemicon International Inc., Temecula, Calif.) diluted 1/18,000 in PBS containing 10% FCS, 10% NRS, 2% NHS, and 1% T20 for 25 min. Finally, 100 μl of a 42 mM 2′,2′,4′,4′-tetramethylbenzidine substrate solution in a 0.1 M citrate-acetate buffer (pH 6.0) containing 1 to 3 mM H_2O_2 was added to each well, and the plate was left to stand at room temperature in the dark for 20 min. The reaction was stopped by the addition of 50 μl of 2 M H_2SO_4 to each well, and the optical density at 450 nm (620-nm reference) ($OD_{450/620}$ was immediately measured with a Labsystems iEMS plate reader. Between each stage of the assay the wells were washed five times with PBS-T20 (0.05% T20) with a Denley well wash 4 Mk2. Included as controls in eight wells each were sera strongly positive for RV-specific IgG, NHS, and the WHO 80-IU/ml international standard diluted in NHS to give 15 IU/ml.

Determination of cutoff value. To provide a wide dynamic range and to allow for interassay variation, the results of the GACELISA were expressed as a corrected percentage of the absorbance of the positive control serum included in each assay by the following formula: corrected percentage = [($OD_{450/620}$ of the sample − $OD_{450/620}$ of the negative control)/($OD_{450/620}$ of the positive control − $OD_{450/620}$ of the negative control)] × 100 when $OD_{450/620}$ is based upon the mean negative result plus 2 standard deviations by using saliva samples from 26 subjects negative for serum RV-specific IgG antibody by the Behring Enzygnost ELISA, a cutoff value of 2.7% was determined: samples with corrected percentages of ≥2.7 were considered RV-specific IgG positive, and those with corrected percentages of <2.7 were considered RV-specific IgG negative.

Statistical methods. The assays were evaluated by three methods.

(i) Kappa statistic. The kappa statistic evaluates the degree of agreement between two measurements obtained by two different assays and is used when neither assay is universally accepted as a "gold standard." A kappa statistic of 1 indicates perfect agreement, one of 0 corresponds to a level of agreement expected by chance, and one of −1 indicates perfect negative agreement. The 95% confidence intervals around the kappa statistic were also calculated. If this interval does not straddle 0, we can conclude that the methods show more agreement than expected by chance. Kappa statistics (*K*) were calculated by the formula $K = (P_{obs} - P_{exp})/(1 - P_{exp})$, where P_{obs} is the observed proportion of agreement between the two methods and P_{exp} is the proportion of agreement expected by chance (7).

(ii) Spearman's rank correlation. Spearman's rank correlation is a nonparametric measure of the degree of association between two variables. The values of each variable are independently ranked, and the measure is based on the differences between the pairs of ranks of the two variables. Spearman's rank correlation (r_s) was calculated by the formula $r_s = 1 - [6\Sigma d^2/n(n^2 - 1)]$, where d is the difference between each pair of ranks and n is the number of subjects. A value of 1 corresponds to perfect agreement between the ranks of the two variables, 0 corresponds to no relationship, and −1 corresponds to a perfect inverse agreement between the ranks. The 95% confidence intervals around r_s were also calculated.

(iii) Exact binomial test. The binomial distribution is used to calculate the *P* value for comparison of the agreement of the two assays with saliva with the matching result for serum (1).

RESULTS

International antibody standard. A titration of the WHO 80-IU/ml standard diluted in NHS showed that 6.7 IU/ml corresponded with the 2.7% cutoff level of the amplification-based GACELISA.

Serum-saliva panels. The results for all three serum-saliva panels are presented in Table 1. Overall, the amplification-based GACELISA showed a higher level of agreement than the GACRIA with the Behring ELISA by the kappa statistic and rank correlation. The exact binomial *P* value of 0.011 suggests that the amplification-based GACELISA was significantly different from the GACRIA when the results of those assays were compared with those of the Behring ELISA. This was mainly due to the results for panel 3a but was partly due to the greater correlation of the results of the amplification-based GACELISA than those of the GACRIA with the results of the Behring ELISA for panel 1. Compared to the Behring ELISA result for serum from all the serum-saliva pairs, the GACELISA was both more sensitive (82%) and specific (100%) than GACRIA, whose sensitivity and specificity were 74.4 and 97.3%, respectively. The positive predictive values (PPVs) for both the amplification-based GACELISA and GACRIA were high, being 100 and 99%, respectively. The negative predictive value (NPV), however, was low for both assays with saliva due to the inclusion of the results for panel 3a, although the NPV was higher for the amplification-based GACELISA (56.4%) than the GACRIA (46.8%).

Panel 1. All of the 97 serum samples from panel 1 were positive for RV-specific IgG by the Behring ELISA. Of the 97 saliva samples, 92 were RV-specific IgG positive by the amplification-based amplified GACELISA and 93 were positive by

TABLE 1—*Continued*

Kappa statistic (95% CI) for agreement with B[c]		Rank correlation (95% CI) with B		Exact binomial *P* value[d]
G	R	G	R	
NA	NA	0.74 (0.63–0.82)	0.55 (0.40–0.68)	1.00
NA	NA	0.21 (−0.21–0.56)	−0.06 (0.45–0.35)	1.00
0.16 (0.01–0.31)	0.06 (−0.01–0.12)	0.58 (0.37–0.73)	0.35 (0.10–0.57)	0.0001
0.81 (0.57–1.00)	0.90 (0.72–1.00)	0.74 (0.44–0.88)	0.74 (0.46–0.89)	1.00
0.63 (0.51–0.74)	0.51 (0.39–0.62)	0.68 (0.60–0.75)	0.45 (0.33–0.56)	0.011

the GACRIA, giving sensitivities of 94.8 and 95.8%, respectively. The results of the amplification-based GACELISA showed a better correlation with those of the Behring ELISA than the results of the GACRIA did. The exact binomial *P* value, however, showed that there was no significant difference in the agreement of the two assays with saliva with the Behring ELISA with serum.

Panel 2. All 24 serum samples from panel 2 were RV-specific IgG negative by the Behring ELISA. By the amplication-based GACELISA and GACRIA, the 24 corresponding saliva samples were also RV-specific IgG negative, giving a specificity of 100% for both assays with saliva.

Panel 3a. Of 55 serum samples in panel 3a, 51 were RV-specific IgG positive and 4 were negative by the Behring ELISA. The four saliva samples corresponding to the four negative serum samples were all RV-specific IgG negative by both the amplification-based GACELISA and GACRIA. Of the 51 serum samples which were positive, 15 of the corresponding saliva samples were positive by both the amplification-based GACELISA and GACRIA, 14 were positive by the amplification-based GACELISA only, and 22 were negative by both assays. The sensitivities of both salivary assays were low compared to the results of the Behring ELISA with serum, but the sensitivity was considerably higher for the amplification-based GACELISA (60.8%) than for the GACRIA (29.4%) (Table 1). The specificity and PPV were 100% for both assays with saliva, although the NPV was very low for both assays. The kappa statistic for agreement with the Behring ELISA was higher for the amplification-based GACELISA than for the GACRIA but was low for both assays (Table 1). The rank correlation was also higher for the amplification-based GACELISA, and the exact binomial *P* value showed that the results of the amplified GACELISA agreed significantly more than the results of the GACRIA with the results of the Behring ELISA with serum ($P = 0.0001$) (Table 1).

Panel 3b. Of 21 serum samples in panel 3b, 12 were RV-specific IgG positive and 9 were negative by the Behring ELISA. All nine saliva samples corresponding to the nine negative serum samples were negative by the amplification-based GACELISA and one saliva sample tested weakly positive by GACRIA. For the 12 serum samples which were positive, 10 corresponding saliva samples were positive by both assays, 1 was negative by the amplification-based GACELISA only, and 1 was negative by both assays. Both assays with saliva had similar sensitivities (90.9%), but the specificity of the amplification-based GACELISA (100%) was higher than that of the GACRIA (90%) (Table 1). The PPV and NPV for both assays were high, being slightly higher for the amplification-based GACELISA than for the GACRIA (Table 1). The kappa statistic was high for both assays, showing good agreement with the Behring ELISA results, and the rank correlation was 0.74 for both the amplification-based GACELISA and GACRIA (Table 1). The exact binomial *P* value showed that there was no significant difference in the agreement of the results of the two assays with saliva with those of the Behring ELISA with serum.

Panels 1 to 3. Overall, 29 serum-saliva pairs from all three panels gave discordant results by the amplification-based GACELISA. All serum samples were RV-specific IgG positive by the Behring ELISA and all saliva samples were negative by the amplification-based GACELISA, with a geometric mean titer (GMT) of 38.3 IU/ml for serum RV-specific IgG. Twenty-two of 29 serum-saliva pairs with discordant results were from panel 3a (subject ages, 17 years or older). For serum-saliva pairs with concordant positive results, the RV-specific IgG GMT in serum was higher, being 60.2 IU/ml ($P < 0.05$ by comparison of the log titer by the *t* test).

DISCUSSION

In order to develop assays for salivary RV-specific IgG that could be more widely used than the previously described GACRIA (13), it was decided that a corresponding IgG-capture ELISA should be developed. Because initial studies (data not shown) showed that simply substituting a horseradish peroxidase conjugate for a ^{125}I-labeled conjugated antibody did not result in an ELISA with sufficient sensitivity, it was decided that the FITC–anti-FITC amplification system should be used (15). The performance of the amplification-based GACELISA was compared to that of the previously described GACRIA (13). The performances of both capture assays for saliva testing were also assessed by examining matching serum samples by a sensitive indirect ELISA (Behring) capable of detecting as little as 4 IU of RV-specific IgG per ml. By the kappa statistic and rank correlation for most of the individual serum-saliva panels and overall, the results of the amplification-based GACELISA with saliva had a higher level of agreement with the results of the Behring ELISA with corresponding serum samples than the results of the GACRIA with saliva did (Table 1). In addition, the overall sensitivity and specificity of the amplification-based GACELISA with saliva relative to the results of the Behring assay with serum were higher than those of the GACRIA (Table 1).

With serum-saliva panels 1, 2, and 3b (the ages of the subjects who provided samples for panel 3b were ≤10 years), the results of the amplification-based GACELISA compared favorably to those of the Behring ELISA with serum, with a sensitivity of 94.4%, a specificity and a PPV of 100%, and an NPV of 85%, and were similar to those obtained by GACRIA.

By contrast, with panel 3a (comprising samples from an Indian population of subjects whose ages were ≥17 years), the results of the amplification-based GACELISA showed significantly better agreement with those of the Behring ELISA with serum than those of the GACRIA. Also, although both assays with saliva had low sensitivities compared to the results of the assay with serum, with this panel, the sensitivity of the amplification-based GACELISA (60.8%) was considerably higher than that of the GACRIA (29.4%) (Table 1).

The reason for the low sensitivities of assays with saliva samples from panel 3a may be due to the older ages of the subjects who provided samples for this panel and the type of assay format used. Reactivity in antibody-capture assays depends on the proportion of antibody specific for the antigen under test. The proportion of IgG specific for RV may decrease with age as a consequence of an increase in the levels of exposure to other antigens, so this may explain the lower sensitivity of capture assays for RV-specific IgG in older subjects. This is supported by the finding that the majority of paired samples giving discordant results (the serum is positive and the saliva is negative) were from adults, and of these, the RV-specific IgG GMT in sera was significantly lower than the RV-specific IgG GMT in sera from paired samples giving concordant positive results. A similar finding of a decrease in the sensitivity of detection of RV-specific IgG in saliva with age was made by Nokes et al. (11), who used samples from a rural Ethiopian community. There is therefore a need for further investigation of the factors affecting the performance of antibody-capture assays with saliva, particularly in respect to the lack of sensitivity for subjects in older age groups. Since in this study the only saliva samples representative of an adult population came from India, this issue may be addressed by further age-stratified studies with samples from both Western and Third World populations and could incorporate the detection of IgG to viral antigens other than RV in saliva. More basic investigations into the constituents of saliva and their effects on the performance of virus-specific antibody assays are also required. For example, a further important consideration may be the local production of IgG in saliva. Cutts et al. (4) suggest that increased local production of IgG in saliva may reduce the proportion of total antibody that is specific and may therefore lead to a decrease in reactivity in capture assays.

The sensitivity, specificity, and predictive values of the amplification-based GACELISA for the detection of RV-specific IgG (Table 1) have important implications for its use. In adult populations the sensitivity and NPV of the amplification-based GACELISA were low (although they were higher than those of the GACRIA), and there is thus a high probability of false-negative results. This may compromise the accurate identification of, for example, immunity in women of childbearing age, which is of prime importance when screening adult populations for immunity to RV. For pediatric populations (ages, <14 years), however, the sensitivity and predictive values of the amplification-based GACELISA with saliva samples closely matched those of the sensitive Behring ELISA with serum. The majority of susceptible individuals are found in this age group, and these individuals make up the primary transmission group for RV. The results therefore suggest that the amplification-based GACELISA could reliably be used for the screening of children for immunity to RV. The measles-mumps-rubella is targeted to children, with the aim being to eliminate congenital rubella by the year 2000 (9, 20). The amplification-based GACELISA has now been successfully introduced for routine use with saliva samples from the United Kingdom rubella surveillance program (14), in which a high proportion of saliva samples examined (92% in 1997 [13a]) are from children under the age of 14 years.

This assessment of the amplification-based GACELISA for the detection of RV-specific IgG showed its performance to be superior to that of the previously described GACRIA, with the advantage of a substantially shorter running time, in addition to all the benefits of a nonradioactive assay. Although the amplification-based GACELISA for the detection of RV-specific IgG with saliva, like the GACRIA, was not as sensitive as the Behring ELISA for the detection of RV-specific IgG with serum, particularly when samples from adults were tested, a sensitivity approaching that of the sensitive Behring ELISA with serum was achieved when saliva from children were tested. Moreover, because the results of the amplification-based GACELISA correlated better to the results of the ELISA with serum and overall was more sensitive than the GACRIA for the detection of RV-specific IgG in saliva, it is a candidate assay that could be used for wider testing of saliva.

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