Non-Invasive Detection of Viral Antibodies Using Oral Flocked Swabs

Running Title: Oral flocked swabs to detect viral antibodies

Word Counts: Abstract: 173 Text Body: 3,709

David J. Speicher[1,2,3,4,#], Kathy Luinstra[2], Emma J. Smith[5], Santina Castriciano[6], Marek Smieja[1,2]

[1]Department of Pathology & Molecular Medicine, McMaster University, Ontario, Canada

[2]Department of Laboratory Medicine, St. Joseph's Healthcare Hamilton, Ontario, Canada

[3]Menzies Health Institute Queensland, Griffith University, Queensland, Australia

[4]M.G. DeGroote Institute for Infectious Disease Research, Department of Biochemistry and Biomedical Sciences, DeGroote School of Medicine, McMaster University, Hamilton, Ontario, Canada

[5]Department of Mathematics and Statistics, University of Guelph, Ontario, Canada

[6]Copan Italia, Brescia, Italy

[#]**Corresponding author:** Dr. David J. Speicher; speichdj@mcmaster.ca
Department of Biochemistry & Biomedical Sciences
McMaster University
1200 Main Street West, Room HSC 4N59
Hamilton Ontario L8N 3Z5, Canada
Phone: 905-525-9140 x21663
FAX 905-522-9033

Keywords: oral flocked swabs, saliva, diagnostics, Cytomegalovirus, Epstein-Barr virus, Varicella-Zoster virus, Measles, Mumps

Highlights

- Oral flocked swabs are an easy, self-collection method for measuring viral antibodies.
- Viral IgG is stable on dried oral flocked swabs for at least two years.
- Oral swabs are highly sensitive for CMV, VZV, and EBV IgG.
- Oral swabs are potentially useful for surveillance and clinical microbiology.

Abstract

Salivary antibodies are useful in surveillance and vaccination studies. However, low antibody levels and degradation by endonucleases are problematic. Oral flocked swabs are a potential non-invasive alternative to blood for detecting viral antibodies. Serum and saliva collected from 50 healthy volunteers were stored at -80^{o}C; dried swabs at room temperature. Seroprevalence for Cytomegalovirus (CMV), Varicella-Zoster virus (VZV), Epstein-Barr virus (EBV), Measles and Mumps IgG antibodies were determined using commercial ELISAs and processed on an automated platform. For each antibody, swabs correlated well with saliva. For CMV IgG, VZV IgG, and EBV EBNA-1 IgG and VCA IgG, the swab sensitivities compared to serum were 95.8%, 96%, 92.1% and 95.5% respectively. For Measles IgG, swab sensitivity was 84.5%. Mumps IgG displayed poor sensitivity for oral swabs (60.5%) and saliva (68.2%). Specificities for IgG antibodies were 100% for CMV, EBV and Mumps. Specificities for VZV and Measles could not be determined due to seropositive volunteers. As oral flocked swabs correlate well with serum, are easy to self-collect and stable at room temperature further research is warranted.

1. Introduction

Immunological screening for viral antibodies (primarily IgG) in serum to assess past infection or vaccine immunity is routinely performed via commercial enzyme immunoassays (EIA) on closed platforms. Serum is the gold standard for determining immune status but is invasive to collect. Saliva has considerable diagnostic potential: it is non-invasive, abundant, easily collected, and representative of oral and systemic health. Salivary diagnostics is rapidly emerging, especially defining biomarkers for point-of-care testing of infectious diseases (1). Salivary antibodies are primarily secretory IgA from the salivary glands, while IgG and IgM are derived from serum plasma cells and passively diffused into the oral cavity via gingival crevicular fluid (2, 3). Salivary IgG (sIgG) is systemically representative and strongly correlates with serum levels, but loads are approximately 1:800 that of serum (4, 5). This is problematic for typical closed testing systems that incorporate a 1:100 dilution step. Despite low levels, salivary antibodies are utilized in the U.S. Food and Drug Administration approved OraQuick ADVANCE® Rapid HIV-1/2 Antibody Test (OraSure Technologies, Inc., USA) and OraQuick® HCV Test (OraSure Technologies, Inc.) (1). However, many commercial assays are cost-prohibitive for resource-limited settings.

Saliva collection can be difficult in children and hyposalivators, such as immunocompromised patients. Salivary endonucleases are detrimental, remaining active at -80^{o}C, necessitating special handling, or storage in proteolytic stabilizers unsuitable for antibody preservation (6). To overcome these limitations, procedures for viral antibody detection were optimized on an open commercial platform for dried oral flocked swabs, after room temperature storage. The efficiency of oral flocked swabs to detect viral antibodies has yet to be determined. Our method

was initially optimized for Cytomegalovirus (CMV) IgG due to its importance in hematopoietic stem cell, solid organ, and haploidentical transplantations as well as prenatal patients (7, 8). We then assessed the procedures' potential application to detect Varicella-Zoster virus (VZV), Epstein-Barr virus (EBV), Measles and Mumps IgG.

2. Materials and Methods

2.1. Study Population:

Following Hamilton Integrated Research Ethics Board (HiREB #14-658) approval and written informed consent, two oral swabs, unstimulated saliva, and blood were collected. Optimisation of pre-analytic and analytic procedures was performed on 10 healthy volunteers with known CMV seropositivity (5 positive, 5 negative), and expanded to 50 healthy volunteers for the diagnostic accuracy study. Laboratory staff (15 males:35 females) from St. Joseph's Healthcare Hamilton with an average age of 43.4 years (range: 18-65 years) voluntarily provided all sample types, except for one who could not produce a saliva sample. Two oral swabs (FLOQSwabs® #520C, Copan Italia S.p.A., Brescia, Italy) were collected consecutively by moistening the flocked swab on the tongue and then rotating between the gums and cheek three to five times. Swabs were then dried for an hour inside a biosafety cabinet and stored inverted in a microcentrifuge tube at room temperature prior to elution and at -20°C following elution. Whilst circadian rhythm was not accounted for as swabs were collected at times convenient for the volunteer, participants were asked to refrain from eating or drinking 60-minutes prior to collection. Cell-free unstimulated saliva was collected by expectorating 2-5mL into a sterile 50mL Falcon tube, centrifuging at 2,800 *x g* for 10-minutes and aspirating the supernatant (9).

Supernatant was aliquoted into 1mL portions and stored at -80C; the cell pellet was discarded. Serum was obtained from a 5mL blood collection via venipuncture, allowed to clot, centrifuged at 3,000 *x g* for 10-minutes, and stored at -80^{o}C.

2.2. Development and Optimisation of pre-analytic and analytic procedures:

The first optimisation to determine the optimal dilution for testing oral swabs was performed with a CMV IgG EIA on the ThunderBolt® ELISA Analyzer; both from Gold Standard Diagnostics (GSDx, Davis, CA, USA). To elute viral antibodies, 250µL PBS was added to a dried swab head, vortexed for 30-seconds, incubated at room temperature for 10-minutes, and centrifuged at 14,000 *x g* for one minute, before discarding the swab. Serial dilutions (2-fold serially from neat to 1:16) were prepared in PBS and 100µL tested on the ThunderBolt® ELISA Analyzer as per manufacturer's instructions. Repeated measures one-way ANOVA was performed in R3.5.0 in combination with polynomial contrasts to assess the nature and significance of the relationship between dilutions and optical density (O.D.) values (10).

The second optimisation determined the effect of tube shape and elution volume on O.D. values and status. Various volumes of PBS (150µL, 200µL, and 250µL) were added to swabs stored in two shapes of microcentrifuge tubes: 2.0mL flat-bottomed, screw cap tubes (SCT-200-Y, Axygen Scientific, Union City, CA, USA) and 1.5mL conical microcentrifuge tubes (MCT-150-C, Axygen Scientific). The volume of PBS recovered was measured for each. To assess the relationship between O.D. values and both tube shape and volume, a linear mixed effects model was fit. O.D. values were treated as the response and volume added and tube type were

considered as the main effects. As sample manipulations could affect positivity only positive patients were considered.

The third optimization determined the effect pelleted buccal cells had on O.D. values and positivity of five salivopositive and five salivonegative samples using three methods: 1. Pellet, measuring the supernatant O.D. values with undisturbed pelleted buccal cells at the bottom of the tube; 2. Supernatant, measuring the supernatant O.D. values after transfer to a new tube without disturbing the pellet; 3. Resuspended; measuring the O.D. values after complete resuspension of the buccal cells. To determine the relationship between resuspension methods, an initial ANOVA followed by paired testing was used. Further pairwise comparisons between O.D. values of the three methods (pellet, supernatant, and resuspended) were constructed using paired t-tests.

The fourth optimisation determined the stability of viral antibodies in dried flocked swabs over time by measuring O.D. values from five salivopositive samples at baseline and after two months stored at room temperature. Paired t-tests were used to compare average O.D. values at both time points.

2.3. Diagnostic Accuracy Study

A cross-sectional diagnostic accuracy study was performed to compare oral flocked swabs and unstimulated saliva versus serum as a reference standard, for the detection of various viral-specific IgG antibodies. As results were similar between two consecutively collected swabs

(data not shown) swab eluates were pooled to facilitate automated testing of multiple analytes. The assays utilized included: CMV IgG EIA, EBV EBNA IgG EIA, EBV VCA IgG EIA, Measles IgG EIA, and Mumps IgG EIA from GSDx; RIDASCREEN® VZV IgG (K5621), RIDASCREEN® Measles IgG (K5421), and RIDASCREEN® Mumps IgG (K5521) from R-Biopharm AG (Darmstadt, Germany). These commercial EIAs are optimised for serum but were used off-label for oral fluids. All assays were performed as per manufacturer's protocol using 100µL 1:100 sera, but 100µL undiluted oral fluid. For each assay, the mean O.D. and standard deviation (SD) were calculated from swabs and saliva specimens corresponding to "true-negative" subjects, i.e. serum test negative subjects. We then defined cut-off values as: Non-reactive if less than two SDs above the average O.D. values; Reactive if greater than 3 SDs above the average O.D.; all other O.D. values considered indeterminate. As all samples were seropositive for VZV and measles the cut-off O.D. values for these analytes were extrapolated from the cut-off O.D. values of other assays from the same manufacturer. For each, the diagnostic test accuracy (sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV), and overall accuracy), and misclassification rates were determined. Kappa statistics were calculated in a pairwise fashion to quantify the agreement beyond chance between oral swabs, unstimulated saliva, and serum for all antibodies of interest.

3. Results

3.1. Optimisation of pre-analytic procedures:

To determine the effect of sample dilutions, O.D. values were measured at five, two-fold dilution points. Dilutions significantly decrease O.D. values (p=0.013) and affected positivity: 4-fold and

157 8-fold dilutions yielded 2/5 (40%) and 4/5 (80%) false non-reactive samples, respectively
158 (Figure 1). Pairwise comparison showed that dilutions significantly decreased O.D. values in a
159 linear relationship (F=17.758, p=0.014). Therefore, swabs were used undiluted to ensure that
160 weakly reactive samples did not become falsely non-reactive.

161

162 To determine the effect of tube shape and establish the optimal elution volume, O.D. values were
163 measured at three volumes of PBS and two tube shape combinations. The conical tube shape
164 caused eluate to be reabsorbed by the swab , significantly reduced recovery volume (conical vs
165 flat-bottomed: 60.66 ± 4.38% vs 98.63 ± 0.78%; p=0.017), O.D. values (p=0.003), and
166 potentially affected the positivity of weakly reactive samples (Figure 2). The O.D. values
167 slightly decreased with increased volume added (150µL to 250µL), but this difference was not
168 significant (p=0.184) and did not affect positivity. In the non-reactive samples, the average O.D.
169 values of the flat-bottomed tubes (0.080 ± 0.008) were slightly higher than the conical tubes
170 (0.059 ± 0.021), but this difference was not significant (p=0.323). Therefore, subsequent testing
171 was performed by eluting with 250µL PBS in flat-bottomed tubes to facilitate two tests per swab.

172

173 To determine the most efficient sample handling procedure to yield the highest O.D. values,
174 three methods were evaluated. In the reactive samples, there was little difference between the
175 O.D. values: undisturbed pellet (0.688 ± 0.434); supernatant (0.545 ± 0.222); resuspended pellet
176 (0.531 ± 0.214). One sample was a weak reactive in the pellet method (O.D. 0.258) but became
177 indeterminate in the supernatant (O.D. 0.214) and resuspended pellet (O.D. 0.197) method.
178 Pairwise comparison revealed no significant differences in O.D. values between the sample

handing methods (pellet:supernatant, p=0.312; pellet:resuspended, p=0.288; supernatant:resuspended, p=0.515), but fractioning the sample could produce an indeterminate result from a weakly reactive sample and was more labour intensive. Therefore, subsequent testing was performed using the whole sample following centrifugation.

To determine the stability of oral swab collections at room temperature, O.D. values were measured from dried oral swabs the day of collection and after two months of storage at room temperature. The average O.D. value did not significantly differ between baseline (O.D. 0.575 ± 0.284) and two months (O.D. 0.568 ± 0.188). The mean difference in O.D. values was 0.008 (p=0.946). No change is salivopositivity was observed.

3.2. Diagnostic Accuracy Study

To determine the correlation between oral flocked swabs, unstimulated saliva and serum a cross-sectional diagnostic accuracy study was conducted on 50 volunteers for CMV, VZV, EBV EBNA-1 and VCA, Measles, and Mumps IgG. For CMV IgG, the seropositivity using serum was 24/50 (48.0%). The cut-off O.D. values for swabs and saliva were non-reactive <0.179 and reactive >0.221; and non-reactive <0.220 and reactive >0.253, respectively (Table 1). Using the new cut-off O.D. values the sensitivity of swabs and saliva were 23/24 (95.8%; 95% CI: 78.1%, 100%) and 24/24 (100%; 95% CI: 83.7%, 100%), respectively (Table 2). Specificity of both swabs and saliva were 100%. One swab was indeterminate. The agreement beyond chance was very good between oral swabs and both serum (K=0.88; 95% CI: 0.76, 1.000) and saliva

(K=0.85; 95% CI: 0.71, 1.00), and perfect between saliva and serum (K=1.00; 95% CI: 0.86, 1.00) (Figure 3).

For VZV IgG, all participants were seropositive. There was excellent correlation between both swabs and saliva to serum: 48/50 (96.0%; 95% CI: 85.7%, 99.7%) and 46/49 (93.9%; 95% CI: 82.9%, 98.5%), respectively. As there were no seronegative participants specificity could not be determined and Cohen's kappa coefficient was poor for both sera to swab and saliva (K=0), and fair for swabs vs saliva (K=0.37; 95% CI: -0.189, 0.928).

For EBV, fewer participants were seropositive for EBNA-1 IgG than VCA IgG: 38/50 (76.0%) and 44/50 (88.0%), respectively. The sensitivity of swabs and saliva were comparable for both EBNA-1 IgG [swabs: 35/38 (92.1%; 95% CI: 78.5%, 98.0%); saliva: 34/38 (91.9%; 95% CI: 75.3%, 96.4%)] and VCA IgG [swabs: 42/44 (95.5%; 95% CI: 84.0%, 99.6%); saliva: 42/43 (97.7%; 95% CI: 86.8%, 100%)]. However, sensitivity for the composite measure of EBNA-1 IgG *or* VCA IgG equated to 43/44 (97.7%; 95% CI: 87.1%, 100%) and 44/44 (100%; 95% CI: 90.4%, 100%) for swabs and saliva, respectively. The agreement for EBNA-1 IgG was very good for both swab (K = 0.85; 95% CI: 0.68, 1.00) and saliva (K=0.85; 95% CI: 0.68, 1.00) compared to serum, and perfect between swabs and saliva (K=1. 000; 95% CI: 0.90, 1.00). The agreement for VCA IgG was very good for both swab (K=0.83; 95% CI: 0.61, 1.000) and saliva (K=0.91; 95% CI: 0.74, 1.00) compared to serum, and good between swabs and saliva (K=0.76; 95% CI: 0.50, 1.00).

222 For Measles and Mumps IgG the correlation between oral swabs, unstimulated saliva and serum
223 as well as between two EIAs was determined. For Measles IgG, all participants were
224 seropositive by both assays. The sensitivity of swabs and saliva was much higher for the GSDx
225 assays for both swabs [41/48 (85.4%; 95% CI: 72.5%, 93.1%) vs 24/49 (48.9%; 95% CI: 35.6%,
226 62.5%)] and saliva [46/49 (93.9%; 95% CI: 82.9%, 98.5%) vs 34/49 (69.4%; 95% CI: 55.4%,
227 80.6%)]. However, the agreement for both assays between swab and saliva compared to sera
228 was poor (K=0), and poor for swab compared to saliva (GSDx: K=0.24; 95% CI: -0.08, 0.56; R-
229 Biopharm: K=0.27; 95% CI: -0.09, 0.46). Further analysis showed the R-Biopharm assay had a
230 much larger misclassification rate for both swab [25/50 (50.0%; 95% CI: 36.6%, 63.4%) vs 5/50
231 (10.0%; 95% CI: 3.9%, 21.8%)] and saliva [15/49 (30.6%; 95% CI: 19.5%, 44.6%) vs 3/49
232 (6.1%; 95% CI: 2.1%, 16.5%)], suggesting that the GSDx assay performs better for measuring
233 measles salivopositivity.

234

235 For Mumps IgG, both assays produced comparable seroprevalence of 45/50 (90.0%) and 46/50
236 (92.0%) for the GSDx and R-Biopharm assays, respectively. The sensitivity of both assays was
237 poor for both swabs [GSDx: 26/43 (60.5%; 95% CI: 45.6%, 73.7%); R-Biopharm: 28/45 (62.2%;
238 95% CI: 47.6%, 74.9%)] and saliva [GSDx: 30/44 (68.2%; 95% CI: 53.4%, 80.1%); R-
239 Biopharm: 33/45 (73.3%; 95% CI: 58.4%, 84.2%)]. …(11). The agreement was only fair for
240 both assays between swab (GSDx: K=0.20; 95% CI: 0.00, 0.40; R-Biopharm: K=0.21; 95% CI:
241 0.03-0.40) and saliva (GSDx: K=0.25; 95% CI: 0.02, 0.47; R-Biopharm: K=0.32; 95% CI: 0.07,
242 0.56) compared to sera, as well as swabs compared to saliva (GSDx: K=0.25; 95% CI: 0.03,
243 0.47; R-Biopharm: K=0.38; 95% CI: 0.16, 0.60). Further analysis showed that both assays had a
244 large misclassification rate for both swab [GSDx: 18/44 (40.9%; 95% CI: 27.7%, 55.6%); R-

Biopharm: 17/45 (37.8%; 95% CI: 25.1%, 52.4%)] and saliva [GSDx: 15/45 (33.3%; 95% CI: 21.3%, 48.0%); R-Biopharm: 12/45 (26.7%; 95% CI: 15.8%, 41.2%)], suggesting that neither assay is ideal for measuring mumps salivopositivity.

4. Discussion

Oral fluids contain IgG profiles highly similar to those in serum for a range of antigens and diseases regardless of anatomical location (12). Their non-invasive collection is utilized in several point-of-care assays to detect viral infections and immunity. To avoid degradation by salivary endonucleases, diagnostic assays either utilize unadulterated gingival crevicular fluid or whole saliva either chilled immediately following collection and stored frozen or stored in biological stabilizers (4, 6). Studies for viral IgG utilize the Oracol saliva collection system (Malvern Medical Developments, UK), which collects 1mL saliva into 1mL transport medium allowing transportation at room temperature (13, 14). However, as samples must be stored frozen for long-term preservation this is not ideal for field studies and resource-limited settings (15). Therefore, as FLOQSwabs® are routinely used for bacteriology and molecular microbiology (16, 17), we developed a simple pre-analytic and analytic method for detecting viral sIgG on an open commercial platform using dried FLOQSwabs® that can be stored at room temperature for up to 2-months. This was not an epidemiological study, but our method is applicable to point-of-care or home testing, surveillance, clinical epidemiology and clinical microbiology.

Our protocol was developed on a convenient cohort of healthy volunteers and appears promising as swabs can be self-collected and diagnostic accuracy is comparable to saliva and serum for

most viruses tested. The similarity between swabs and saliva is due to cell-free viral IgG secreted via the gingival crevicular fluid into the mouth and absorbed onto the swab regardless of oral location swabbed. As swab absorption volume is much lower than the volume of saliva in the mouth it is possible to collect multiple swabs consecutively at any time of day without a reduction in positivity. Storing swabs dried maintains sample integrity and restricts activity of salivary endonucleases for at least 2-months post-collection. Ideally, swabs could be collected and shipped, but further investigation is needed to assess drying time on sample stability. Nevertheless, a few procedural steps must be heeded. Whilst cut-off O.D. values vary between assays, platforms and/or cohort tested, it is essential to elute swabs into flat-bottomed tubes to prevent reabsorption and then test undiluted eluate. Elution with 250µL PBS permits each sample to be tested twice. Further work is needed to (i) optimize elution and testing volumes to maximize testing and increase O.D. values to resolve indeterminate samples, (ii) determine sample integrity by comparing the absolute amount of human IgG, and (iii) compare manual testing vs automation to determine the robustness of the protocol.

Our swab protocol was initially developed for CMV due to interest from blood banks and transplant programs. CMV is readily shed in saliva, the viral load is 100-fold higher, and the limit of detection is 10-fold lower in saliva collected with a sterile swab than urine (18-20). CMV antibody profiles in serum also strongly correlate with CMV infection and oral shedding (8, 21, 22), and, based on our study, CMV IgG can be detected accurately in oral swabs. Swabs displayed similar diagnostic accuracy to saliva and serum. The overall O.D. values in oral fluids was lower than serum with swabs slightly lower than saliva but displayed a linear comparison in O.D. values between serum and oral fluids. The slightly lower swab O.D. values compared to

saliva results from a fraction of whole saliva diluted in eluate. The one swab that was equivocal was strongly positive in sera and saliva suggesting further optimisation is warranted. Whilst our protocol was developed for CMV IgG in healthy volunteers and may not be reflective of assay performance in hospital or immunocompromised patient populations, it worked well for herpesviruses due to maintained immunity from latent infections, but less than optimal for measles and mumps possibly due to waning vaccine-induced immunity.

Few publications examined salivary IgG for VZV and EBV. In the only study on VZV IgG in oral fluids, Talukder *et al.* examined 1,092 participants and reported a sensitivity and specificity of 93% and 95.7%, respectively (14). The sensitivity is comparable to saliva (93.9%) and swabs (96.0%) in our study, but our sample size was insufficient to determine assay specificity. In a hospital laboratory volunteer study, virtually all employees have either received screening and vaccination for vaccine-preventable illnesses or are old enough to have natural immunity. Based on the diagnostic accuracy our procedure could be studied as a screening method for preschool children susceptible to chicken pox.

Epidemiological screening for EBV, the aetiological agent of infectious mononucleosis and nasopharyngeal carcinoma, is performed by screening for EBV VCA IgM, VCA IgG, and EBNA-1 IgG via IFA or EIAs to distinguish acute from past infection (23). Both Vyse *et al.* and Crowcroft *et al.* utilized oral fluids to determine EBV immune status (24, 25). Vyse *et al.* used a 'G' antibody capture radioimmunoassay for EBV VCA IgG and reported a sensitivity and specificity of 93.5% and 100%, respectively (25). Their assay was less sensitive than IFA as

312 samples with a total IgG <2 mg/L yielded false positive reactions due to the monoclonal
313 antibody binding non-specifically to unsaturated anti-human IgG on the solid phase. Although
314 we didn't examine the total IgG in our samples, our protocol produced higher sensitivities for
315 both swabs and saliva with no known false positives. As 5% of people do not produce EBNA-1
316 IgG after EBV infection we validated both EBV EBNA-1 IgG and VCA IgG (26). Combining
317 both assays our protocol yielded a sensitivity of 97.7% and 100% for swabs and saliva,
318 respectively, and may be useful for surveillance studies and could be preferred over serum
319 heterophile antibody testing for acute infectious mononucleosis work-up.

320

321 As Measles and Mumps are vaccine-preventable infections several studies have used oral fluids
322 as a non-invasive alternative for monitoring the efficacy of vaccination programs. For Measles
323 studies have used off-label either the commercialized Measles IgG Capture EIA (Microimmune
324 Ltd., UK), and the Enzygnost® Anti-Measles Virus/IgG (Siemens Health Care Diagnostics
325 GmbH, Germany) with mixed results (5). Whilst Hayford *et al*., reported that oral fluids are not
326 suitable to detect immunity for Measles due to poor sensitivity (60.2%) and specificity (75.7%)
327 (27, 28) others reported sensitivity of 90.0-92.0% and a specificity of 77.8-100% using the
328 Microimmune Ltd. assay (29-32). Our study also produced mixed results between the GSDx and
329 R-Biopharm assays. Whilst the R-Biopharm assay produced poorly, the GSDx assay was
330 comparable to the Microimmune Ltd. assay. Further optimization is required to increase the
331 sensitivity of our assay, but in its present stage may be adequate for epidemiological studies. If
332 the efficacy of vaccination is essential, confirmatory testing on serum should be performed for
333 non-reactive oral samples.

334

For Mumps, Vainio *et al.*, assessed the Mumps IgG Capture EIA (Microimmune Ltd.) and reported low detection of Mumps IgG in oral samples (76% sensitivity) and recommended that the Microimmune assay not be used for surveillance studies (33). In our study, both the GSDx and R-Biopharm assays produced similarly low sensitivities for both swabs and saliva, but possibly for different reasons. The R-Biopharm assay produced low O.D. values for both oral fluids and serum suggestive of an issue with the assay. The GSDx assay yielded a range of O.D. values for serum similar in range to the other assays, but much lower (<0.5) for swabs. Whilst it is possible that Mumps IgG is not excreted into the mouth, vaccine-derived immunity to Mumps also wanes especially into adulthood and maybe too low to detect in oral fluids (34).

Whilst the use of oral fluids is not novel, we developed a simple non-invasive protocol for detecting viral IgG using oral flocked swabs. Based on the high sensitivity and excellent correlation with serum, oral samples are ideal for CMV, EBV, and VZV, adequate for Measles, but poor for Mumps. As samples can be stored dried for a few months, they are a viable option for home and field collection. Future work should investigate the utility of oral swabs for viral IgM as well as Rubella. Studies using oral fluids for Rubella IgM look promising with 79-96.9% sensitivity and 90-100% specificity (32, 35). Nonetheless, oral swabs appear promising for surveillance studies, transplant screening programs, and clinical microbiology.

5. CRediT Author Statement

David J. Speicher: Conceptualization, Investigation, Writing - Original Draft; Kathy Luinstra: Methodology, Writing - Review & Editing; Emma J. Smith: Formal analysis; Santina Castriciano: Conceptualization, Resources, Funding acquisition; Marek Smieja: Supervision

6. Acknowledgements

This study was supported in part by an award from The Research Institute of St. Joe's Hamilton. We are grateful for the donation of FLOQSwabs® (#520C) from Copan Italia, and commercial EIA kits from Gold Standard Diagnostics and R-Biopharm AG. We thank Gold Standard Diagnostics for lending the ThunderBolt® ELISA Analyzer. This study was based on the initial CMV IgG testing by Dr Milena Furione and presented at the Clinical Virology Symposium (CVS 2016; Poster #230). Santina Castriciano is an employee of Copan Italia. The other authors have no financial or other conflicts of interest to declare. Whilst this manuscript was shared with Copan Italia and Gold Standard Diagnostics prior to submission these collaborators had no influence on the data analysis or publication. We are thankful for the support from Jalees Nasir for the statistical help. We are grateful to the many volunteers at St. Joseph's Healthcare Hamilton who provided sera, saliva, and oral swabs for testing.

7. References

1. Khan RS, Khurshid Z, Yahya Ibrahim Asiri F. 2017. Advancing Point-of-Care (PoC) Testing Using Human Saliva as Liquid Biopsy. Diagnostics (Basel) 7.

2. Brandtzaeg P. 2007. Do salivary antibodies reliably reflect both mucosal and systemic immunity? Ann N Y Acad Sci 1098:288-311.

3. Parry JV, Perry KR, Mortimer PP. 1987. Sensitive assays for viral antibodies in saliva: an alternative to tests on serum. Lancet 2:72-5.

4. Saccoccio FM, Gallagher MK, Adler SP, McVoy MA. 2011. Neutralizing activity of saliva against cytomegalovirus. Clin Vaccine Immunol 18:1536-42.

5. Dimech W, Mulders MN. 2016. A review of testing used in seroprevalence studies on measles and rubella. Vaccine 34:4119-4122.

6. Speicher DJ, Wanzala P, D'Lima M, Johnson KE, Johnson NW. 2015. Detecting DNA viruses in oral fluids: evaluation of collection and storage methods. Diagn Microbiol Infect Dis 82:120-7.

7. Ljungman P, Hakki M, Boeckh M. 2011. Cytomegalovirus in hematopoietic stem cell transplant recipients. Hematol Oncol Clin North Am 25:151-69.

8. Dollard SC, Keyserling H, Radford K, Amin MM, Stowell J, Winter J, Schmid DS, Cannon MJ, Hyde TB. 2014. Cytomegalovirus viral and antibody correlates in young children. BMC Res Notes 7:776.

9. Henson BS, Wong DT. 2010. Collection, storage, and processing of saliva samples for downstream molecular applications. Methods Mol Biol 666:21-30.

10. R Core Team. 2018. R: A language and environment for statistical computing., R Foundation for Statistical Computing, Vienna, Austria. https://www.R-project.org/.

394 11. Upper D. 1974. The unsuccessful self-treatment of a case of "writer's block". J Appl
395 Behav Anal 7:497.
396 12. Hettegger P, Huber J, Passecker K, Soldo R, Kegler U, Nohammer C, Weinhausel A.
397 2019. High similarity of IgG antibody profiles in blood and saliva opens opportunities for
398 saliva based serology. PLoS One 14:e0218456.
399 13. Furuta Y, Ohtani F, Aizawa H, Fukuda S, Kawabata H, Bergstrom T. 2005. Varicella-
400 zoster virus reactivation is an important cause of acute peripheral facial paralysis in
401 children. Pediatr Infect Dis J 24:97-101.
402 14. Talukder Y, Gopal R, Andrews N, Glenn M, Breuer J, Brown D. 2005. Development and
403 evaluation of Varicella zoster virus ELISA for oral fluid suitable for epidemiological
404 studies. J Virol Methods 128:162-7.
405 15. Rosenbaum S, Gettler LT, McDade TW, Belarmino NM, Kuzawa CW. 2018. The effects
406 of collection and storage conditions in the field on salivary testosterone, cortisol, and
407 sIgA values. Ann Hum Biol 45:428-434.
408 16. Coorevits L, Vanscheeuwijck C, Traen A, Binge L, Ryckaert I, Padalko E. 2015.
409 Evaluation of Copan FLOQSwab for the molecular detection of Chlamydia trachomatis
410 by Abbott RealTime CT PCR. Acta Clin Belg 70:398-402.
411 17. Larios OE, Coleman BL, Drews SJ, Mazzulli T, Borgundvaag B, Green K, Group ST-FS,
412 McGeer AJ. 2011. Self-collected mid-turbinate swabs for the detection of respiratory
413 viruses in adults with acute respiratory illnesses. PLoS One 6:e21335.
414 18. Cannon MJ, Stowell JD, Clark R, Dollard PR, Johnson D, Mask K, Stover C, Wu K,
415 Amin M, Hendley W, Guo J, Schmid DS, Dollard SC. 2014. Repeated measures study of

416 weekly and daily cytomegalovirus shedding patterns in saliva and urine of healthy
417 cytomegalovirus-seropositive children. BMC Infect Dis 14:569.
418 19. Barkai G, Ari-Even Roth D, Barzilai A, Tepperberg-Oikawa M, Mendelson E,
419 Hildesheimer M, Kuint J. 2014. Universal neonatal cytomegalovirus screening using
420 saliva - report of clinical experience. J Clin Virol 60:361-6.
421 20. Stowell JD, Mask K, Amin M, Clark R, Levis D, Hendley W, Lanzieri TM, Dollard SC,
422 Cannon MJ. 2014. Cross-sectional study of cytomegalovirus shedding and
423 immunological markers among seropositive children and their mothers. BMC Infect Dis
424 14:568.
425 21. Cardoso ES, Jesus BL, Gomes LG, Sousa SM, Gadelha SR, Marin LJ. 2015. The use of
426 saliva as a practical and feasible alternative to urine in large-scale screening for
427 congenital cytomegalovirus infection increases inclusion and detection rates. Rev Soc
428 Bras Med Trop 48:206-7.
429 22. Wang JB, Adler SP. 1996. Salivary antibodies to cytomegalovirus (CMV) glycoprotein B
430 accurately predict CMV infections among preschool children. J Clin Microbiol 34:2632-
431 4.
432 23. De Paschale M, Clerici P. 2012. Serological diagnosis of Epstein-Barr virus infection:
433 Problems and solutions. World J Virol 1:31-43.
434 24. Crowcroft NS, Vyse A, Brown DW, Strachan DP. 1998. Epidemiology of Epstein-Barr
435 virus infection in pre-adolescent children: application of a new salivary method in
436 Edinburgh, Scotland. J Epidemiol Community Health 52:101-4.

437 25. Vyse AJ, Knowles WA, Cohen BJ, Brown DW. 1997. Detection of IgG antibody to
438 Epstein-Barr virus viral capsid antigen in saliva by antibody capture radioimmunoassay. J
439 Virol Methods 63:93-101.
440 26. De Paschale M, Agrappi C, Manco MT, Mirri P, Vigano EF, Clerici P. 2009.
441 Seroepidemiology of EBV and interpretation of the "isolated VCA IgG" pattern. J Med
442 Virol 81:325-31.
443 27. Hayford KT, Al-Emran HM, Moss WJ, Shomik MS, Bishai D, Levine OS. 2013.
444 Validation of an anti-measles virus-specific IgG assay with oral fluid samples for
445 immunization surveillance in Bangladesh. J Virol Methods 193:512-8.
446 28. Hayford KT, Shomik MS, Al-Emran HM, Moss WJ, Bishai D, Levine OS. 2013. Measles
447 vaccination coverage estimates from surveys, clinic records, and immune markers in oral
448 fluid and blood: a population-based cross-sectional study. BMC Public Health 13:1211.
449 29. Hutse V, Van Hecke K, De Bruyn R, Samu O, Lernout T, Muyembe JJ, Brochier B.
450 2010. Oral fluid for the serological and molecular diagnosis of measles. Int J Infect Dis
451 14:e991-7.
452 30. Kremer JR, Muller CP. 2005. Evaluation of commercial assay detecting specific
453 immunoglobulin g in oral fluid for determining measles immunity in vaccinees. Clin
454 Diagn Lab Immunol 12:668-70.
455 31. Warrener L, Slibinskas R, Chua KB, Nigatu W, Brown KE, Sasnauskas K, Samuel D,
456 Brown D. 2011. A point-of-care test for measles diagnosis: detection of measles-specific
457 IgM antibodies and viral nucleic acid. Bull World Health Organ 89:675-82.
458 32. Nokes DJ, Enquselassie F, Nigatu W, Vyse AJ, Cohen BJ, Brown DW, Cutts FT. 2001.
459 Has oral fluid the potential to replace serum for the evaluation of population immunity

levels? A study of measles, rubella and hepatitis B in rural Ethiopia. Bull World Health Organ 79:588-95.

33. Vainio K, Samdal HH, Anestad G, Wedege E, Skutlaberg DH, Bransdal KT, Mundal R, Aaberge IS. 2008. Detection of measles- and mumps-specific IgG antibodies in paired serum and oral fluid samples from Norwegian conscripts. Eur J Clin Microbiol Infect Dis 27:461-5.

34. Lewnard JA, Grad YH. 2018. Vaccine waning and mumps re-emergence in the United States. Sci Transl Med 10.

35. Vijaylakshmi P, Muthukkaruppan VR, Rajasundari A, Korukluoglu G, Nigatu W, Warrener LA, Samuel D, Brown DW. 2006. Evaluation of a commercial rubella IgM assay for use on oral fluid samples for diagnosis and surveillance of congenital rubella syndrome and postnatal rubella. J Clin Virol 37:265-8.

Figures

Figure 1. Two-fold dilutions of the swab eluate in PBS with detection for CMV IgG. Dashed lines indicate the reactive (upper; O.D. = 0.291) and non-reactive (lower; O.D. = 0.222) cut-off O.D. values.

Figure 2. Graphical representation showing how flat-bottom (solid circles and line) and conical (empty circles and dashed line) tube shape affects (A) volume recovered and (B) CMV sIgG loads.

Figure 3. Comparison of viral-specific sIgG in paired flocked swab versus serum samples from 50 healthy volunteers for the following targets: 1. CMV; 2. VZV; 3. EBV; 4. Measles; 5. Mumps. For EBV both (a) EBNA-1 and (b) VCA were tested. For Measles and Mumps assays from (a) Gold Standard Diagnostics and (b) R-Biopharm AG were investigated. Positive cut-off values (dotted blue line) and negative cut-off values (dotted red line) were determined for each assay.

Tables

Table 1. Cut-off O.D. values for viral-specific IgG assays. For each assay the cut-off O.D. values were determined as follows from the average O.D. values of all negative samples: Non-reactive = O.D. < mean (non-reactive) + 2 St. Dev; Indeterminate = > mean (non-reactive) + 2 St Dev but < mean (non-reactive) + 3 St Dev; Reactive = O.D. > mean (non-reactive) + 3 St Dev.

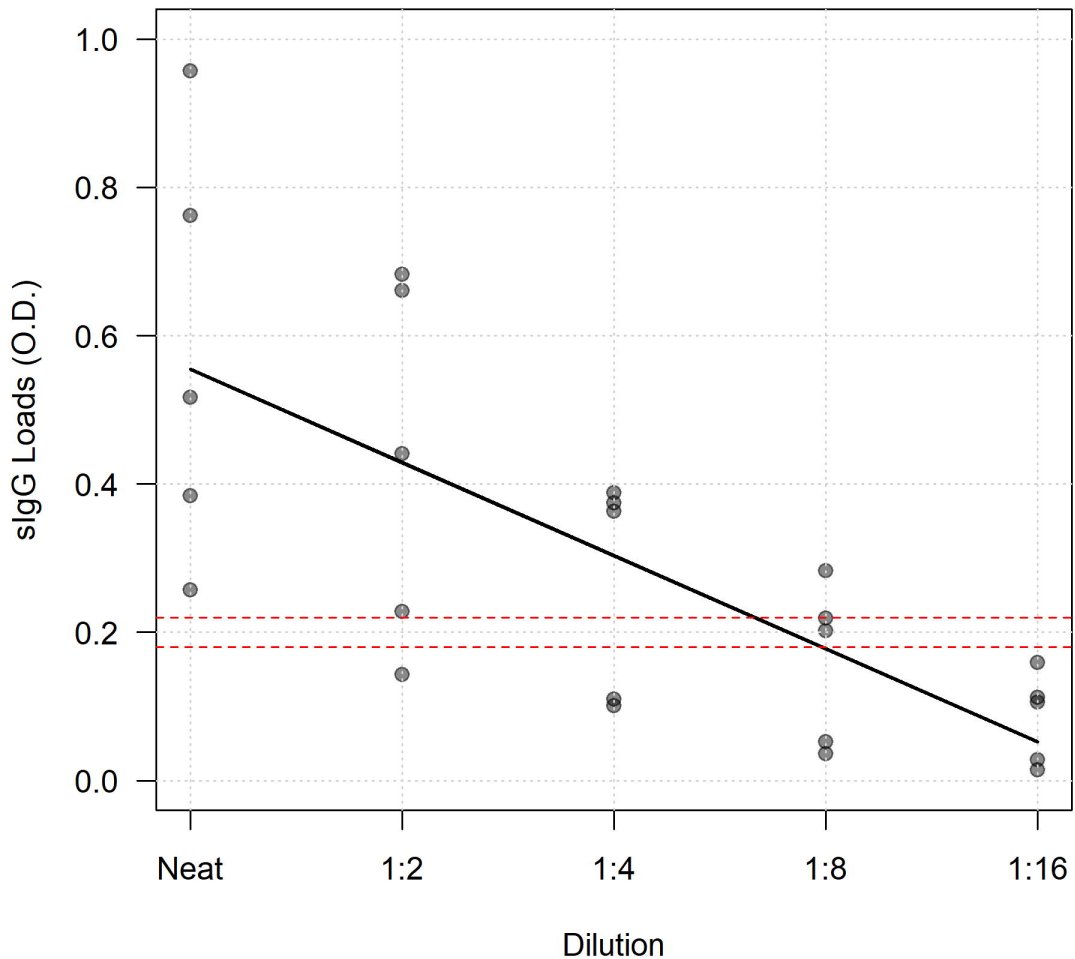
		Serum		Flocked Swab		Saliva	
Target	Company	Non-Reactive	Reactive	Non-Reactive	Reactive	Non-Reactive	Reactive
CMV IgG	GSDx	0.222	0.291	0.178	0.221	0.219	0.253
VZV IgG	R-Biopharm	0.220	0.420	0.100	0.116	0.087	0.091
EBV EBNA-1 IgG	GSDx	0.449	0.609	0.067	0.084	0.084	0.105
EBV VCA IgG	GSDx	0.324	0.438	0.116	0.150	0.193	0.229
Measles IgG	R-Biopharm	0.200	0.250	0.081	0.100	0.102	0.125
Measles IgG	GSDx	0.275	0.400	0.101	0.116	0.115	0.135
Mumps IgG	R-Biopharm	0.155	0.174	0.109	0.137	0.113	0.135
Mumps IgG	GSDx	0.532	0.635	0.086	0.108	0.112	0.13

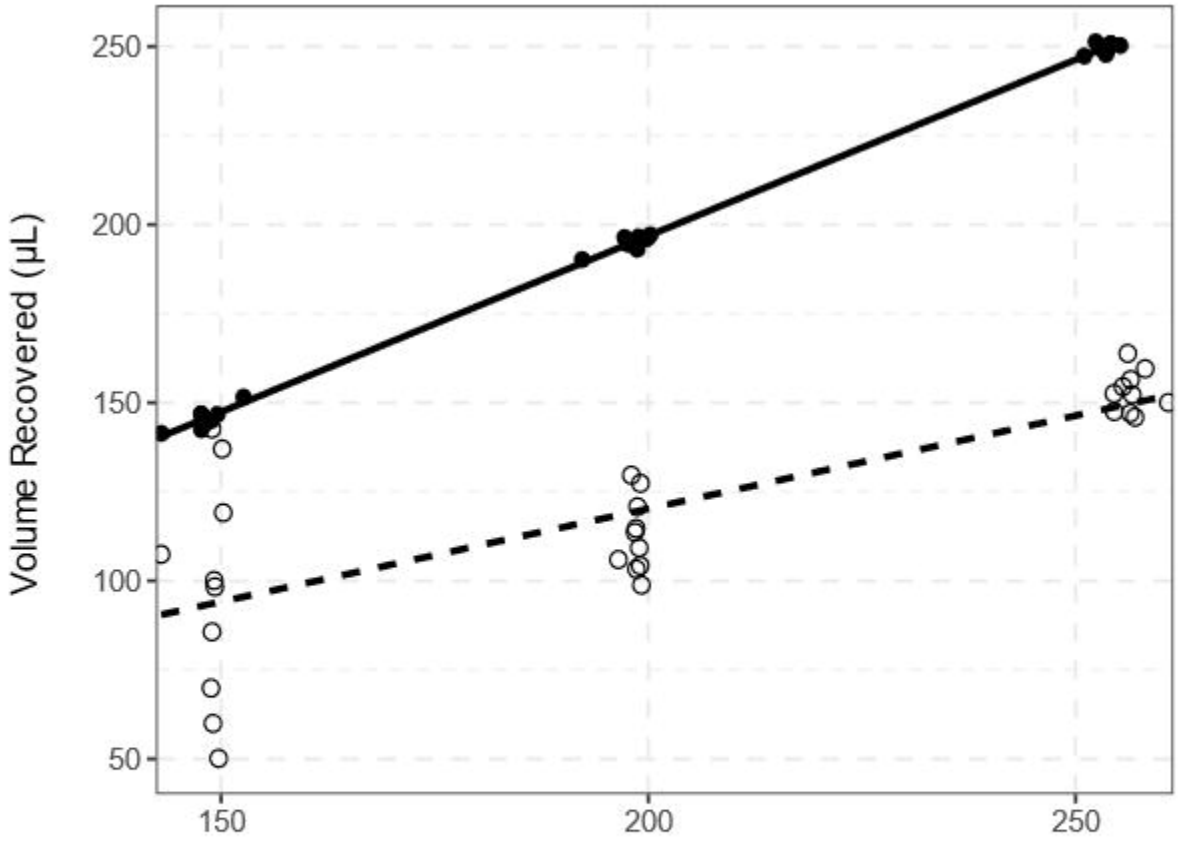
GSDx=Gold Standard Diagnostics

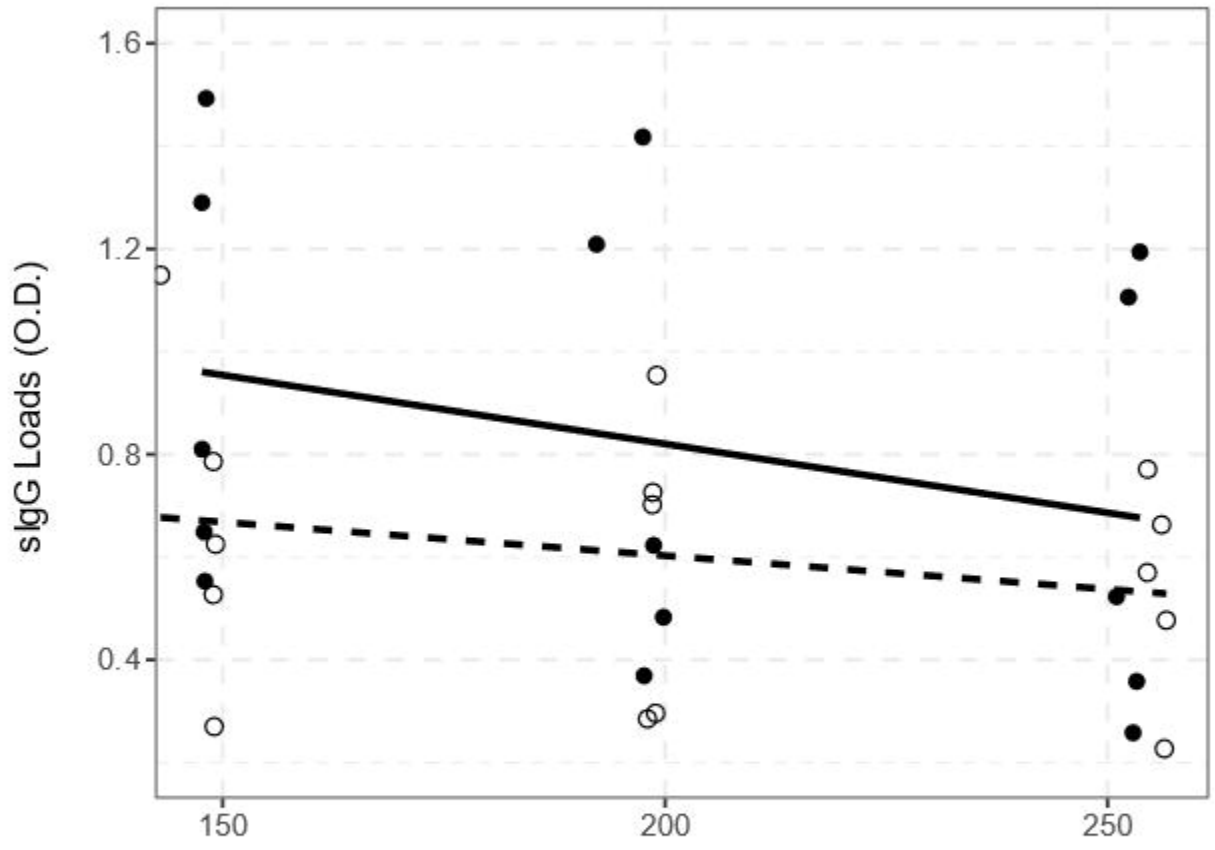
Table 2. Diagnostic accuracy for viral-specific IgG assays in swabs and saliva compared to sera. For all samples were seropositive for VZV and Measles so the specificity could not be determined.

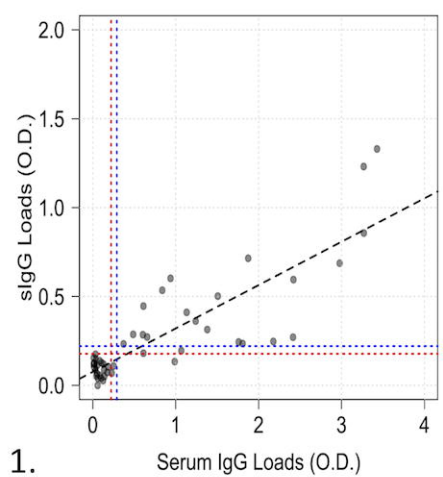
	Cytomegalovirus		Varicella Zoster Virus		Epstein-Barr Virus				Measles				Mumps			
Assay	**GSDx CMV IgG**		**R-Bio VZV IgG**		**GSDx EBNA-1 IgG**		**GSDx VCA IgG**		**GSDx MeV IgG**		**R-Bio MeV IgG**		**GSDx MuV IgG**		**R-Bio MuV IgG**	
Specimen	Swabs	Saliva	Swabs	Saliva	Swabs	Saliva	Swabs	Saliva	Swabs	Saliva	Swabs	Saliva	Swabs	Saliva	Swabs	Saliva
Sample (n=)	50	49	50	49	50	49	50	49	50	49	50	49	50	49	50	49
Positives*	23/24	24/24	48/50	46/49	35/38	34/38	42/44	42/44	41/50	46/50	24/50	34/50	27/45	31/45	28/46	33/46
Sensitivity	95.8%	100.0%	96.0%	93.9%	92.1%	91.9%	95.5%	97.7%	85.4%	93.9%	49.0%	69.4%	60.5%	68.2%	62.2%	73.3%
Specificity	100%	100%	ND	ND	100%	100%	100%	100%	ND	ND	ND	ND	80.0%	80.0%	100.0%	100.0%
PPV	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	96.3%	96.8%	100.0%	100.0%
NPV	96.3%	100%	ND	ND	80.0%	80.0%	75.0%	85.7%	ND	ND	ND	ND	19.0%	22.2%	19.0%	25.0%
Accuracy	98.0%	100%	96.0%	93.9%	94.0%	93.9%	96.0%	98.0%	85.4%	93.9%	49.0%	69.4%	62.5%	69.4%	65.3%	75.5%
Misclassification	4.0%	0.0%	4.0%	6.1%	6.0%	6.1%	4.0%	2.0%	10.0%	6.1%	50.0%	30.6%	40.9%	33.3%	37.8%	26.7%
Agreement (K)	0.88	0.85	0.00	0.00	0.85	0.85	0.83	0.91	0.00	0.00	0.00	0.00	0.20	0.25	0.21	0.32
Linearity (Adj. R^2)	0.713	0.622	0.248	0.218	0.315	0.178	0.232	0.266	0.308	0.351	0.554	0.316	0.142	0.221	0.005	0.017

*# of swab or saliva positives/# serum positive samples; PPV = Positive Predictive Value; NPV = Negative Predictive Value; GSDx = Gold Standard Diagnostics; R-Bio = R-Biopharm AG; ND = Not Determined.

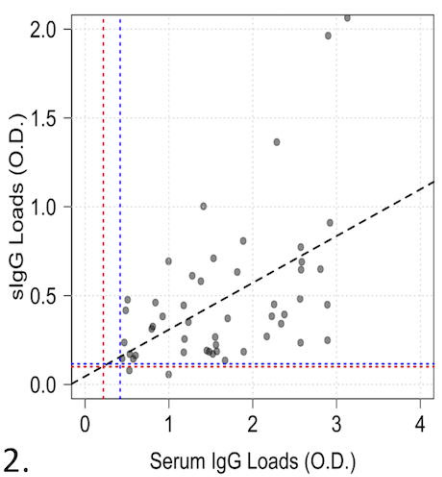

1.0
0.8
0.6
0.4
0.2
0.0
sIgG Loads (O.D.)
Neat
1:2
1:4
1:8
1:16
Dilution


250
200
150
100
50
Volume Recovered (µL)
150
200
250
Volume Added (µL)
Conical
Flat

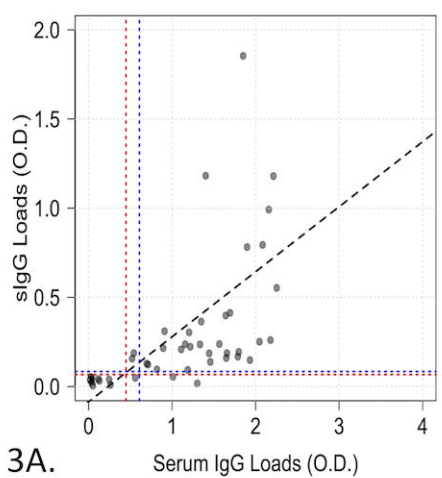

1.6
1.2
0.8
0.4
sIgG Loads (O.D.)
150
200
250
Volume Added (µL)
Conical
Flat



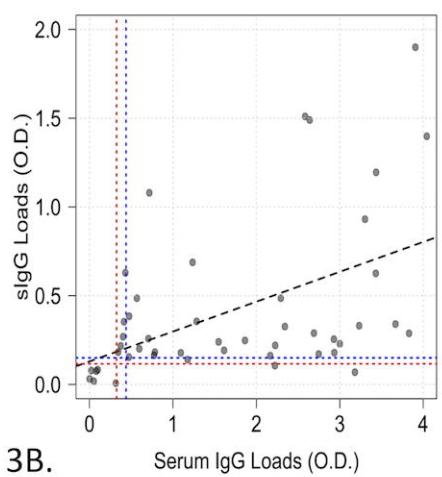
1.



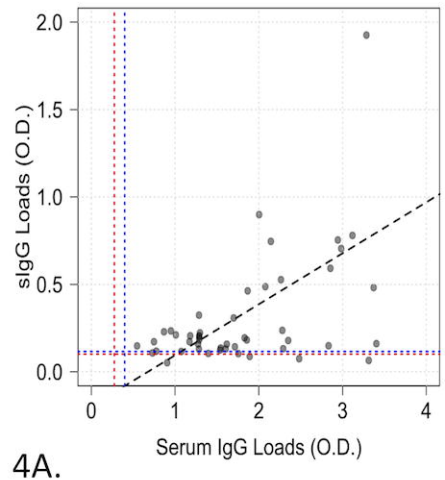
2.



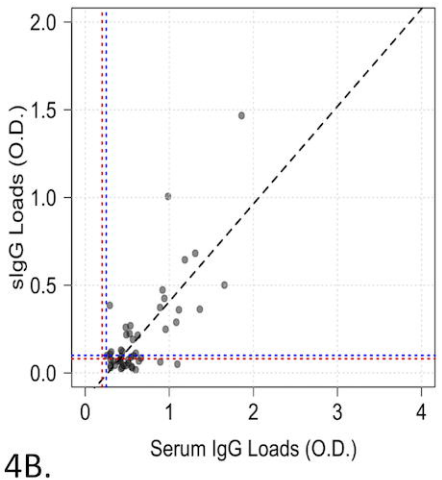
3A.



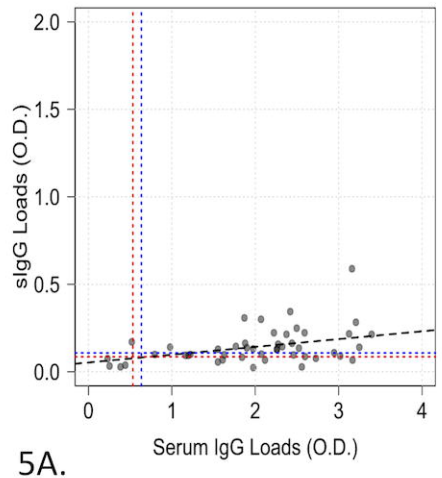
3B.



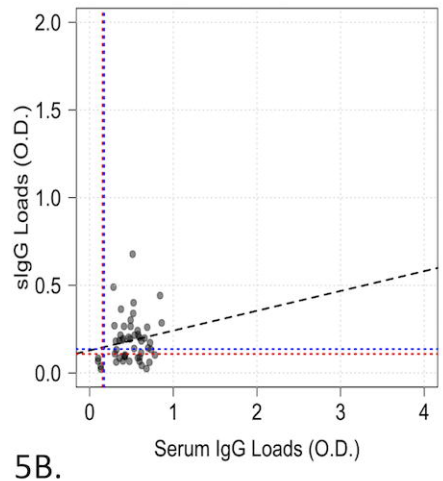
4A.



4B.



5A.



5B.