Non-Invasive Detection of Viral Antibodies Using Oral Flocked Swabs

Running Title: Oral flocked swabs for the detection of viral antibodies

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Abstract

Saliva contains antibodies potentially useful for determining serostatus for surveillance and vaccination studies. However, antibody levels are low, and degradation by endonucleases is problematic. As a simple, non-invasive alternative to blood we developed a method for detecting viral antibodies from oral flocked swabs. Serum, saliva, and oral swabs were collected from 50 healthy volunteers. Sera and saliva were stored at -80°C. Dried swabs were stored at room temperature. Seroprevalence for Cytomegalovirus (CMV), Varicella Zoster virus (VZV), Epstein-Barr virus (EBV), Measles and Mumps IgG antibodies were determined using commercial ELISA assays and processed on the ThunderBolt® Analyzer (Gold Standard Diagnostics). For each antibody, swabs correlated well with saliva. For CMV IgG, the swab sensitivity and specificity compared to serum were 95.8% and 100%, respectively. For VZV IgG, swab and saliva sensitivity were 96.0% and 93.9%, respectively. As all volunteers were seropositive for VZV and Measles, specificity could not be determined. For EBV EBNA-1 IgG and VCA IgG, swab and saliva sensitivity were 92.1% and 95.5%, respectively; specificities were 100%. For Measles IgG, swab and saliva sensitivity were 84.5% and 93.9%, respectively. Mumps IgG displayed poor sensitivity for oral swabs (60.5%) and saliva (68.2%), but both were 100% specific. Dried oral swabs correlated well with serum for CMV, VZV, EBV and Measles antibodies with excellent sensitivity and specificity, but had poor sensitivity for detecting antibodies to Mumps. As oral flocked swabs are easy to self-collect and can be stored at room temperature they are an ideal tool for seroprevalence studies.

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 sample for determining immune status but is invasive to collect. Saliva has considerable diagnostic potential: it is abundant, and collection is easy and non-invasive. Saliva is representative of oral and systemic health, and the field of salivary diagnostics is rapidly emerging, especially the identification and validation of biomarkers for point-of-care testing of infectious diseases (1). Detection of antibodies in oral fluids has been utilized in the U.S. Food and Drug Administration (FDA) approved OraQuick ADVANCE® Rapid HIV-1/2 Antibody Test (OraSure Technologies, Inc., USA) and OraQuick® HCV Test (OraSure Technologies, Inc.), have advanced the development of microfluidic systems to detect host anti-HIV antibodies and viral RNA simultaneously, and could aid vaccination, transplantation and donor programs (1, 2). Salivary antibodies are primarily secretory IgA from the salivary glands, while IgG and IgM are derived from plasma cells in the serum and passively diffused into the oral cavity via gingival crevicular fluid (3, 4). Whilst salivary IgG is systemically representative and strongly correlates with serum levels, loads are approximately 1:800 that of serum (5, 6); ergo making antibody testing on commercial platforms problematic as most are closed systems designed for testing sera and incorporate a 1:100 dilution step.

Viral antibodies can be detected in saliva, but collection can be difficult in children and hyposalivators, such as immunocompromised patients. Salivary endonucleases, which remain active at -80°C, are detrimental and require special sample handling, or storage in proteolytic stabilizers unsuitable for antibody preservation (7). Therefore, we developed and optimised pre-

analytic and analytic procedures that allows oral nylon flocked swabs to be used for detecting viral antibodies on an open commercial platform. This 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 (8, 9). The optimized procedure was then applied to determine the diagnostic accuracy of detecting Varicella Zoster virus (VZV), Epstein-Barr virus (EBV), Measles and Mumps IgG. Whilst oral flocked swabs are a non-invasive and simple alternative to blood and saliva for detecting viral shedding, their efficiency to detect viral antibodies had yet to be determined.

Materials and Methods

Study Population:

Following approval from the Hamilton Integrated Research Ethics Board (HiREB #14-658) and obtaining 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. Volunteers consisted of laboratory staff (15 males:35 females) from St. Joseph's Healthcare Hamilton; average age was 43.4 years (range: 18-65 years). All sample types were collected from the volunteers, except for one who could not produce a saliva sample. Oral swabs (FLOQSwabs® #520C, Copan Italia S.p.A., Brescia, Italy) were collected by moistening the flocked swab on the tongue and then rotating between the gums and cheek three to five times, dried for an hour inside a biosafety cabinet, and then stored inverted in a microcentrifuge tube at room temperature. 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. Two swabs were collected consecutively. 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 (10). The supernatant was aliquoted into 1mL portions and stored at -80C; the cell pellet was discarded. Serum was obtained by collecting 5mL blood via venipuncture in a serum tube, allowed to clot for 30 minutes, centrifuged at 3,000 *x g* for 10 minutes, and stored at -80ºC.

Development and Optimisation of pre-analytic and analytic procedures:

Optimisation of pre-analytic and analytic procedures was performed using the CMV IgG EIA, on the ThunderBolt® ELISA Analyzer both from Gold Standard Diagnostics (GSDx, Davis, CA, USA). The first optimisation determined the optimal dilution for testing oral swabs on swabs from five seropositive individuals. To elute viral antibodies, 250µL PBS was added to a dried swab head, vortexed for 30 seconds, incubated at room temperature for 10-minutes, centrifuged at 14,000 *x g* for one minute, and then the swab was removed with forceps. Serial dilutions (2-fold serially from neat to 1:16) were prepared in PBS. 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 (11).

As preliminary testing with conical microcentrifuge tubes produced inconsistent results (data not shown) 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). For each combination the volume of PBS recovered was measured. To determine the nature of 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 O.D. values when the pelleted buccal cells were left undisturbed at the bottom of the microcentrifuge tube; 2. Supernatant, measuring the O.D. values on the supernatant after it was transferred to a new tube without disturbing the pellet; 3. Resuspended; measuring the O.D. values after the pellet was completely resuspended by vortexing for 30 seconds. To determine the relationship between patient observations, an initial ANOVA followed by a 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 stored in dried flocked swabs over time by measuring the O.D. values of five salivopositive samples at baseline and after two months stored at room temperature. Paired t-tests were also used to compare average O.D. values at baseline and after two months of storage.

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 consecutively collected swabs (data not shown) swab eluates were often pooled to allow for more testing from a single sample. The commercial 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). For each assay, we calculated the mean O.D. and standard deviation (SD) from swabs and saliva specimens corresponding to "true-negative" subjects whose serum was test negative. We then defined cut-off values as: Non-reactive if < mean O.D. + 2 SD; Reactive if > mean O.D. + 3 SD; or indeterminate if the values fell between mean +2 SD and +3 SD. 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.

Results

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

To determine the effect of tube shape and establish the optimal elution volume, O.D. values were measured at three volumes of PBS and two tube shape combinations. The conical microcentrifuge tubes had a significant decrease in volume recovered and lower recalculated O.D. values due to the conical shape extending above the swab shaft causing the eluate to be reabsorbed and retained in the swab head (Figure 2). The average percent of volume of PBS recovered from the conical microcentrifuge tubes was 60.66 ± 4.38%, but for the flat-bottomed tubes was 98.63 ± 0.78%. Statistical analysis confirmed that tube shape greatly affects the volume recovered (p=0.017), O.D. values (p=0.003) and could possibly affect the status of weakly reactive samples. In the non-reactive samples, the average O.D. values of the flat-bottomed tubes (O.D. values: 0.080 ± 0.008) were slightly higher than the conical tubes (O.D. values: 0.059 ± 0.021), but this difference did not affect positivity. Therefore, subsequent testing was performed by eluting with 250µL PBS in flat-bottomed tubes, and to facilitate two tests per swab.

To determine the most efficient sample handling procedure to yield the highest O.D. values, three methods were evaluated. In the reactive samples, the average O.D. values ± SD for the undisturbed pellet, supernatant, and resuspended pellet were 0.688 ± 0.434; 0.545 ± 0.222; and 0.531 ± 0.214, respectively. One sample was a weak reactive (O.D. 0.258) in the pellet method but became indeterminate in the supernatant (O.D. 0.214) or resuspended pellet (O.D. 0.197) method. 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). In the non-reactive samples, the average O.D. ± SD values for the three methods, pellet, supernatant, and resuspended, were 0.077 ± 0.047; 0.059 ± 0.045; and 0.061 ± 0.062, respectively. For both reactive and non-reactive samples, the sample handling procedure did not significantly affect O.D. values but fractioning the sample could produce an indeterminate result from a weakly reactive sample. Therefore, subsequent testing was performed using the whole sample following centrifugation. This method was also simpler and reduced hands on time.

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 from baseline (O.D. 0.575 ± 0.284) to after two months of storage (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.

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). Based on the 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 = 0.848; 95% CI: 0.71, 1.00), and 100% agreement 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 EBV 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 EBV 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 EBV 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 EBV 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 EBV 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).

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

For Mumps IgG, both assays produced comparable seroprevalence of 45/50 (90.0%) and 46/50 (92.0%) for the GSDx and R-Biopharmassays, respectively. The sensitivity of both assays was poor for both swabs [GSDx: 26/43 (60.5%; 95% CI: 45.6%, 73.7%); R-Biopharm: 28/45 (62.2%; 95% CI: 47.6%, 74.9%)] and saliva [GSDx: 30/44 (68.2%; 95% CI: 53.4%, 80.1%); R-Biopharm: 33/45 (73.3%; 95% CI: 58.4%, 84.2%)]. …(12). The agreement was only fair for both assays between swab (GSDx: K = 0.20; 95% CI: 0.00, 0.40; R-Biopharm: K = 0.21; 95% CI: 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, 0.56) compared to sera, as well as swabs compared to saliva (GSDx: K = 0.25; 95% CI: 0.03, 0.47; R-Biopharm: K = 0.38; 95% CI: 0.16, 0.60). Further analysis showed that both assays had a 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.

Discussion

Salivary diagnostic assays for viral infections and immunity have great potential. However, saliva as a diagnostic fluid can be problematic as samples must be quickly chilled and stored frozen to slow degradation, and salivary IgG loads are approximately 1:800 that of serum (5, 7). Therefore, we developed and optimised pre-analytic and analytic procedures for the detection of viral IgG on an open commercial platform using dried flocked oral swabs stored at room temperature, which performed well for detecting CMV, VZV, and EBV EBNA-1 and VCA IgG, adequately for Measles IgG, and poorly for Mumps IgG. This is the first report of oral swabs used to detect CMV

IgG. Based on the high sensitivity and excellent correlation with serum, oral samples are ideal for non-invasive, high-throughput screening of transplant donors. Whilst this study only tested IgG, as salivary IgM is more concentrated than IgG (i.e. 1:400 that of serum) this procedure could be applied to acute diagnosis but further testing is required (4).

Optimisation of pre-analytic and analytic procedures for flocked swabs was initially performed for CMV due to interest from blood banks and transplant programs as a screen for CMV positivity. 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 (13-15). CMV antibody profiles in serum also strongly correlate with oral CMV shedding (9, 16), and, based on this study, CMV IgG can accurately be detected in oral swabs. Our procedure appears promising, but a few procedural steps must be heeded. Whilst cut-off O.D. values might vary between assays, platforms and/or cohort tested, it is essential to elute swabs into appropriate tubes to prevent reabsorption and to use undiluted eluate. As testing volume only allows two tests per swab it is possible to collect multiple swabs consecutively at any time of day without a reduction in positivity. Swabs collected from the same individual yielded the same positivity regardless of daytime collection time (data not shown). Further work is needed to optimize elution and testing volumes to maximize testing and increase O.D. values to sort out indeterminate samples as well as compare manual testing vs automation to see if this method is ideal for resource-constrained settings.

The few publications examining salivary IgG for VZV, EBV, Measles and Mumps utilized the Oracol saliva collection system (Malvern Medical Developments, UK) coupled with an EIA,

indirect immunofluorescent assays (IFA), or antibody capture radioimmunoassays performed manually (17, 18). Whilst the Oracol system collects 1 mL saliva and can be transported at room temperature once eluted in 1 mL transport medium, samples must be frozen prior to processing and testing. In 2005, Talukder *et al.* developed a VZV ELISA for oral fluids on 1,092 participants with a sensitivity and specificity of 93% and 95.7%, respectively (18). Whilst our sample size was insufficient to determine assay specificity, the sensitivity of saliva and swabs in our study is comparable to that reported by Talukder *et al*. suggesting that 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 (19). Both Vyse *et al.* (1997) and Crowcroft *et al.* (1998) utilized oral fluids to determine EBV immune status (20, 21). Vyse *et al.* measured EBV immune status by coupling the Oracol system with a 'G' antibody capture radioimmunoassay to detect EBV VCA IgG and reported a sensitivity and specificity of 93.5% and 100%, respectively (21). However, Vyse *et al.*, reported that their method was less sensitive than IFA as samples with a total IgG <2 mg/L yielded false positive reactions due to the monoclonal antibody binding non-specifically to unsaturated anti-human IgG on the solid phase. Compared to Vyse *et al*., our method produced higher sensitivities for both nylon flocked swabs and saliva with no false positives. As 5% of people do not produce EBNA-1 IgG after EBV infection we validated both EBV EBNA-1 IgG and VCA IgG (22). Combining both EBV EBNA-1 and VCA IgG our procedure yielded a sensitivity of 97.7% and 100% for swabs and saliva, respectively, and may be useful for epidemiological screening.

Several seroprevalence studies have used oral fluids as a non-invasive alternative for monitoring the efficacy of vaccination programs for Measles with most studies using the Oracol system coupled with either the commercialized Measles IgG Capture EIA (Microimmune Ltd., UK), and the Enzygnost® Anti-Measles Virus/IgG (Siemens Health Care Diagnostics GmbH, Germany) (6). As cut-off O.D. values and equivocal ranges differed between studies and some studies used the Microimmune assays on saliva and the Enzygnost assay as the comparative "gold standard" on serum, it is difficult to compare results between studies (6). Whilst Hayford *et al*., reported that oral fluids are not suitable to detect immunity for Measles due to poor sensitivity (60.2%) and specificity (75.7%) (23, 24) others reported sensitivity of 90.0-92.0% and a specificity of 77.8-100% using the Microimmune Ltd. assay (25-28). Our study compared the GSDx and R-Biopharm assays for Measles IgG, and whilst the R-Biopharm assay produced poor sensitivity and specificity with oral fluids, the GSDx assay was comparable to the Microimmune Ltd. assay. Further optimization is required to increase the sensitivity of our assay, but in its present stage may be adequate for epidemiological studies. If the efficacy of vaccination is essential, confirmatory testing on serum should be performed for non-reactive oral samples.

For Mumps, Vainio *et al.*, utilized the Oracol system coupled with the Mumps IgG Capture EIA (Microimmune Ltd., Clin-Tech Limited, Guildford, UK) and reported low detection of Mumps IgG in oral samples and recommended that the Microimmune assay not be used for serosurveillence studies (29). Our study also reported a poor sensitivity for detecting Mumps IgG with both the GSDx and R-Biopharm assays. As the Microimmune, GSDx and R-Biopharm assays performed well in serum, the decrease in salivopositivity may be biological rather than assay

dependent, but further optimisation is required to devise a method for the accurate detection of Mumps IgG in oral fluids.

One limitation of our study is that our sampling did not include enough non-reactive participants for Measles and VZV to determine specificity. In a hospital laboratory volunteer study, virtually all employees have received screening and vaccination for these vaccine-preventable illnesses. Further work is also needed to optimise oral flocked swabs for the detection of Measles and Mumps IgG. It is possible that low sensitivity of detecting measles and mumps is due to the lack of virus specific IgG in oral fluids. Whilst the level of IgG in saliva is 1:800 that of serum it would be interesting to compare the absolute amount of IgG in serum compared to swabs and saliva using the Total Human IgG EIA (Clin-Tech Limited). Future work should also investigate the utility of oral swabs for Rubella IgM as previous studies look promising with 79-96.9% sensitivity and 90-100% specificity (28, 30). Nevertheless, oral swabs performed remarkably well for the detection of CMV, VZV, EBV EBNA-1 and VCA IgG. As oral flocked swabs, unlike saliva, can be easily collected and stored long-term at room temperature without degradation of sample integrity, making them ideal for field studies. We suggest that oral swabs could be considered for the non-invasive, high-throughput screening of organ donors for CMV IgG and epidemiological seroprevalence studies for CMV, VZV, EBV and Measles.

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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. Chen Z, Zhu H, Malamud D, Barber C, Ongagna YY, Yasmin R, Modak S, Janal MN, Abrams WR, Montagna RA. 2016. A Rapid, Self-confirming Assay for HIV: Simultaneous Detection of Anti-HIV Antibodies and Viral RNA. J AIDS Clin Res 7.
3. Brandtzaeg P. 2007. Do salivary antibodies reliably reflect both mucosal and systemic immunity? Ann N Y Acad Sci 1098:288-311.
4. Parry JV, Perry KR, Mortimer PP. 1987. Sensitive assays for viral antibodies in saliva: an alternative to tests on serum. Lancet 2:72-5.
5. Saccoccio FM, Gallagher MK, Adler SP, McVoy MA. 2011. Neutralizing activity of saliva against cytomegalovirus. Clin Vaccine Immunol 18:1536-42.
6. Dimech W, Mulders MN. 2016. A review of testing used in seroprevalence studies on measles and rubella. Vaccine 34:4119-4122.
7. 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.
8. Ljungman P, Hakki M, Boeckh M. 2011. Cytomegalovirus in hematopoietic stem cell transplant recipients. Hematol Oncol Clin North Am 25:151-69.
9. 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.

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

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

12. Upper D. 1974. The unsuccessful self-treatment of a case of "writer's block". J Appl Behav Anal 7:497.

13. Cannon MJ, Stowell JD, Clark R, Dollard PR, Johnson D, Mask K, Stover C, Wu K, Amin M, Hendley W, Guo J, Schmid DS, Dollard SC. 2014. Repeated measures study of weekly and daily cytomegalovirus shedding patterns in saliva and urine of healthy cytomegalovirus-seropositive children. BMC Infect Dis 14:569.

14. Barkai G, Ari-Even Roth D, Barzilai A, Tepperberg-Oikawa M, Mendelson E, Hildesheimer M, Kuint J. 2014. Universal neonatal cytomegalovirus screening using saliva - report of clinical experience. J Clin Virol 60:361-6.

15. Stowell JD, Mask K, Amin M, Clark R, Levis D, Hendley W, Lanzieri TM, Dollard SC, Cannon MJ. 2014. Cross-sectional study of cytomegalovirus shedding and immunological markers among seropositive children and their mothers. BMC Infect Dis 14:568.

16. Cardoso ES, Jesus BL, Gomes LG, Sousa SM, Gadelha SR, Marin LJ. 2015. The use of saliva as a practical and feasible alternative to urine in large-scale screening for congenital cytomegalovirus infection increases inclusion and detection rates. Rev Soc Bras Med Trop 48:206-7.

17. Furuta Y, Ohtani F, Aizawa H, Fukuda S, Kawabata H, Bergstrom T. 2005. Varicella-zoster virus reactivation is an important cause of acute peripheral facial paralysis in children. Pediatr Infect Dis J 24:97-101.

18. Talukder Y, Gopal R, Andrews N, Glenn M, Breuer J, Brown D. 2005. Development and evaluation of Varicella zoster virus ELISA for oral fluid suitable for epidemiological studies. J Virol Methods 128:162-7.

19. De Paschale M, Clerici P. 2012. Serological diagnosis of Epstein-Barr virus infection: Problems and solutions. World J Virol 1:31-43.

20. Crowcroft NS, Vyse A, Brown DW, Strachan DP. 1998. Epidemiology of Epstein-Barr virus infection in pre-adolescent children: application of a new salivary method in Edinburgh, Scotland. J Epidemiol Community Health 52:101-4.

21. Vyse AJ, Knowles WA, Cohen BJ, Brown DW. 1997. Detection of IgG antibody to Epstein-Barr virus viral capsid antigen in saliva by antibody capture radioimmunoassay. J Virol Methods 63:93-101.

22. De Paschale M, Agrappi C, Manco MT, Mirri P, Vigano EF, Clerici P. 2009. Seroepidemiology of EBV and interpretation of the "isolated VCA IgG" pattern. J Med Virol 81:325-31.

23. Hayford KT, Al-Emran HM, Moss WJ, Shomik MS, Bishai D, Levine OS. 2013. Validation of an anti-measles virus-specific IgG assay with oral fluid samples for immunization surveillance in Bangladesh. J Virol Methods 193:512-8.

24. Hayford KT, Shomik MS, Al-Emran HM, Moss WJ, Bishai D, Levine OS. 2013. Measles vaccination coverage estimates from surveys, clinic records, and immune markers in oral fluid and blood: a population-based cross-sectional study. BMC Public Health 13:1211.

25. Hutse V, Van Hecke K, De Bruyn R, Samu O, Lernout T, Muyembe JJ, Brochier B. 2010. Oral fluid for the serological and molecular diagnosis of measles. Int J Infect Dis 14:e991-7.

26. Kremer JR, Muller CP. 2005. Evaluation of commercial assay detecting specific immunoglobulin g in oral fluid for determining measles immunity in vaccinees. Clin Diagn Lab Immunol 12:668-70.

27. Warrener L, Slibinskas R, Chua KB, Nigatu W, Brown KE, Sasnauskas K, Samuel D, Brown D. 2011. A point-of-care test for measles diagnosis: detection of measles-specific IgM antibodies and viral nucleic acid. Bull World Health Organ 89:675-82.

28. Nokes DJ, Enquselassie F, Nigatu W, Vyse AJ, Cohen BJ, Brown DW, Cutts FT. 2001. 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.

29. 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.

30. 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) Recalculated O.D. values for CMV Ig.

Figure 3. Comparison of viral-specific IgG (Recalculated O.D. values) 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

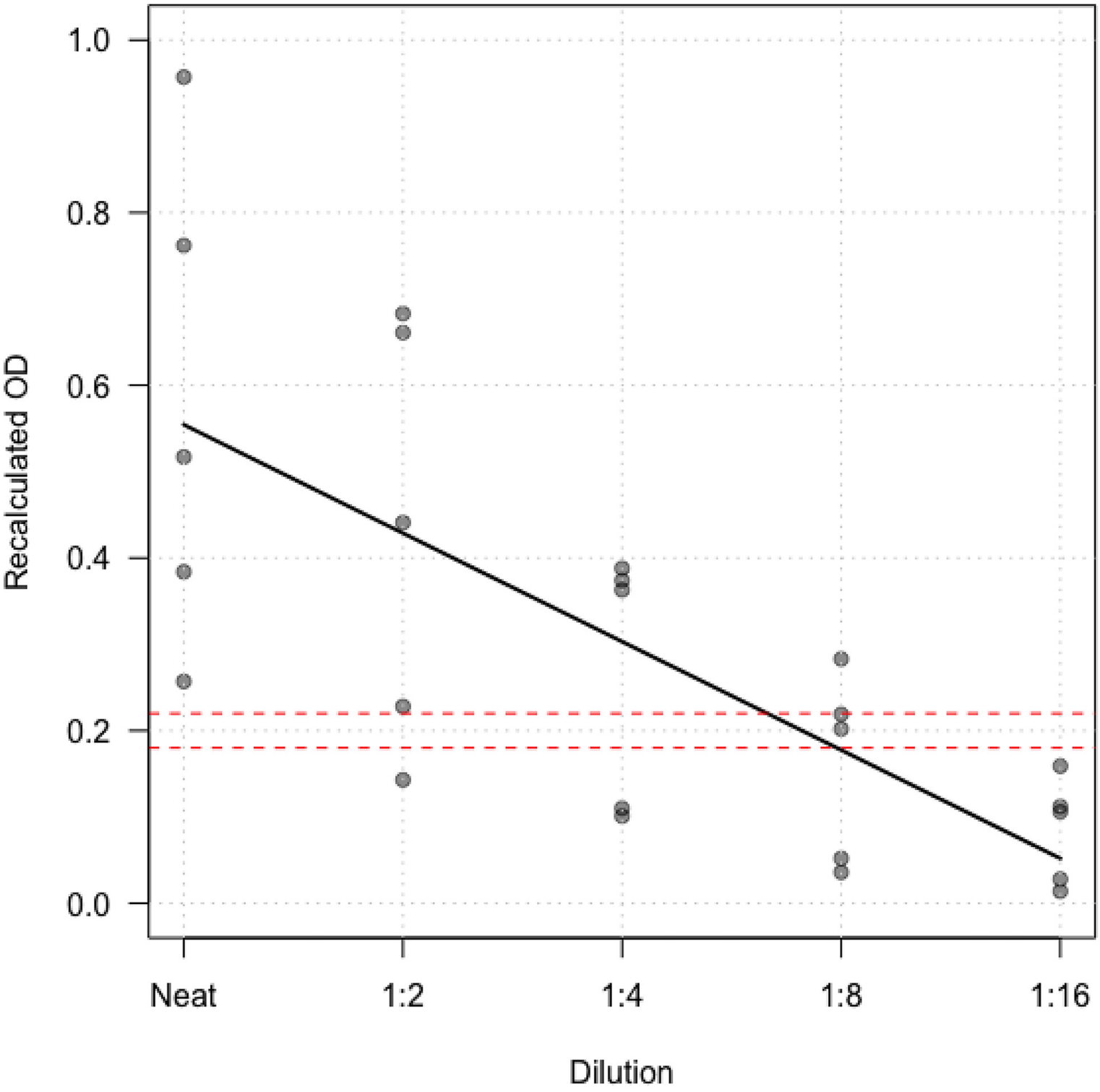
Table 1. Cut-off O.D. values for viral-specific IgG assays. For each assay the cut-off O.D. values were determined as follows from the average O.D. values of all negative samples: Non-reactive = O.D. < mean (non-reactive) + 2 St. Dev; Indeterminate = > mean (non-reactive) + 2 St Dev but < mean (non-reactive)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

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		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	95.80%	100%	96.00%	93.90%	92.10%	91.90%	95.50%	97.70%	85.40%	93.90%	49.00%	69.40%	60.50%	68.20%	62.20%	73.30%
Specificity	100%	100%	ND	ND	100%	100%	100%	100%	ND	ND	ND	ND	80.00%	80.00%	100%	100%
PPV	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	96.30%	96.80%	100%	100%
NPV	96.30%	100%	ND	ND	80.00%	80.00%	75.00%	85.70%	ND	ND	ND	ND	19.00%	22.20%	19.00%	25.00%
Accuracy	98.00%	100%	96.00%	93.90%	94.00%	93.90%	96.00%	98.00%	85.40%	93.90%	49.00%	69.40%	62.50%	69.40%	65.30%	75.50%
Misclassification	4.00%	0%	4.00%	6.10%	6.00%	6.10%	4.00%	2.00%	10.00%	6.10%	50.00%	30.60%	40.90%	33.30%	37.80%	26.70%

*# 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.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1:4
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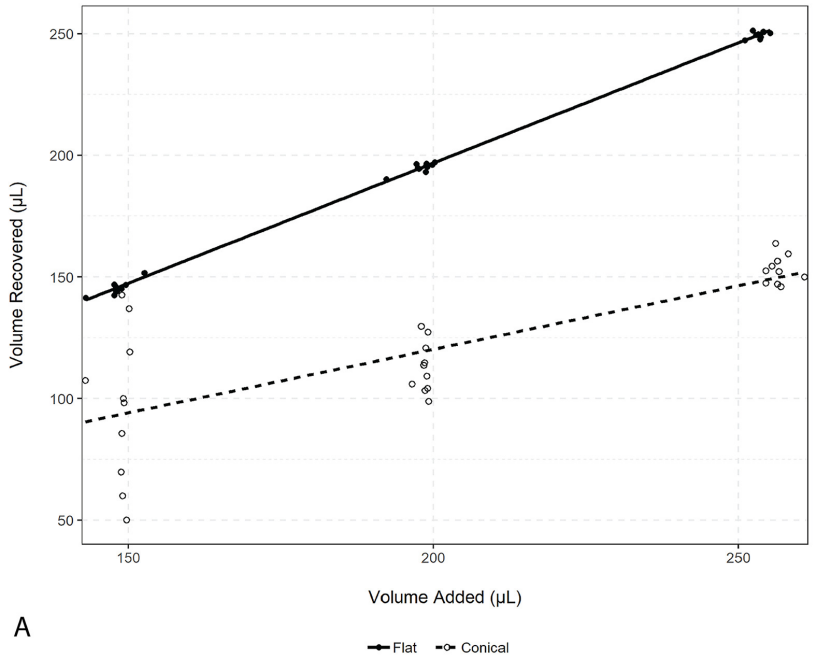
Dilution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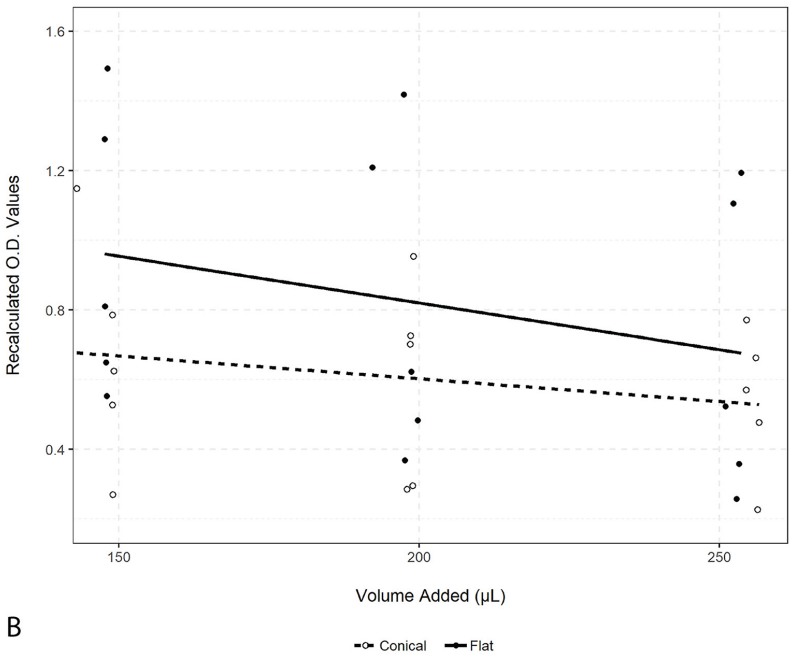
200
150
100
50
150
200
250
Volume Added (µL)
A
Flat
Conical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250
Volume Added (µL)
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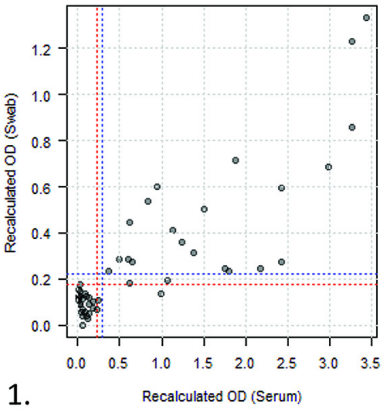
Conical
Flat



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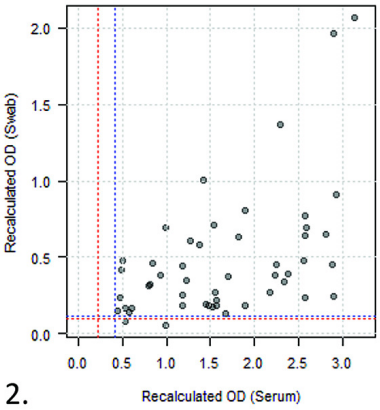

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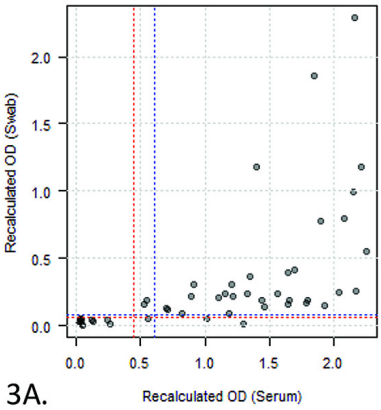

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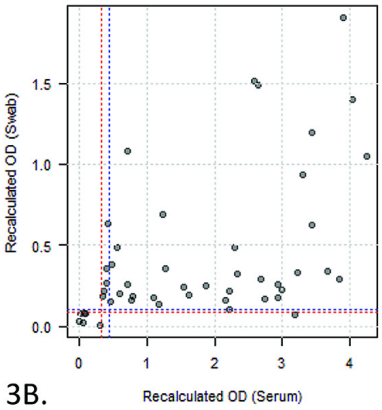

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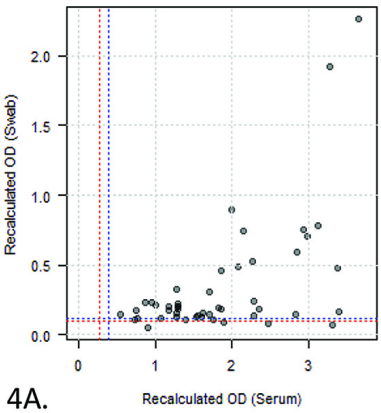

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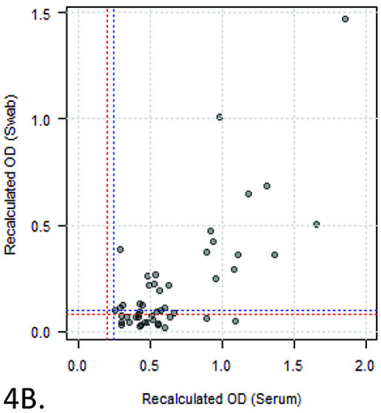

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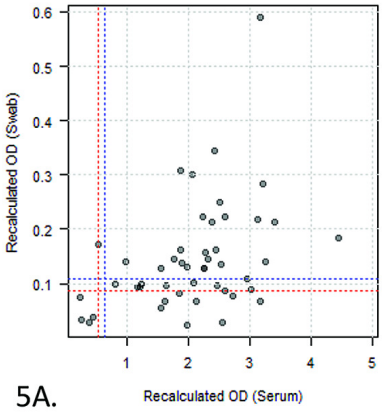

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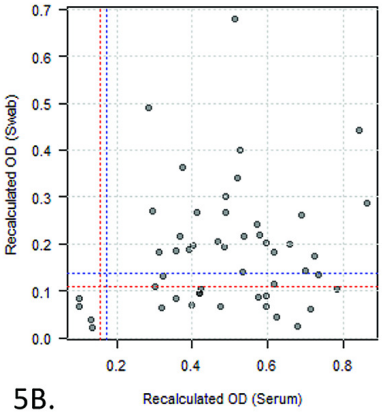

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