Detection of Nucleocapsid Antibodies Associated with Primary SARS-CoV-2 Infection in Unvaccinated and Vaccinated Blood Donors

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Nucleocapsid antibody assays can be used to estimate SARS-CoV-2 infection prevalence in regions implementing spike-based COVID-19 vaccines. However, poor sensitivity of nucleocapsid antibody assays in detecting infection after vaccination has been reported. We derived a lower cutoff for identifying previous infections in a large blood donor cohort (N = 142,599) by using the Ortho VITROS Anti-SARS-CoV-2 Total-N Antibody assay, improving sensitivity while maintaining specificity >98%. We validated sensitivity in samples donated after self-reported swab-confirmed infection diagnoses. Sensitivity for first infections in unvaccinated donors was 98.1% (95% CI 98.0–98.2) and for infection after vaccination was 95.6% (95% CI 95.6–95.7) based on the standard cutoff. Regression analysis showed sensitivity was reduced in the Delta compared with Omicron period, in older donors, in asymptomatic infections, ≤30 days after infection, and for infection after vaccination. The standard Ortho N antibody threshold demonstrated good sensitivity, which was modestly improved with the revised cutoff.

In the United States, as in many countries, convenience sample serosurveillance studies (e.g., in blood donors) have demonstrated that most of the population has SARS-CoV-2 antibodies from vaccination, infection, or both (1–3). Therefore, continued surveillance using serologic tests requires robust detection of first infections in vaccinated persons (infection after vaccination) and reinfections to yield meaningful estimates of infection incidence. Serologic detection of nucleocapsid antibodies has been a critical tool to detect previous SARS-CoV-2 infection and discriminate between vaccine- and infection-induced antibody reactivity in the context of spike-based vaccines.

The National Blood Donor Cohort (NBDC), a longitudinal study sponsored by the Centers for Disease Control and Prevention (CDC), was conducted in partnership with the 2 largest US blood collectors (Vitalant and the American Red Cross) and their central testing laboratory Creative Testing Solutions (M. Stone et al., unpub. data). We classified participating donors into 4 groups on the basis of infection and vaccination status as of mid-2021: not previously infected or vaccinated, previously infected, previously vaccinated, or both previously infected and vaccinated. An earlier iteration of this program (the National Blood Donor Serosurvey) conducted serial monthly cross-sectional

serosurveys during July 2020–December 2021 (*4*–*6*) to provide population-weighted seroprevalence estimates. However, because vaccination rates increased in 2021, the percentage of donations with vaccine-induced, infection-induced, or both vaccine-induced and infection-induced spike antibody reactivity approached 95%, and rates of infection-induced N antibody reactivity exceeded 75% in the United States by the end of 2022 (*1*,*7*), thus diminishing the value of cross-sectional serosurveillance. Important objectives of the NBDC included continued monitoring of SARS-CoV-2 infection incidence and vaccine- and infection-induced seroprevalence in the context of endemic SARS-CoV-2 transmission and increasing frequency of infection after vaccination and reinfections (M. Stone et al., unpub. data).

Several studies have suggested that the sensitivity of nucleocapsid antibody serologic tests for detecting previous SARS-CoV-2 infection is reduced in vaccinated persons compared with unvaccinated persons (*8*–*10*; H.J. Whitaker et al., unpub. data, https://doi.org/10.1101/2021.10.25.21264964). Significantly reduced nucleocapsid antibody reactivity has been reported in previously vaccinated persons with PCR-confirmed infections compared with infections in unvaccinated persons (H.J. Whitaker et al., unpub. data). Moderna mRNA vaccine trial data showed lower rates of nucleocapsid antibody seropositivity after PCR-confirmed infection among vaccine recipients compared with placebo recipients (40.4% vs. 93.4%) (*8*). A blunted nucleocapsid antibody response for infection after vaccination, and consequently reduced sensitivity of nucleocapsid antibody serology, may result from suppression of viral replication attributable to existing spike antibodies and an associated anamnestic response (*11*). In addition, studies relying on nucleocapsid IgG detection (*12*) may be confounded by rapidly waning antibodies below the limit of detection (i.e., seroreversion) (*13*–*16*).

We previously demonstrated good performance of the Ortho VITROS Anti-SARS-CoV-2 Total N Antibody (Ortho nucleocapsid antibody) assay (Quidel Ortho, https://www.quidelortho.com) and Roche Elecsys NC Anti-SARS-CoV-2 (Roche nucleocapsid antibody) assay (Roche, https://www.roche.com) for serosurveillance applications, without differentiating infections in vaccinated and unvaccinated persons (*17*). In this study, to increase sensitivity while maintaining high specificity for serologic detection of infection after vaccination, we derived a revised reactive versus nonreactive cutoff for those assays. In addition, for the Ortho nucleocapsid antibody assay we sought to validate the sensitivity of both the manufacturer's recommended and our revised cutoff for identifying

first infections in vaccinated and unvaccinated donors who self-reported swab-confirmed infections. We then assessed factors influencing detection of nucleocapsid antibodies and assessed the durability of antibody detection after primary infection.

Materials and Methods

Study Population
We identified repeat blood donors from 2 national blood collection organizations (Vitalant and American Red Cross) who had known prior SARS-CoV-2 infection and COVID-19 vaccination status determined during June 2020–July 2021, when all donations were tested for SARS-CoV-2 antibodies and donors reported vaccination status at the time of donation. Eligible donors were those presenting $\geq 2\times$ during the screening period and meeting all blood donor eligibility criteria. The NBDC includes 142,599 repeat blood donors. We based eligibility screening on donations tested with Ortho VITROS Anti-SARS-CoV-2 (spike) antibody assay and Roche nucleocapsid antibody assay, and we retained all spike antibody–reactive samples (*5*,*6*,*18*,*19*). During follow-up from July 2021 through December 2022, we identified donation specimens in real time and stored them frozen at –20°C. In 2022, we tested 1 donation specimen per donor per quarter by using the Ortho VITROS Anti-SARS-CoV-2 IgG Quantitative Test and the Ortho nucleocapsid antibody assay at Creative Testing Solutions and Vitalant Research Institute. We captured self-reported vaccination status at each donation as part of routine donation procedures. We invited all cohort donors to respond to electronic surveys on vaccination history, infection history, and clinical outcomes of infections; the overall response rate was 46.5%. NBDC seroprevalence estimates have been published (*1*).

Analysis and Statistical Methods
We conducted all analyses by using the SAS System version 9.4 (SAS Institute, https://www.sas.com). In total, we conducted 6 specific statistical analyses.

Derivation of Revised Nonreactive versus Reactive Cutoff for Ortho Nucleocapsid Antibody Assay
To detect infections serologically with optimal sensitivity, we derived a revised cutoff by using receiver operating characteristic (ROC) curve analysis (Appendix, https://wwwnc.cdc.gov/EID/article/30/8/24-0659-App1.pdf). We consequently defined gray zone reactivity as reactivity above the revised threshold and below the standard threshold (0.395$\leq$signal-to-cutoff ratio [S/CO]<1.0).

Effect of Vaccination Status on Nucleocapsid Antibody Reactivity

To assess whether first infections after vaccination were associated with reduced postinfection nucleocapsid antibody reactivity compared with first infections in unvaccinated donors, we evaluated Ortho nucleocapsid antibody assay reactivity distributions after infection in 2 groups: serologically identified putative first infections, defined as the first donation sample for each donor in which nucleocapsid antibody reactivity was above the revised cutoff, among all donors in the NBDC; and first survey-reported swab-confirmed infections. For the first group, we based vaccination status on self-report at the time of donation. For the second group, we defined swab-confirmed infections as infections confirmed by viral antigen or PCR testing or by physician diagnosis (presumed positive swab-based test) and vaccination status at the time of infection on survey-reported vaccinations. For both groups, we stratified donation samples by vaccination status (vaccinated vs. unvaccinated) at the time of infection and by period (the Delta variant era, July–December 2021, vs. the Omicron era, January–December 2022 [20]) and stratified them further by quarter. We calculated the proportions of donation samples with reactivity in the gray zone for each group.

Sensitivity of Manufacturer's Recommended and Revised Cutoffs for Detection of First Infections

To validate sensitivity, we identified survey-reported swab-confirmed first SARS-CoV-2 infections. We classified an infection as occurring in an unvaccinated donor if the donor had not reported any vaccination before the date of diagnosed infection, and we defined an infection as infection after vaccination if it occurred ≥14 days after completion of an approved primary vaccination series (1 dose of the J&J/Janssen vaccine [https://www.jnj.com] or 2 doses of either the Pfizer-BioNtech [https://www.pfizer.com] or Moderna [https://www.modernatx.com] mRNA vaccines). For cases to be included in this analysis, ≥1 donation sample had to have been collected 14–180 days after diagnosis with no prior nucleocapsid antibody reactivity above the standard cutoff. We identified a total of 2,751 swab-confirmed first infections in unvaccinated donors and 8,187 swab-confirmed first infections that were infection after vaccination. For a secondary, more restrictive analysis, we only included infection after vaccination if a postvaccination spike antibody–reactive, nucleocapsid antibody–nonreactive sample had been collected before infection, demonstrating vaccine-induced spike antibodies

seroconversion in the absence of infection-induced nucleocapsid antibodies. We excluded infections occurring after only 1 mRNA vaccination dose or <14 days after completion of a vaccination series. We identified 5,079 infection after vaccination cases for this analysis. We included only 1 postinfection sample per case in either analysis.

We estimated the sensitivity of both the manufacturer's recommended and our revised cutoffs on the Ortho nucleocapsid antibody assay in first donation samples after swab-confirmed infection, stratified by vaccination status of the donor at the time of infection. In addition, we stratified infections according to the variant era (Delta period vs. Omicron period), donor age (<65 years vs. ≥65 years), and whether the infection was associated with ≥1 self-reported symptom. We defined sensitivity as the proportion of samples that were reactive and calculated 95% CIs by using the Wilson score method. We assessed differences in sensitivity for different strata by using the binomial exact test. We assessed differences in sensitivity associated with different cutoffs computed on the same stratum by computing a p value for the difference in the Youden's J statistic associated with each cutoff.

Factors Associated with Nucleocapsid Antibody Seroconversion after Swab-Confirmed Infection

We performed bivariate and multivariable logistic regression to assess the effect of vaccination status, timing of sample collection relative to infection, donor demographics (age and sex), presence of symptoms, and variant era on nucleocapsid antibody detection. We included samples collected <14 days or >180 days after swab-confirmed infection because the model adjusted for time from infection to sample collection. We computed unadjusted odds ratios (ORs) and adjusted odds ratios (aORs) from logistic regression. After assessing ORs, we combined the vaccination status and timing variables for the multivariable regression.

Durability of Nucleocapsid Antibody Detection

We assessed durability of nucleocapsid antibody detection after primary infection in unvaccinated and vaccinated donors by examining the proportion of primary infections detectable by time from swab-confirmed infection to sample collection (0–13, 14–30, 31–60, 61–90, 91–180, 181–365, and >365 days). To account for multiple observations per time bin per donor, we weighted observations so that donors were equally weighted within each time bin, regardless of the number of observations. We computed 95% CIs by using the Wilson score method.

Effect of Adjustment for Nucleocapsid Antibody Sensitivity on Seroprevalence Estimates

To assess the potential effect of imperfect sensitivity and specificity on estimates of infection rates among vaccinated persons, we compared adjusted and unadjusted estimates of the proportion of vaccinated donors (not previously infected) who experienced infection after vaccination during 3 periods in the NBDC: quarter (Q) 2 2021–Q1 2022, Q1–Q2 2022, and Q2–Q3 2022. We cannot estimate the proportion of infections that were asymptomatic from survey data because diagnostic testing is largely driven by the presence of symptoms; therefore, for the purposes of this model, we used an estimated proportion of infections after vaccination that are asymptomatic of 32.4% (*21*). We then adjusted for a weighted average of symptomatic- and asymptomatic-specific sensitivity estimates and for specificity estimated using prepandemic samples. We computed the adjusted proportion of persons infected during each period by using the estimator derived by Rogan and Gladen (*22*), and we based 95% CIs on parametric bootstrapping (10,000 iterations) of the proportion of tests that were reactive, sensitivity, and specificity (treated as binomially distributed and incorporating the uncertainty arising from limited sample size).

Ethics Considerations

All blood donors consented to use of deidentified, residual specimens for further research purposes. Consistent with the policies and guidance of the University of California–San Francisco Institutional Review Board, Vitalant Research Institute self-certified the use of deidentified donations in this study as not meeting the criteria for human subjects research. CDC investigators reviewed and relied on this determination as consistent with applicable federal law and CDC policy. The donor surveys conducted by Vitalant Research Institute and American Red Cross were conducted under protocols supervised and approved by the Advarra and American Red Cross institutional review boards, respectively, and linked to biospecimens in deidentified form.

Results

Revised Cutoff for Detecting Previous Infection with Ortho Nucleocapsid Antibody Assay

The nonreactive versus reactive threshold on the Roche nucleocapsid antibody cutoff index that maximized Youden's J statistic was $\geq$0.205, and this optimized cutoff was used in defining cases for the Ortho ROC curve analysis. The ROC-optimized threshold on the Ortho nucleocapsid antibody assay was S/CO $\geq$0.395, which had a sensitivity of 98.7% and a specificity of 98.7% in the Ortho optimization sample set. The area under the ROC curve was 0.994 (Appendix).

Effect of Vaccination Status on Nucleocapsid Antibody Reactivity

We calculated distributions of Ortho nucleocapsid antibody assay S/COs in first longitudinal samples with reactivity above the revised cutoff (S/CO $\geq$0.395 [i.e., putative first infections]) from previously uninfected donors (based on negative Ortho nucleocapsid antibody results [S/CO <0.395] in all previous longitudinal samples), by vaccination status and variant era (Figure 1).

During the Delta era, 35.2% of serologically identified infections after vaccination showed gray zone reactivity (0.395$\leq$S/CO< 1.0) compared with 7.5% of serologically identified primary infections in unvaccinated donors, declining to 3.8% for infections after vaccination and 2.7% for primary infections in unvaccinated donors by Q4 2022 (Omicron period Q4) (Figure 1, panel A). Among survey respondents with swab-confirmation infection, we did not observe a similar increased proportion of gray zone nucleocapsid antibody reactivity in the Delta period; most had reactivity above the standard cutoff (Figure 1, panel B). The donation specimens (Figure 1, panel B) correspond to the specimens used in assessing sensitivity for the 2 cutoffs.

Sensitivity for Detection of Swab-Confirmed Primary Infections

Overall sensitivity of the Ortho nucleocapsid antibody assay manufacturer's cutoff was 98.1% (95% CI 98.0%–98.2%) for detection of first infections in unvaccinated donors and 95.6% (95% CI 95.6%–95.7%) for detection of first infections after vaccination (Table). Sensitivity was increased when using the revised cutoff, to 98.4% (95% CI 98.4%–98.5%) in unvaccinated donors and to 97.0% (95% CI 96.9%–97.0%) for infections after vaccination. Although sensitivity is necessarily increased by reducing the cutoff from the manufacturer's suggestion, Youden's J index is not statistically improved by the reduced cutoff (p = 0.13 based on a 1-tailed test). Sensitivity for detection of infection after vaccination using the standard cutoff was higher during the Omicron era (96.0% [95% CI 95.9%–96.0%) than the Delta era (93.9% [95% CI 93.5%–95.4%]; p = 0.001) and was higher for detecting symptomatic than asymptomatic infections after vaccination (96.2% [95% CI 96.1%–96.2%] vs. 90.1% [95% CI 88.5%–91.7%]; p<0.0001). A secondary

Table. Sensitivity of SARS-CoV-2 nucleocapsid antibody assay for detection of first infections in unvaccinated and vaccinated donors, United States, July 2021–December 2022*

Characteristic	Sensitivity for detection of first infections in unvaccinated donors			Sensitivity for detection of first infections in vaccinated donors		
	No. donors	Manufacturer's cutoff,† % (95% CI)	Revised cutoff,‡ % (95% CI)	No. donors	Manufacturer's cutoff,† % (95% CI)	Revised cutoff,‡ % (95% CI)
Overall	2,751	98.1 (98.0–98.2)	98.4 (98.4–98.5)	8,187	95.6 (95.6–95.7)	97.0 (96.9–97.0)
Delta: Jul–Dec 2021	1,343	98.4 (98.2–98.5)	98.5 (98.4–98.6)	1,349	93.9 (93.5–94.4)	95.3 (94.9–95.6)
Omicron: Jan–Dec 2022	1,408	97.9 (97.7–98.0)	98.4 (98.3–98.5)	6,838	96.0 (95.9–96.0)	97.3 (97.3–97.4)
Age <65 y	2,225	98.2 (98.1–98.2)	98.4 (98.4–98.5)	5,194	96.2 (96.1–96.3)	97.3 (97.2–97.3)
Age ≥65 y	526	97.9 (97.5–98.4)	98.5 (98.2–98.8)	2,993	94.7 (94.5–94.9)	96.5 (96.4–96.6)
Symptomatic§	2,430	98.4 (98.3–98.4)	98.7 (98.7–98.8)	7,416	96.2 (96.1–96.2)	97.5 (97.5–97.6)
Asymptomatic§	208	96.6 (95.0–98.3)	96.6 (95.0–98.3)	627	90.1 (88.5–91.7)	91.2 (89.8–92.6)

*Proportion reactive in the first sample collected after reported swab-confirmed infection, collected 14 to 180 days postinfection.
†Signal-to-cutoff ratio ≥1.000.
‡Signal-to-cutoff ratio >0.395.
§Symptomatic or asymptomatic status could not be ascertained for all infections because of incomplete survey responses.

sensitivity analysis using a more restrictive case definition of infection after vaccination, which required spike antibody seroconversion after vaccination, showed similar patterns (Appendix Table 1).

Factors Associated with Nucleocapsid Antibody Seroconversion after Swab-Confirmed Infection

On first samples collected after first swab-confirmed infection, bivariate logistic regression showed that infection during the Delta era, age <65 years, female sex, symptomatic infection, being unvaccinated at the time of infection, longer time between vaccination and infection, and longer intervals between infection and sample collection were all statistically significantly associated with increased probability of detection (Figure 2; Appendix Table 2). Of note, the percentage detected in all time to sample categories, other than donation samples collected <14 days postinfection (dpi) (48.9%), ranged from 87.7% to 98.1%.

In multivariable logistic regression, infection during the Delta period (vs. Omicron period) remained statistically significant, but the direction of effect changed to reduced detection (aOR 0.80 [95% CI 0.66–0.98]) from increased detection in bivariate analysis (OR 1.51 [95% CI 1.28–1.78]), possibly because variant era (calendar time) is also strongly associated with vaccination status. Younger age groups had higher odds of detection than donors ≥65 years of age, whereas donor sex was not significantly associated with detection in the multivariable analysis. Asymptomatic infection was significantly associated with reduced detection (aOR 0.46, 95% CI 0.37–0.57). Being vaccinated at the time of infection significantly reduced detection compared with being unvaccinated (aORs <1), with the exception of primary vaccination ≤30 days before infection, which was not statistically significant. Vaccination reduced detection compared with no vaccination, and more recent receipt of either a primary vaccination series (31–180 days before infection) or a booster vaccination (≤30 days or 31–180 days before infection) was associated with reduced odds of detection than when infections occurred >180 days since the most recent vaccine. Compared with sample collection 31–90 dpi, sample collection <14 days (aOR 0.04 [95% CI 0.03–0.05]) and 14–30 days (aOR 0.34 [95% CI 0.28–0.40]) after infection were associated with greatly reduced detection, whereas sample collection 3–6 months (aOR 1.79 [95% CI 1.40–2.28]) or ≥7 months (aOR 2.19 [95% CI 1.44–3.35]) after infection were associated with increased detection (Figure 2; Appendix Table 2).

Durability of Nucleocapsid Antibody Detection

Nucleocapsid antibody reactivity was detected in less than half of specimens collected <14 dpi, >80% of specimens collected 14–30 dpi, and >90% of specimens collected >90 dpi in donors who were vaccinated and unvaccinated at the time of infection. We observed no decline in percentage detected in later time bins, including >1 year postinfection. The proportion detected was slightly lower in vaccinated donors and for asymptomatic infections at all times after infection (Figure 3).

Effect of Adjustment for Nucleocapsid Antibody Sensitivity on Seroprevalence Estimates

Estimates of the proportion of vaccinated donors who had become infected in each time period (using the nucleocapsid antibody test), adjusted for sensitivity and specificity, and assuming that 32.4% of infections were asymptomatic, differed little from unadjusted estimates. Adjusted estimated infection rates increased in each period by 0.2–0.9 percentage points, or proportionally by 1.5%–4.0% (Appendix Table 3).

Discussion

Despite reports of sensitivity as low as 40% for serologic detection of infection after vaccination (*8*),
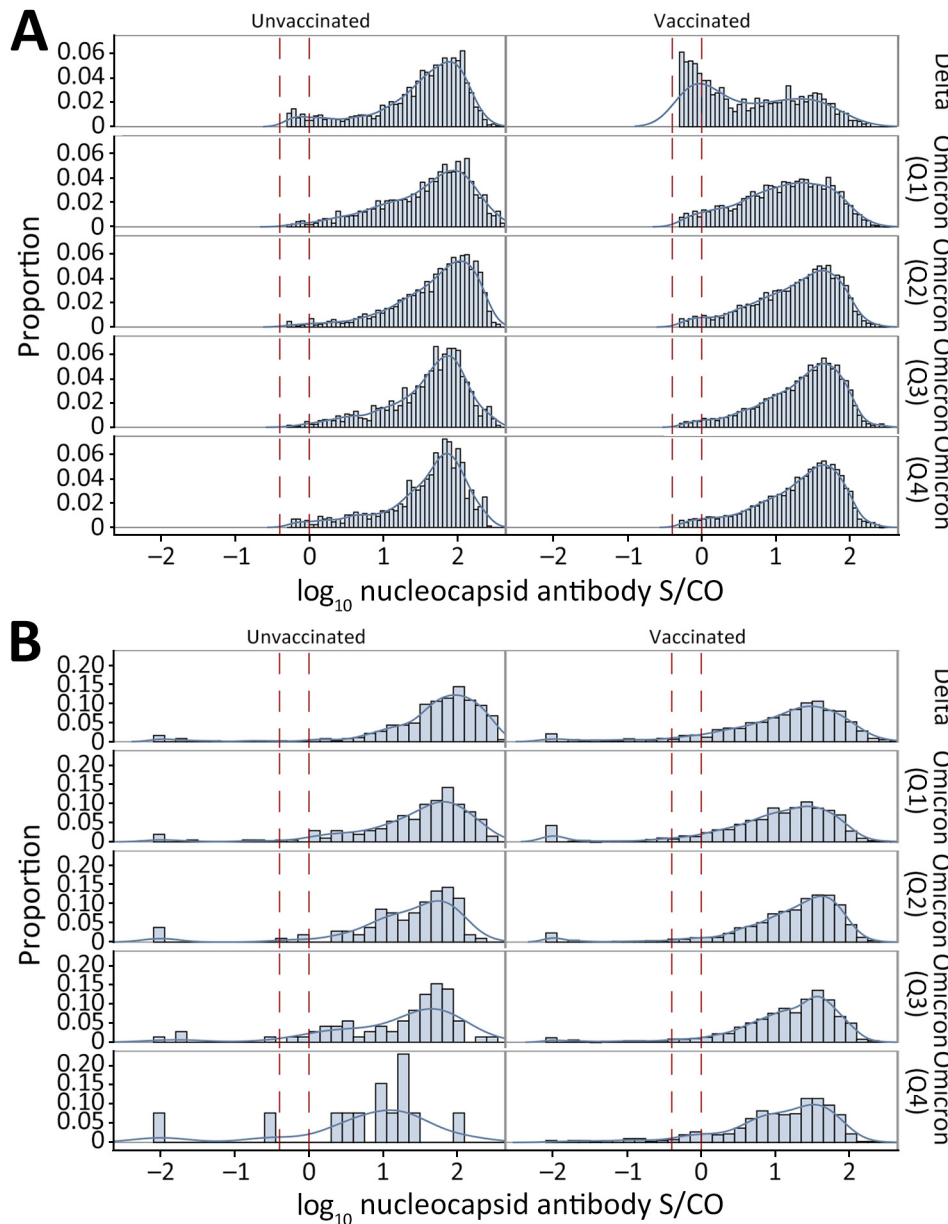
Figure 1. Nucleocapsid antibody signal intensity distributions observed in vaccinated and unvaccinated blood donors after primary SARS-CoV-2 infection, United States, July 2021–December 2022. A) Reactivity of putative serologically identified infections at the first longitudinal sample showing reactivity above the reduced cutoff (gray zone reactivity, S/CO ratio$\geq$0.395$\leq$1), by vaccination status and variant era (6,555 unvaccinated donors [left] and 22,217 vaccinated donors [right]). B) Reactivity at the first sample collected after self-reported swab-confirmed infection (14–80 days postinfection), by vaccination status and variant era (2,751 unvaccinated donors [left] and 8,187 vaccinated donors [right]). Vertical dashed lines indicate the gray zone of nucleocapsid antibodies. Q1, January–March 2022; Q2, April–June 2022; Q3, July–September 2022; Q4, October–December 2022. S/CO, signal-to-cutoff ratio.

our findings demonstrate sensitivity for detection of swab-confirmed first infections >98% among unvaccinated persons and >95% among vaccinated persons and supports use of the manufacturer's recommended cutoff for identifying previous infections in vaccinated and unvaccinated persons. Timing of sample collection after infection affected sensitivity (we observed poorer sensitivity <30 dpi); thus, timing of sample collection must be considered when interpreting previous reports.

In our validation of sensitivity for detection of first infections, the number of infections in vaccinated donors greatly exceeded those in unvaccinated donors in the study period, a function of high donor vaccination rates. This finding demonstrates the importance of sensitive detection of infection after vaccination in SARS-CoV-2 serosurveillance programs.

The revised cutoff for the Ortho nucleocapsid antibody assay offered minimal improvement in sensitivity to detect infection after vaccination compared with the manufacturer's cutoff. Our ROC analysis equally weighted sensitivity and specificity, an approach appropriate for surveillance applications, but potentially less appropriate for clinical applications prioritizing specificity. The revised cutoff was not associated with a statistical improvement in Youden's J statistic and only minimally increased sensitivity. The effect of adjusting seroprevalence estimates in vaccinated NBDC

donors for sensitivity and specificity was modest (Appendix Table 3), not exceeding a proportional effect of 4% on point estimates. This finding indicates that the standard assay cutoff for infection after vaccination detection performed sufficiently.

Recent receipt of primary or booster vaccinations reduced the likelihood of nucleocapsid antibody seroconversion after infection. Multivariable regression showed that recent receipt of an additional vaccine (booster) dose was associated with reduced detection, but the timing of primary vaccination relative to infection affected detection less. A study in Japan showed similar results, indicating reduced sensitivity to detect infection within 1–2 months of a third mRNA COVID-19 vaccine dose (sensitivity 78%) but high sensitivity for infections occurring >3 months after the second or >4 months after the third dose (23). The Moderna vaccine trial data (8) were selected for infections after vaccination occurring soon after vaccination and were collected relatively soon after infection, likely contributing to poor sensitivity in that study (8). Our data confirmed relatively poor sensitivity in specimens collected within 1 month of infection.

Because much of the lack of detection observed in our study occurred at times soon after infection, a longitudinal cohort study probably would detect many of the infections missed at the first postinfection specimen at later timepoints using specimens from those persons. Despite some waning of nucleocapsid antibody levels after infection, longer-term durability of antibody detection >1 year after infection confirmed earlier findings by our group of robust durability of detection using nucleocapsid direct antigen sandwich
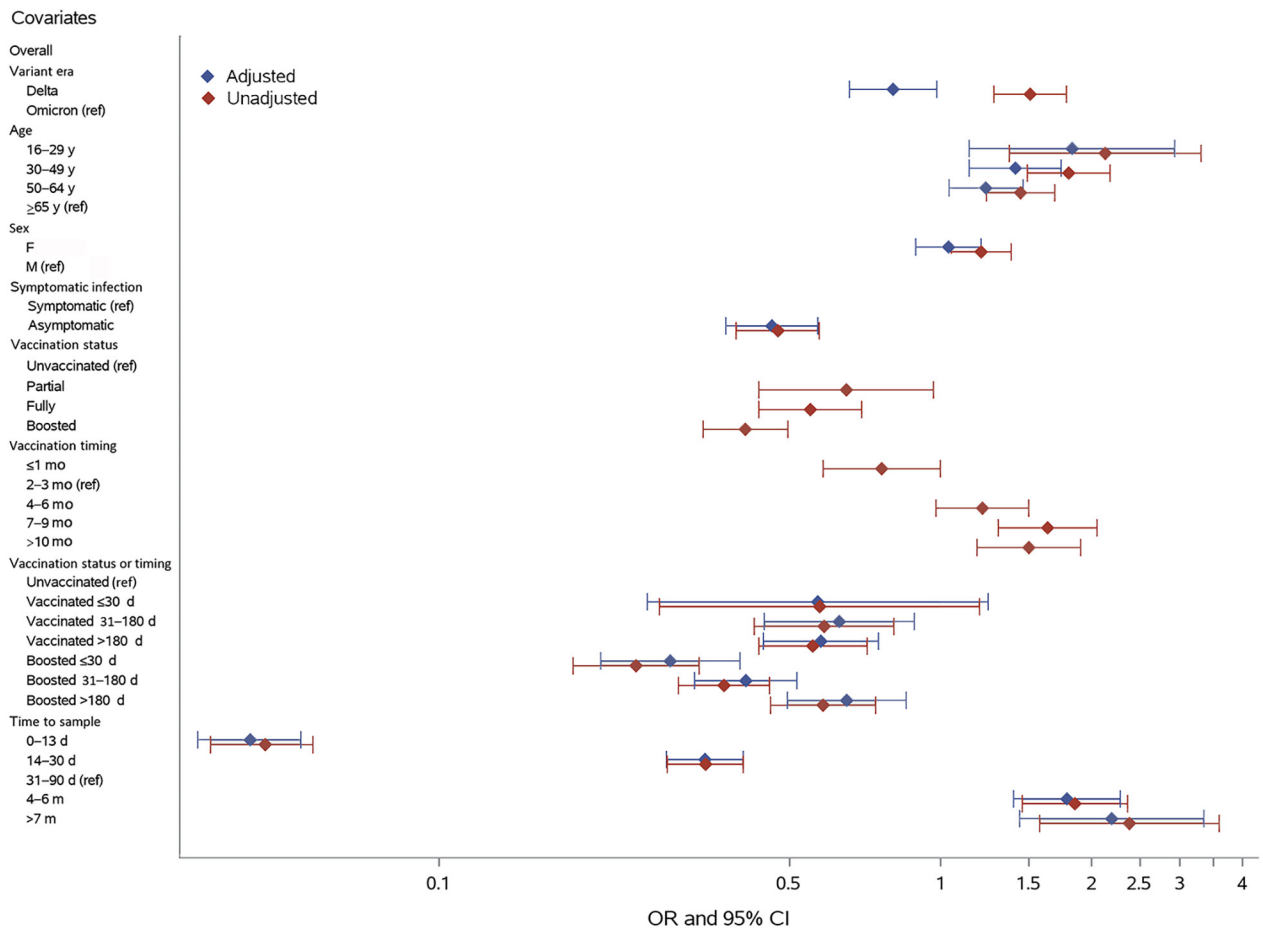

Figure 2. Factors influencing nucleocapsid antibody seroconversion after swab-confirmed first SARS-CoV-2 infections among vaccinated and unvaccinated blood donors, United States, July 2021–December 2022. ORs and 95% CIs from logistic regression are shown. In the multivariable regression model (adjusted ORs), the categories for certain variables have been grouped together; the vaccination status at the time of infection and timing of most recent vaccine before infection were combined in the vaccination status or timing variable, and in the variable for time from infection to tested sample, the groups for samples collected 7–12 months and >1 year postinfection were combined. Number of samples in each group, the proportion of nucleocapsid antibody–reactive samples, and ORs are shown in Appendix Table 2 (https://wwwnc.cdc.gov/EID/article/30/8/24-0659-App1.pdf). OR, odds ratio; ref, referent.

A

No. unvaccinated	45	338	717	722	1,927	2,686	2,407
No. vaccinated	199	1,446	2,879	2,123	5,322	4,040	653

B

No. unvaccinated	6	32	52	48	160	226	259
No. vaccinated	39	306	665	674	1,767	2,460	2,148

C

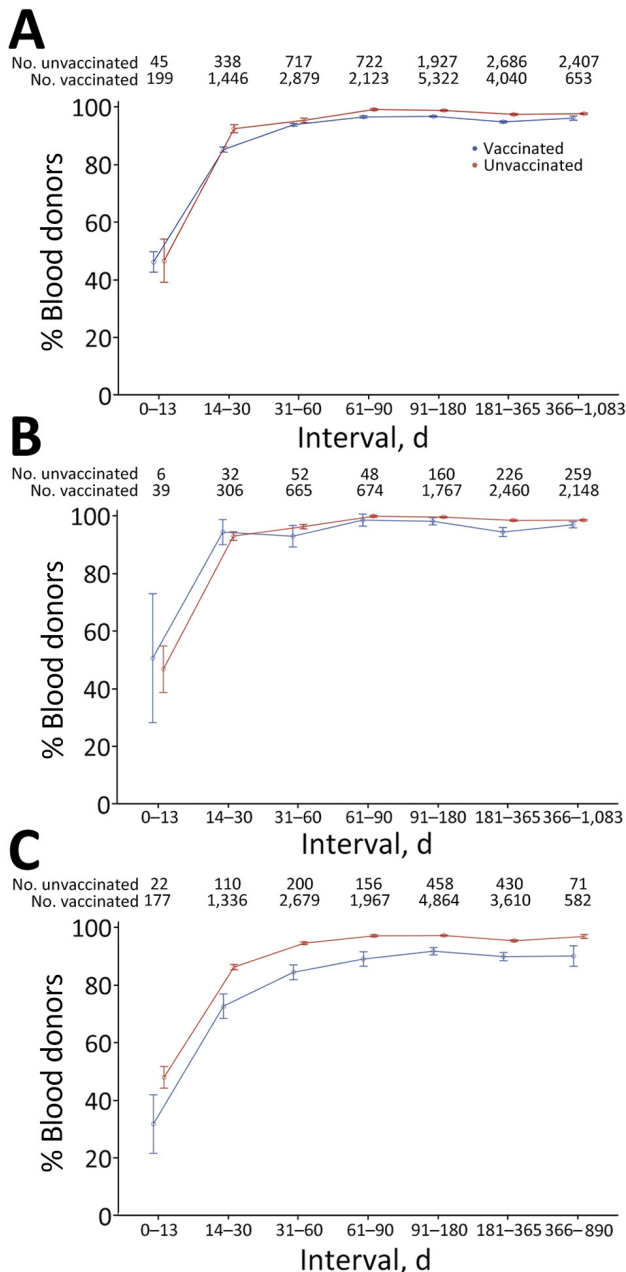| No. unvaccinated	22	110	200	156	458	430	71
No. vaccinated	177	1,336	2,679	1,967	4,864	3,610	582

Figure 3. Sensitivity of nucleocapsid antibody serologic tests by time from swab-confirmed infection to sample collection in vaccinated and unvaccinated blood donors, using the manufacturer's recommended cutoff, United States, July 2021–December 2022. The percentage of donors showing reactivity in first or subsequent samples after swab-confirmed infection is shown. A) Reactive proportions stratified by vaccination status at the time of infection. B) Infections in unvaccinated donors stratified by reported symptoms. C) Reactive proportions in unvaccinated donors with swab-confirmed infections stratified by reported symptoms. To account for multiple observations per time bin, observations were weighted so that donors were equally weighted within each time bin, regardless of the number of observations. Error bars indicate median and maximum durations of follow-up for each group.

assays(*13*).We observed substantially reduced detection in the first month after infection, especially during the first 14 days, when <50% of recently infected persons demonstrated nucleocapsid antibody seroconversion but observed very good detection at later timepoints. Nucleocapsid IgG assays used in numerous serosurveillance studies (*14*, *24*–*28*) show more rapid waning in antibody signal than nucleocapsid total Ig assays (*13*) and thus require adjustments for seroreversion in estimating cumulative incidence (*14*). So-called direct immunoassays (i.e., antigen sandwich format total Ig assays), are more sensitive to increasing antibody affinity than IgG assays, which probably explains the more durable reactivity associated with antibody maturation and persistence postinfection despite waning in IgG concentrations (*29*). Although rapidly waning IgG assays may be less appropriate for serosurveillance aimed at documenting cumulative incidence than total Ig assays, they may have advantages for detecting reinfections based on antibody boosting and as correlates of protection (*30*).

A limitation of this study was that the case definition of infection in the validation data was based on self-reported diagnosed infection, without active surveillance of the cohort for asymptomatic infection. As a result, most of survey-reported swab-confirmed infections in the validation set were associated with COVID-19 symptoms (92%). In contrast, a meta-analysis of Omicron infections estimated that 32.4% of infections were asymptomatic (*31*). This limitation may result in a slight upward bias in our overall sensitivity estimates. However, we found that adjusting for sensitivity to detect symptomatic and asymptomatic infection after vaccination had a modest effect on seroprevalence estimates. A further limitation is that blood donors are not fully representative of the general population; they generally are healthier and more likely to be vaccinated and to receive additional doses (*1*,*32*). Furthermore, vaccination and infection history were self-reported in donor surveys and not confirmed by healthcare records; only 46.5% of cohort participants responded to surveys and could be included in this study, which may have resulted in a biased sample.

Our study demonstrates that detection of first SARS-CoV-2 infections using the Ortho nucleocapsid total Ig antibody assay was robust in vaccinated and unvaccinated donors, indicating overall sensitivities >95%. We also found good durability of nucleocapsid antibody detection for up to >1 year after infection. Seroprevalence studies using this assay can accurately estimate the proportion of persons who have been infected with SARS-CoV-2 ≥1 times. Several factors affect the likelihood of nucleocapsid antibody

seroconversion after first infection, including receipt of primary and additional vaccinations, sampling shortly after infection, and asymptomatic infection, although the effect of these factors was relatively small. Revising the cutoff improved sensitivity only modestly; therefore, use of the manufacturer's recommended cutoff is likely appropriate for most serosurveillance studies.

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Detection of Nucleocapsid Antibodies Associated with Primary SARS-CoV-2 Infection in Unvaccinated and Vaccinated Blood Donors

Appendix

Derivation of a Revised Non-Reactive versus Reactive Cutoff for the Ortho Anti-Nucleocapsid (anti-N) Total Ig Assay

To identify an optimal cutoff for serologic detection of infection using first the Roche anti-N total Ig assay and later the Ortho anti-N total Ig assay, we performed receiver operating characteristic (ROC) curve analyses using samples from blood donors classified as not previously infected with SARS-CoV-2 (controls, 'true negatives') and previously infected with SARS-CoV-2 (cases, 'true positives'). This analysis was performed based on samples collected cross-sectionally in the National Blood Donor Serosurvey (NBDS), without any clinical data or self-reported infection status, as described below.

To determine the optimal cutoff for the Roche anti-N total Ig assay, collected samples that had been tested in parallel with the Ortho anti-S and the Roche anti-N total Ig assays during monthly cross-sectional serosurveillance in the universal screening phase of the NBDS program (June to November 2020) were identified for optimization of the reactive/non-reactive cutoff of the Roche assay. Since samples were collected before widespread availability of vaccines, anti-S total Ig results were treated as independent indication of infection status (both positive and negative). The anti-S total Ig assay was treated as a 'gold standard' in this analysis, based on excellent performance demonstrated in a previous study (sensitivity of 95.8% and specificity of 100%), which was marginally better than the performance of the anti-N total Ig assay (1). Furthermore, the purpose of the analysis was to maximize sensitivity of the anti-N total Ig assay

in the context of VI, which had not been assessed in the prior analysis. A total of 25,065 cases and 30,110 controls were identified using anti-S total Ig results (Roche optimization sample set). The controls were supplemented with 432 samples collected during 2019 before the emergence of SARS-CoV-2. A ROC curve analysis was performed, with optimality defined as maximal Youden's J, i.e., an equal weighting of sensitivity and specificity. The optimal cutoff index (COI) on the Roche anti-N total Ig assay was COI≥0.205.

To determine the optimal cutoff for the Ortho anti-N total Ig assay, a similar sample set tested with the Ortho anti-N total Ig assay from before vaccine roll out was not available. NBDS samples collected after vaccines became available that had been tested with the Ortho anti-S total Ig assay and with both the Roche and Ortho anti-N total Ig assays were identified. For the 'Ortho optimization sample set' we identified 371 donors previously infected with SARS-CoV-2 (cases, i.e., 'true positives') based on reactivity on both anti-S total Ig (manufacturer's cutoff) and Roche anti-N total Ig (revised cutoff derived above to improve sensitivity). For this analysis, controls ('true negatives') were restricted to pre-pandemic samples – 1,248 results supplied by QuidelOrtho from in-house specificity testing performed in support of the assay's Emergency Use Authorization, supplemented with 200 pre-pandemic samples tested as part of a SARS-CoV-2 serology performance study (1). As in the analysis for the Roche assay, Youden's J was used to identify an optimal cutoff. The optimal cutoff on the Ortho anti-N total Ig assay was COI≥0.395.

Appendix Figure shows the distribution of S/CO values in the pre-pandemic control samples and the serologically defined cases (Ortho anti-S total Ig S/CO≥1 and Roche anti-N total Ig COI≥0.205), with vertical lines indicating the optimized cutoff based on our ROC analysis and the manufacturer's recommended cutoff.

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Appendix Table 1. Sensitivity of Ortho anti-nucleocapsid (anti-N) assay for detection of first infections in vaccinated donors. Proportion reactive in the first sample collected after reported swab-confirmed infection, collected 14 to 180 d post-infection, stratified by whether an anti-S reactive/anti-N nonreactive sample was observed after vaccination but before infection

Characteristic	First infections in vaccinated donors (S+/N- observed following vaccination)			First infections in vaccinated donors (No S+/N- following vaccination)		
	No. donors	Manufacturer's cutoff,* % (95% CI)	Revised cutoff,† % (95% CI)	No. donors	Manufacturer's cutoff,* % (95% CI)	Revised cutoff,† % (95% CI)
Overall	5,079	95.5 (95.4–95.6)	97.1 (97.1–97.2)	3,108	95.8 (95.7–96.0)	96.8 (96.7–96.9)
Delta (Jul–Dec 2021)	103	77.7 (56.0–99.4)	82.5 (65.6–99.5)	1,246	95.3 (94.9–95.6)	96.3 (96.0–96.6)
Omicron (Jan–Dec 2022)	4,976	95.9 (95.8–96.0)	97.4 (97.4–97.5)	1,862	96.2 (96.0–96.4)	97.1 (96.9–97.3)
Age <65 y	3,004	96.3 (96.2–96.4)	97.6 (97.6–97.7)	2,190	96.0 (95.9–96.2)	96.8 (96.6–96.9)
Age ≥65 y	2,075	94.4 (94.1–94.7)	96.3 (96.2–96.5)	918	95.3 (94.8–95.8)	96.8 (96.5–97.2)
Symptomatic‡	4,691	96.0 (95.9–96.1)	97.6 (97.6–97.7)	2,725	96.5 (96.4–96.6)	97.4 (97.3–97.5)
Asymptomatic‡	322	89.4 (86.2–92.7)	90.4 (87.4–93.4)	305	90.8 (87.8–93.8)	92.1 (89.6–94.7)

*S/CO ≥1.000.
†S/CO ≥0.395.
‡Symptomatic or asymptomatic status could not be ascertained for all infections because of incomplete survey responses.

Appendix Table 2. Factors influencing anti-nucleocapsid seroconversion following first swab-confirmed SARS-CoV-2 infection. The table shows the proportion of first post-infection samples that were reactive using the standard cutoff on the Ortho assay, the unadjusted odds ratio, and the adjusted odds ratio obtained from multivariable logistic regression.

| Variable	N	% detected	Unadjusted OR (95% CI)	Adjusted OR (95% CI)
Overall	14,094	93.9		
Variant era				
Delta	4,048	95.4	1.51 (1.28–1.78)	0.80 (0.66–0.98)
Omicron	10,046	93.3	ref	ref
Age Group (in years)				
16–29	554	96.0	2.13 (1.37–3.31)	1.83 (1.14–2.93)
30–49	3,582	95.3	1.80 (1.49–2.18)	1.41 (1.14–1.74)
50–64	5,671	94.3	1.44 (1.24–1.69)	1.23 (1.04–1.46)
65+	4,287	91.9	ref	ref
Gender				
Female	7,889	94.4	1.21 (1.05–1.38)	1.04 (0.89–1.20)
Male	6,205	93.3	ref	ref
Symptomatic infection				
Symptomatic	12,613	94.4	ref	ref
Asymptomatic	1,260	88.9	0.47 (0.39–0.57)	0.46 (0.37–0.57)
Vaccination status*				
Unvaccinated	3,927	96.6	ref	
Partially vaccinated	609	94.9	0.65 (0.43–0.97)	
Fully vaccinated	2,705	94.1	0.55 (0.43–0.70)	
Boosted	6,853	92.2	0.41 (0.34–0.50)	
Vaccination timing*				
≤1 mo	797	88.8	0.76 (0.58–1.00)	
2–3 mo	2,022	91.3	ref	
4–6 mo	2,617	92.7	1.21 (0.98–1.50)	
7–9 mo	2,652	94.5	1.64 (1.30–2.05)	
10+ months	2,079	94.0	1.50 (1.18–1.90)	
Vaccination status/timing (in days)				
Unvaccinated	3,927	96.6	ref	ref
Vaccinated (primary) ≤30	140	94.3	0.57 (0.28–1.20)	0.57 (0.26–1.24)
Vaccinated (primary) 31–180	999	94.4	0.59 (0.43–0.81)	0.63 (0.45–0.89)
Vaccinated (primary) >180	2,175	94.1	0.56 (0.43–0.71)	0.58 (0.44–0.75)
Vaccinated (boosted) ≤30	657	87.7	0.25 (0.19–0.33)	0.29 (0.21–0.40)
Vaccinated (boosted) 31–180	3,640	91.4	0.37 (0.30–0.46)	0.41 (0.32–0.52)
Vaccinated (boosted) >180	2,556	94.4	0.58 (0.46–0.74)	0.65 (0.50–0.85)
Time to sample				

Variable	N	% detected	Unadjusted OR (95% CI)	Adjusted OR (95% CI)
0–13 d	378	48.9	0.05 (0.04–0.06)	0.04 (0.03–0.05)
14–30 d	2,109	87.9	0.34 (0.29–0.40)	0.34 (0.28–0.40)
31–90 d	6,769	95.5	*ref*	*ref*
3–6 mo	3,539	97.5	1.85 (1.46–2.36)	1.79 (1.40–2.28)
7+ months	1,299	98.1	2.38 (1.58–3.59)	2.19 (1.44–3.35)

*Variables for vaccination status at time of infection, and for time from most recent vaccination to infection were replaced by a combined vaccination status and timing variable in multivariable logistic regression.

Appendix Table 3. Impact of adjustment for sensitivity and specificity, for detection of VI, on estimated percentage of vaccinated donors who became infected during three time periods in the National Blood Donor Cohort. We assumed that 32.4% of VI were asymptomatic (*2*) and we adjusted for symptom status-specific sensitivity estimates (see Methods).

Time period	No. infected / No. tested* among vaccinated, not previously infected	% infected % (95% CI) (naïve estimate)	% infected % (95% CI) adjusted for Se and Sp	% infected % (95% CI) adjusted for Se and Sp, assuming 32.4% of VI asymptomatic	Difference between adjusted and naïve estimate, % points (proportional difference), assuming 32.4% of VI asymptomatic
Q2 2021 to Q1 2022	7,656 / 33,815	22.64 (22.20–23.09)	23.18 (22.60–23.76)	23.52 (22.91–24.15)	0.88 (3.9%)
Q1 to Q2 2022	3,405 / 27,526	12.37 (11.99–12.76)	12.37 (11.80–12.91)	12.55 (11.96–13.11)	0.18 (1.5%)
Q2 to Q3 2022	5,569 / 24,147	23.06 (22.54–23.60)	23.63 (22.96–24.28)	23.98 (23.28–24.67)	0.92 (4.0%)

*The numerator is the number of donors who were classified as vaccinated and not previously infected in the previous quarter, who were tested and seroconverted anti-N by the following quarter, and the denominator is the number that seroconverted plus the number who did not seroconvert, without any demographic weighting (*3*).
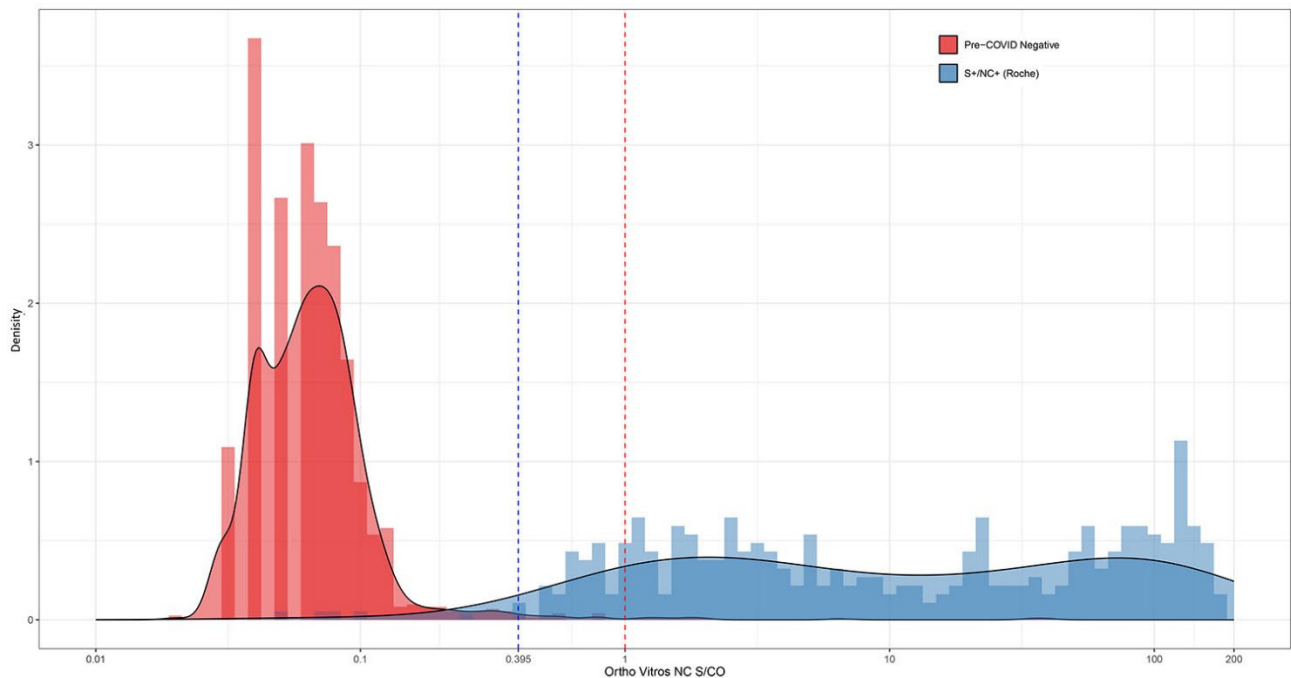
Appendix Figure. Anti-nucleocapsid (anti-N) signal-to-cutoff ratios on the Ortho anti-N total Ig assay, among pre-COVID-19 negative control samples, and samples from previously infected donors identified as anti-S and anti-N reactive using the Ortho anti-S and Roche anti-N total Ig assays. The red dashed line shows the manufacturer's recommended cutoff and the blue dashed line shows revised cutoff identified as optimal using receiver operating characteristic (ROC) curve analysis.