Application of the Euroimmun Anti-SARS-CoV-2-NCP (IgG) and Anti-SARS-CoV-2-QuantiVac-ELISA (IgG) antibody tests to dried blood spots

A previous validation study during the first wave of the RKI-SOEP-study revealed an adapted cutpoint of 0.94 for classifying semiquantitative values of the Euroimmun Anti-SARS-CoV-2-S1-IgG ELISA antibody test in dried blood spot (DBS) samples [1]. For the second wave of the study, different test assays were used. S-antibodies were analysed quantitatively (as opposed to the semiquantitative test in the first wave) using the Anti-SARS-CoV-2-QuantiVac-ELISA (IgG) by Euroimmun. N-antibodies, which were not tested for in the first wave, were analysed semiquantitatively with the Anti-SARS-CoV-2-NCP (IgG) (also by Euroimmun). These tests are commonly used in the analysis of serum samples but were used on DBS in the second wave of the RKI-SOEP study [2] An additional validation study comparing serum with DBS results was therefore conducted in May and June 2022 to potentially optimize the cutpoint for DBS. 244 employees of the RKI took part in the study after an institute-wide call for volunteers was published via email. Blood specimens were taken after participants' informed consent. The samples for all 244 participants could be evaluated. Results were reported back to participants anonymously and no additional information, e.g. on their age, sex, vaccination or infection history, was collected.

Study execution and laboratory methods

For each participant, the study team collected both a venous blood sample, which was processed into serum, and a capillary blood sample, which was processed into DBS. Both samples were then tested for IgG antibodies using the Anti-SARS-CoV-2-QuantiVac-ELISA (IgG) and Anti-SARS-CoV-2-NCP (IgG) (both by Euroimmun AG, Lübeck, Germany). The results of the S-antibody test were quantitative, expressed in binding antibody units (BAU/mL) and classified for serum samples according to the manufacturer's specifications (positive: ≥ 35.2 BAU/mL, indeterminate ≥ 25.6 to < 35.2 BAU/mL, negative: < 25.6 BAU/mL). The results of the N-antibody test were semiquantitative ratio values which were classified for serum samples using the manufacturer-supplied cutpoints (positive: ratio ≥ 1.1; indeterminate: 0.8 ≤ ratio< 1.1, negative: ratio < 0.8). The quantitative assay was rerun with diluted samples for values above the upper detection limit, 27 samples remained above the upper detection limit even after dilution. After analysis, all samples were discarded.

Statistical analysis

The aim of the analyses was to assess the test characteristics of the IgG test assays based on DBS compared to serum samples and, if appropriate, to derive an adapted cutpoint and, in case of the quantitative S-antibody test, a correction formula for DBS results so that the

seroprevalence and quantitative measures based on DBS are comparable to those based on serum samples. Results of the serum measurement were regarded as the gold standard for this analysis.

For the S-antibody test, a linear regression model was run for the log-transformed values in order to determine whether a correction formula was needed to predict quantitative serum values from DBS values. Values above the upper detection limit were set to the value of the upper detection limit. For sensitivity analysis, a model without the observations above the detection limit was run. Bland-Altman-Plots [3] were used to check for agreement visually. For this plot, the difference between the (log) DBS value and the (log) DBS value is plotted against the mean of the two (log) values.

For both the quantitative S-antibody and the semiquantitative N-antibody test, the categorised values were examined for agreement and equal marginal frequencies between serum and DBS classifications using McNemar's test [4].The categorisation used was 'positive' versus 'non-positive' (negative or indeterminate) using the manufacturer-supplied cutpoints. The categorisation for S-antibodies showed perfect agreement between serum and DBS results in our sample (see Table S1), so further analysis of S-antibody misclassification was not necessary.

Result of Serum Sample	Result of DBS		Total
	Positive	Non-positive	
Positive	241 (98.8 %	0 (0 %)	241 (98.8 %)
Non-Positive	0 (0 %)	3 (1.2 %)	3 (1.2 %)
Total	241 (98.8 %)	3 (1.2 %)	244 (100 %)

Table S 1: Categorised IgG S-antibody measurement in serum vs. DBS using the manufacturer-supplied cutpoint, unweighted absolute and cell percentages

The N-antibody results, however, showed discordant classification (see Table S2). An adapted cutpoint for DBS values was determined using the discordant proportion ratio [5], the ratio of the percentage of false positives to the percentage of false negatives. The null hypothesis of McNemar's test can be expressed as a discordant proportion ratio of 1. In our application this would indicate that the DBS result is not systematically biased towards false positives or false negatives, compared to the serum sample. For this purpose, cutpoints in the range of 0.80-1.50 were used to classify the DBS values. For each cutpoint, the proportion of misclassified DBS test results in comparison to serum results was determined and the ratio of false-positive to false-negative results was calculated. The cutpoint that led to the discordant proportion ratio closest to 1 was chosen as the adapted cutpoint.

In the analyses of the N-antibody results, weights were used to account for difference in seroprevalence (the proportion of positive N-antibody results) between the validation study and the main study. This procedure ensures that the results of the validation study are applicable to the main study, since it makes the marginal probabilities for positive and negative DBS test results identical to those observed in the main study. N-antibody positive observations were weighted with the ratio of the raw proportion of positive test results (5.2% in the main study to 17.6% in the validation study resulting in a weight <1) and negative ones with the respective proportions for negatives (94.8% in the main study, 82.4% in the validation study resulting in a weight >1). McNemar's test, however, was conducted on the unweighted data. As a further check of the adapted cutpoint the weighted discordant proportion difference before and after cutpoint adaptation was tested against the null hypothesis of a difference equal to zero [8].

Confidence intervals for the proportion of misclassified DBS test results were calculated using the Wilson score method [6, 7]. The logit method with survey procedures was used for the confidence intervals of weighted percentages .

Results

The linear regression models and Bland-Altman plots (see Supplemental figure S1) indicated good agreement between serum and DBS values for the S-antibody test. For the S-antibody test, the explained variance (R^2) for the DBS values was 97.0%. The intercept (-0.078) had a standard error of 0.064 and was therefore not significantly different from zero. The slope parameter (1.012) had a standard error of 0.011 and was not significantly different from 1. The residual standard error was 0.205 on 242 degrees of freedom. Since neither the intercept nor the slope was significantly different from 0 and 1, respectively, no correction formula was derived. Due to a slightly skewed Bland-Altman plot for the full sample, the analysis was repeated for the S-antibody positive observations only (lower row of figure S1), showing good agreement. Excluding the observations above the upper detection limit did not change this result, either.

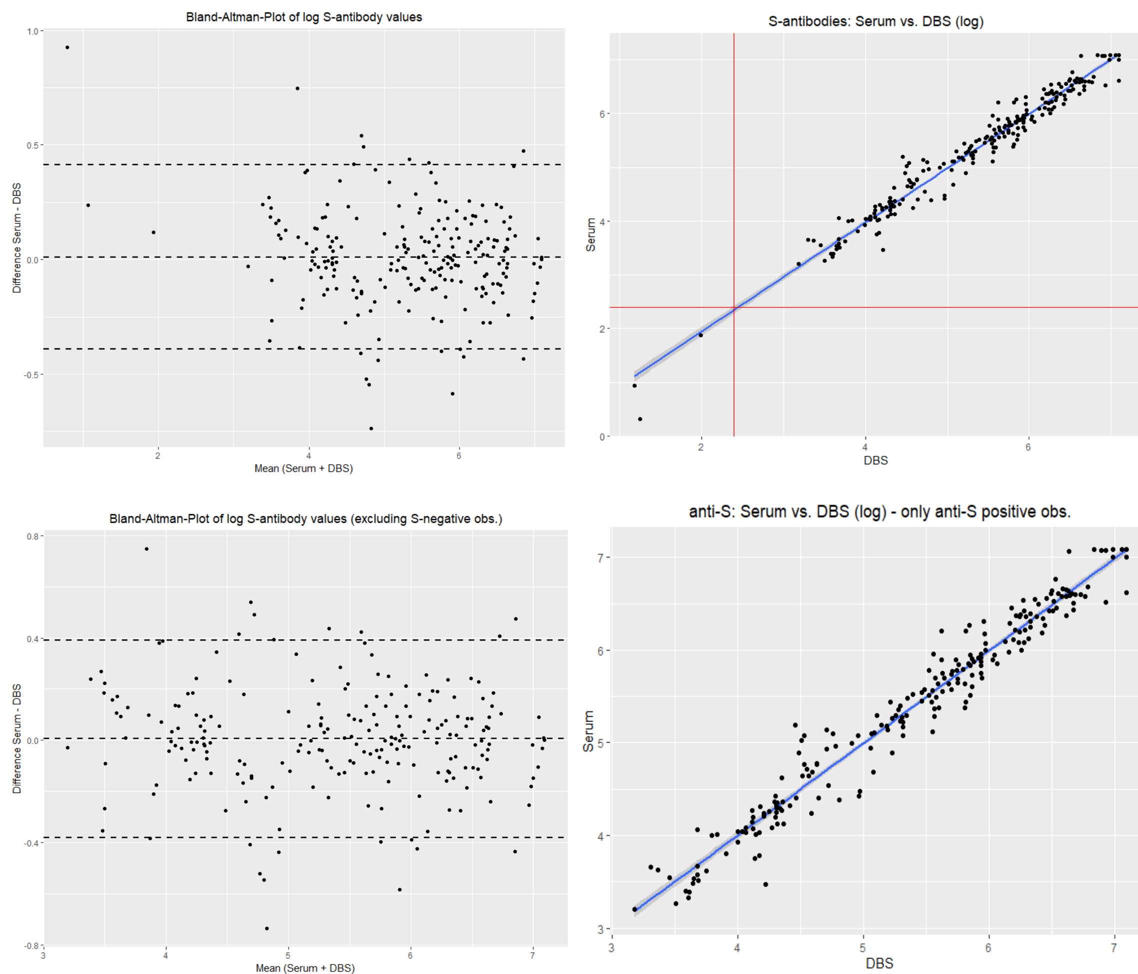

*Figure S 1: **Left panel** Bland-Altman plot of the difference between the quantitative serum log S1-IgG value and the corresponding DBS log value against the mean of the two log values. The dashed lines show the limits of agreement (± 1.96 standard deviations). Plots in the lower row exclude S-antibody negative observations. **Right panel**: Data points and regression line for the regression of quantitative serum log S1-IgG values on DBS log S1-IgG values. The red lines indicate the cutpoint between positive and non-positive categorisation.*

For the categorised N-antibody test results, the proportion of DBS samples misclassified was 6.6% compared to the corresponding serum sample, applying the manufacturer-supplied cutpoint of 1.1 to the DBS samples (16 of 244 dried blood samples were misclassified, 95% CI 4.1 – 10.4%) (see Supplemental Table S2). There were both false negative and false positive categorisations whereby 12 (4.9%) positives in serum were false negatives (95% CI 2.8-8.4%) and 4 (1.6%) negatives in serum were false positives in DBS (95% CI 0.6-4.1%).

McNemar's test without continuity correction provided support against the null-hypothesis of equal marginal frequencies (Pr>ChiSq=0.0455), suggesting a statistical difference between the serum and DBS classifications. The estimated difference of discordant proportions was 5.2% (Pr >|t|=0.0015) supporting this interpretation. Adapting the cutpoint for positivity for DBS-values was therefore considered appropriate. The discordant proportion ratio closest to

1 was reached with a cutpoint of 0.95. When re-applying McNemar's test to the marginal frequencies after cutpoint adaptation, the null hypothesis could no longer be rejected on any meaningful significance level (Pr>ChiSq=0.4227). The estimated difference of discordant proportions then was at -0.5% (Pr>|t|=0.7478). The use of this cutpoint led to an unweighted overall misclassification of 5.7% (14 of 244 samples misclassified, 95% CI 3.4-9.4%) and false positive and false negative misclassifications occurring with similar weighted frequency of 2.8% and 2.4% (5.2 %, 12.7 of 244 samples misclassified, 95% CI 3.0-9.0%) (see Supplemental Table S2).

DBS - Cutpoint: 1.1			
Serum / DBS	Positive	Non-Positive	Total
Positive	39 (4.8%)	4 (0.5%)	43 (5.2%)
Non-Positive	12 (5.7%)	189 (89.1%)	201 (94.8%)
Total	51 (10.4%)	193 (89.6%)	N=244
DBS - Cutpoint: 0.95			
Serum / DBS	Positive	Non-Positive	Total
Positive	46 (8.1%)	9 (2.8%)	55 (10.9%)
Non-Positive	5 (2.4%)	184 (86.7%)	189 (89.1%)
Total	51 (10.4%)	193 (89.6%)	N = 244

Table S 2: Categorised IgG N-antibody measurement in serum vs. categorised IgG N-antibody measurement in dried blood spot using the manufacturer-supplied cutpoint (1.1) and adapted cutpoint (0.95), unweighted absolute numbers and weighted cell percentages

Implementation in the analysis of the seroprevalence study

For IgG S-antibodies, the values of the quantitative Euroimmun QuantiVac ELISA were not found to differ significantly between serum and dried blood samples. For IgG N-antibodies, however, an adapted cutpoint of 0.95 was obtained for classifying dried blood spot samples as N-antibody positive. This cutpoint was therefore used in the analysis of the second wave of the RKI-SOEP study [2] to classify the semiquantitative values of the Euroimmun IgG N-antibody test in dried blood spot samples.

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