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2 **seroconversion and controls for pre-existing seasonal human coronavirus antibody**
3 **cross-reactivity**
4

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ABSTRACT

With growing concern of persistent or multiple waves of SARS-CoV-2 in the United States,
sensitive and specific SARS-CoV-2 antibody assays remain critical for community and hospital-
based SARS-CoV-2 surveillance. Here, we describe the development and application of a
multiplex microsphere-based immunoassay (MMIA) for COVD-19 antibody studies, utilizing
serum samples from non-human primate SARS-CoV-2 infection models, an archived human
sera bank and subjects enrolled at five U.S. military hospitals. The MMIA incorporates prefusion
stabilized spike glycoprotein trimers of SARS-CoV-2, SARS-CoV-1, MERS-CoV, and the
seasonal human coronaviruses HCoV-HKU1 and HCoV-OC43, into a multiplexing system that
enables simultaneous measurement of off-target pre-existing cross-reactive antibodies. We
report the sensitivity and specificity performances for this assay strategy at 98% sensitivity and
100% specificity for subject samples collected as early as 10 days after the onset of symptoms.
In archival sera collected prior to 2019 and serum samples from subjects PCR negative for
SARS-CoV-2, we detected seroprevalence of 72% and 98% for HCoV-HKU1 and HCoV-0C43,
respectively. Requiring only 1.25 μL of sera, this approach permitted the simultaneous
identification of SARS-CoV-2 seroconversion and polyclonal SARS-CoV-2 IgG antibody
responses to SARS-CoV-1 and MERS-CoV, further demonstrating the presence of conserved
epitopes in the spike glycoprotein of zoonotic betacoronaviruses. Application of this serology
assay in observational studies with serum samples collected from subjects before and after
SARS-CoV-2 infection will permit an investigation of the influences of HCoV-induced antibodies
on COVID-19 clinical outcomes.

INTRODUCTION

Severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2) is a novel zoonotic positive-sense, single-stranded, RNA virus responsible for the third viral pandemic of the 21st century, and the third zoonotic coronavirus outbreak in the past 20 years (1, 2). At this time, SARS-CoV-2 has globally caused 34 million COVID-19 cases and over 1 million COVID-19 related deaths. A major concern of the ongoing SARS-CoV-2 pandemic has been the frequent reports of waning virus-specific antibody levels, with several studies reporting decay to undetectable levels within just a few months after infection (3-5). While this is a measurable feature of antibody response, it is also possible that current assays lack the sensitivity required to detect lower levels of SARS-CoV-2 specific antibodies. To date, a variety of antibody tests have been developed with 38 tests granted Emergency Use Authorization (EUA) by the U.S. Food and Drug Administration. The majority of these tests assess for antibodies against the coronavirus spike (S) envelope glycoprotein, the primary target of virus-neutralizing antibodies (6), in either its native-like oligomer conformation, or against one of its protein subunits or domains. In general, most S glycoprotein antigen-based assays report the ability to detect antibodies in 65-70% of infected individuals 8 – 14 days after symptom onset, with positivity rates over 90% not occurring until 2 – 3 weeks after symptom onset (7).

In this study, we describe the development, characterization, and utility of a betacoronavirus (β-CoV) multiplex microsphere-based immunoassay (MMIA) for COVID-19 serology studies. To optimize sensitivity and specificity for measuring SARS-CoV-2 spike reactive antibodies, the MMIA included prefusion stabilized S glycoprotein ectodomain trimers of SARS-CoV-2, SARS-CoV-1, MERS-CoV, and the seasonal human coronaviruses (HCoV), HCoV-HKU1 and HCoV-OC43. The MMIA enabled the simultaneous measurement of relative antibody quantities against each of these medically-relevant betacoronaviruses. We hypothesized that this approach would potentially result in a highly sensitive and specific assay

91   for detecting SARS-CoV-2 specific antibodies through two mechanisms. First, the Luminex

92   xMAP-based platform has a large dynamic range and has been shown to be more sensitive

93   than ELISA for the detection of antibodies to other viral infections (8-10). Second, given the high

94   seroprevalence of the common human betacoronaviruses (11-13), cross-reactive antibodies

95   present in subject samples (14, 15) could be concurrently measured and accounted for in a

96   multiplex approach. By testing for S glycoprotein reactive antibodies to SARS-CoV-2 in the

97   presence of HKU1 and OC43 S glycoproteins, the MMIA assay controls for off-target pre-

98   existing cross-reactive betacoronavirus antibodies, thus enhancing specific SARS-CoV-2

99   antibody detection. Additionally, the simultaneous incubation of serum with S glycoproteins from

100   all the relevant betacoronaviruses may enable a lower threshold for SARS-CoV-2 antibody

101   positivity.

102   Utilizing serum samples from an experimentally challenged non-human primate (NHP)

103   model, together with human sera from subjects confirmed to have SARS-CoV-2 infection and

104   from subjects confirmed to have other coronavirus infections collected prior to 2018, we report

105   the sensitivity and specificity performances for this assay strategy. Serum samples from rhesus

106   macaques experimentally infected with SARS-CoV-2 demonstrated that SARS-CoV-2 S

107   glycoprotein IgG seroconversion was detectable by 10 days post infection (dpi), consistent with

108   other reports demonstrating anti-S glycoprotein IgG seroconversion between 3 and 14 dpi (16-

109   19). As a result, we evaluated serum samples from SARS-CoV-2 positive subjects collected 10

110   days after symptom onset and report 98% sensitivity for SARS-CoV-2 S glycoprotein IgG

111   antibody detection in humans at that time point. We also examined differences in SARS-CoV-2

112   antibody reactivity between widely used antigens: SARS-CoV-2 prefusion stabilized S

113   glycoprotein ectodomain trimer and the receptor-binding domain (RBD). High seroprevalence of

114   seasonal HCoV OC43 and HKU1, ranging from 97 – 98% and 55 – 89%, respectively, was

115   observed across both archival sera and SARS-CoV-2 negative subject serum samples. Through

116   this MMIA strategy we aim to investigate the interplay of pre-existing seasonal HCoV antibodies

117   on SARS-CoV-2 IgG duration, COVID-19 symptom presentation, and disease severity.

118   Preliminary data we have obtained using this multiplex serology strategy demonstrates that

119   SARS-CoV-2 antibody can be detected be detected early after the onset of symptoms and that

120   SARS-CoV-2 infection can stimulate an IgG antibody response that is cross-reactive with

121   SARS-CoV-1 and MERS-CoV S glycoproteins.

122

123   **RESULTS**

124   *Comparison of MMIA and ELISA for SARS-CoV-2 IgG antibody detection*

125   We first established our ability to detect SARS-CoV-2 IgG and monitor SARS-CoV-2

126   seroconversion with sera collected from SARS-CoV-2 infected NHP. Purified IgG from SARS-

127   CoV-2 infected NHP collected 21 dpi were pooled and spiked into NHP negative sera, and the

128   MMIA was quantitatively characterized for IgG polyclonal reactivity revealing SARS-CoV-2 spike

129   antibody MFI curve linearity between 0.625 – 5.0 µg/ml or 3690 – 20,354 MFI (Figure 1).

130   Positive MMIA saturation occurs within 20,000 – 30,000 MFI. To investigate the effects of the

131   increased dynamic range facilitated by Luminex xMAP-based multiplexing systems on MMIA

132   sensitivity, we compared end-point titers by both ELISA and MMIA. In an ELISA, SARS-CoV-2

133   positive NHP sera end-point titers ranged from 1,000 to 2,000 (Figure 2A), consistent with

134   reported ELISA titers for these animals (16). In the MMIA, the ability to detect serially diluted

135   IgG antibodies was 4- to 8-fold greater than ELISA with end-point titers ranging from 4,000 (n=

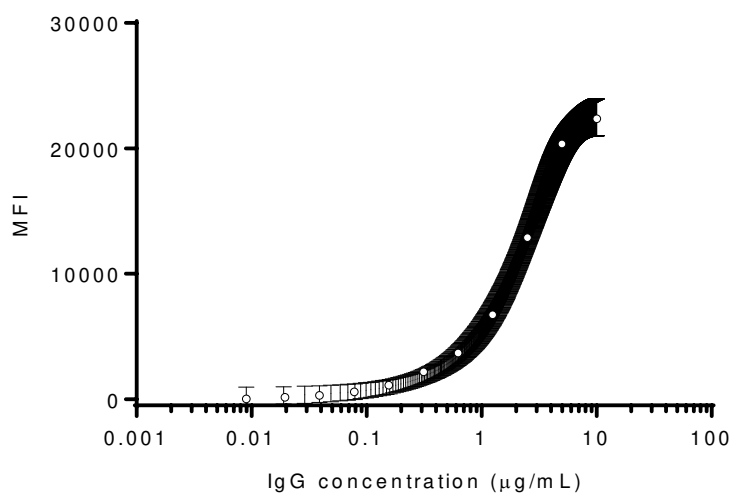136   2) to 16,000 (n= 1) (Figure 2B).

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Figure 1. SARS-CoV-2 spike protein reactivity as a function of IgG concentration. A

sigmoidal curve was used to fit the MEAN±SEM of two independent experiments performed in

technical triplicates. MFI, median fluorescence intensities.

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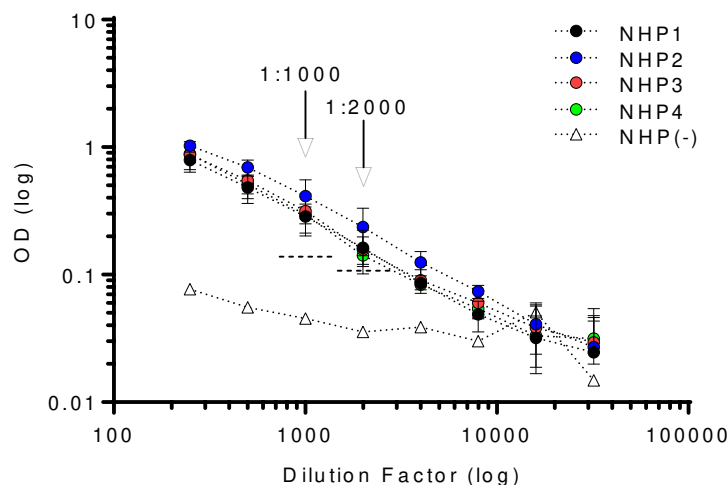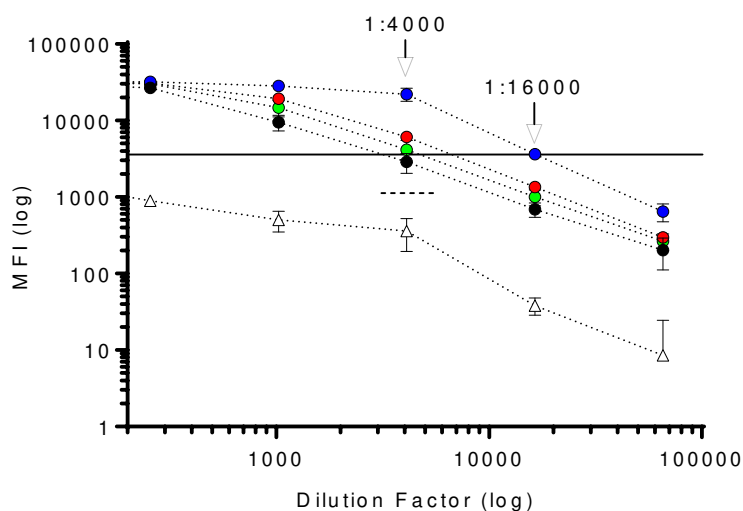
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Figure 2. MMIA displays enhanced sensitivity for SARS-CoV-2 IgG detection. Serum

samples from SARS-CoV-2 infected NHP collected 21 dpi were tested by SARS-CoV-2 spike

protein **(A)** ELISA and **(B)** MMIA. **(A)** Dashed lines indicate 3-fold MFI above the NHP(-) serum

sample(s) diluted 1:1000 and 1:2000. **(B)** A solid line indicates the lower limit of MFI linearity

and a dashed line indicates 3-fold MFI above the NHP(-) serum sample diluted 1:4000. Positive

samples are those above both the MFI level for curve linearity and 3-fold change in NHP(-)

serum. MFI values represent MEAN±SD of two independent experiments performed in technical

triplicates.

186   *Seroconversion in a non-human primate model*

187   Next, we monitored SARS-CoV-2 seroconversion with longitudinal NHP serum samples.

188   SARS-CoV-2 spike protein reactive IgG antibody seroconversion was observed in all four NHP

189   10 dpi (Figure 3A). We also investigated SARS-CoV-2 IgM antibody seroconversion and

190   detected IgM level above baseline in two NHP by 7 dpi; all four NHP had detectable IgM by 10

191   dpi (Figure 3B). Notably, IgG antibody from SARS-CoV-2 challenged NHP did not significantly

192   react with spike proteins from betacoronaviruses, SARS-CoV-1, MERS-CoV or HCoVs, included

193   in the MMIA (Figure 3A), whereas, a varying degree of IgM cross-reactivity was observed

194   (Figure 3B). Additionally, high baseline IgM reactivity to SARS-CoV-2 RBD at 0 dpi inhibited our

195   ability to ascertain the dpi where seroconversion could be observed with this SARS-CoV-2

196   antigen (Figure 3B).

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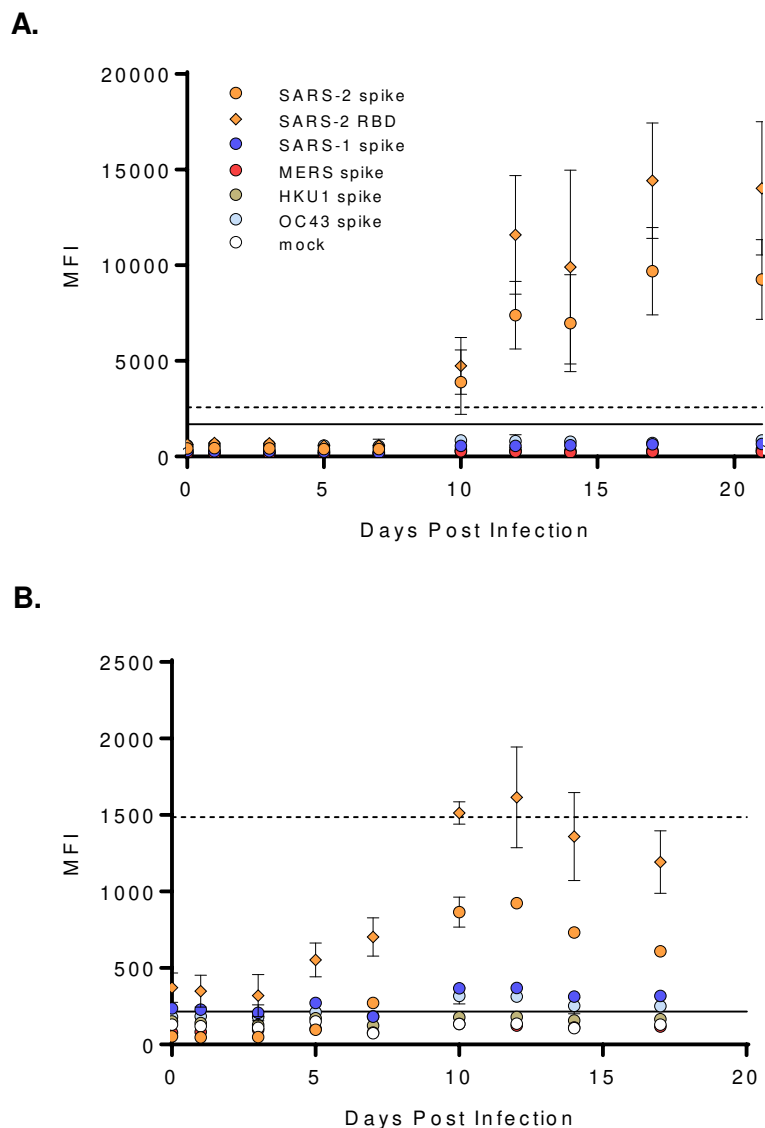204

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Figure 3. SARS-CoV-2 seroconversion in a non-human primate model. Sera from SARS-CoV-2 infected NHP were screened for SARS-CoV-2 spike protein reactive **(A)** IgG and **(B)** IgM. Graphs represent the MEAN±SD of four SARS-CoV-2 challenged NHP screened in two independent experiments performed in technical duplicates. A solid line indicates a 4-fold rise in SARS-CoV-2 spike protein MFI from baseline (0 dpi) and was used as a threshold cutoff for SARS-CoV-2 IgG and IgM seroconversion; a dashed line indicates a 4-fold rise in SARS-CoV-2 RBD protein MFI from baseline.

238   *Archival sera from subjects with PCR-confirmed seasonal coronaviruses exhibit cross-reactivity*

239   *with SARS-CoV-2 spike protein*

240   Despite low sequence similarity and identity between SARS-CoV-2 spike protein and

241   seasonal HCoV spike proteins, antibody cross-reactivity with SARS-CoV-2 proteins has been

242   observed (14, 15). To determine whether prior infection with seasonal HCoV induces antibodies

243   that cross-react with SARS-CoV-2, we assayed archival (pre-2019) serum from human subjects

244   with PCR-confirmed seasonal HCoVs. When setting a cut-off for positivity at three times the

245   mean MFI obtained for a mock antigen preparation-coupled microsphere, we observed that

246   8.89% (4/45) of archived serum samples from HCoV PCR-positive subjects cross-reacted with

247   SARS-CoV-2 spike protein (Figure 4A-D). Cross-reactivity between HCoV-induced antibodies

248   with SARS-CoV-2 spike protein was observed in subjects that were PCR-positive for OC43

249   (1/16), HKU1 (1/6) and 229E (2/10) infection.

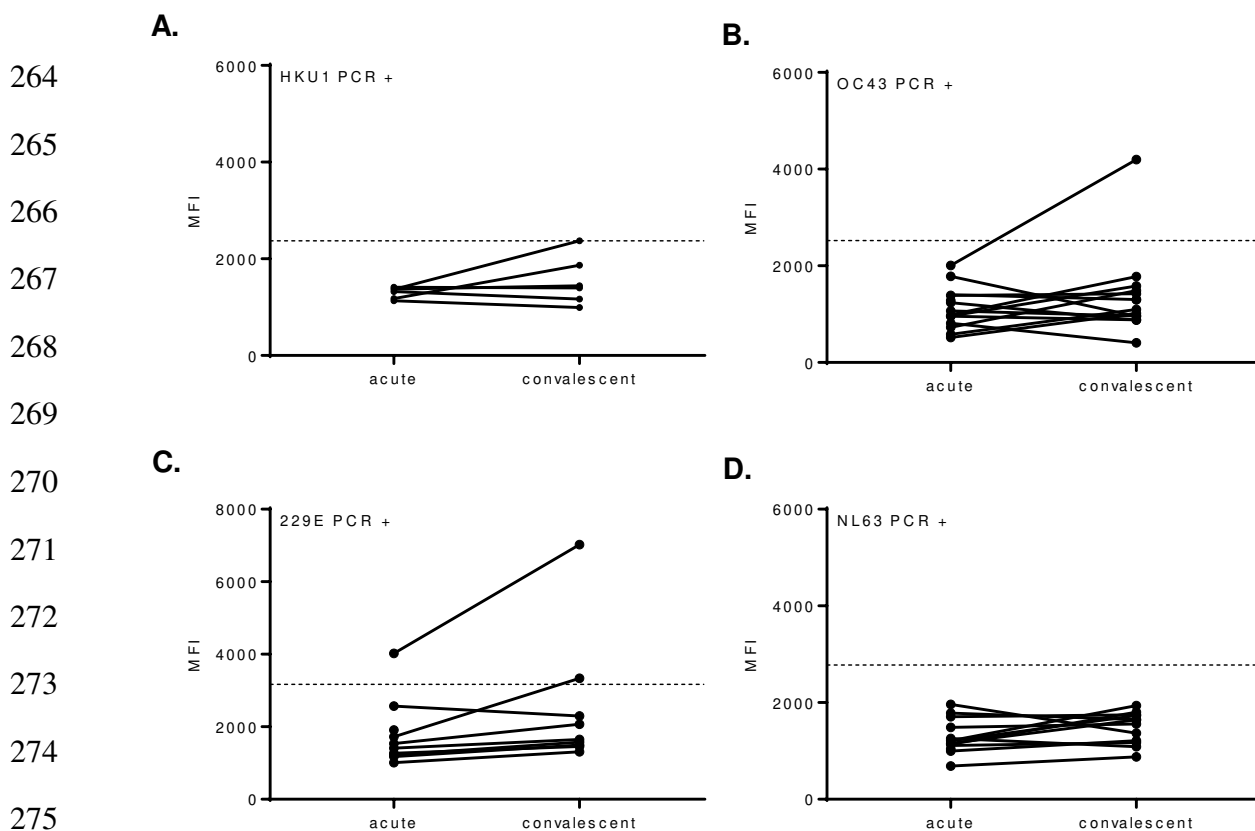250

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Figure 4. Archival sera from subjects with seasonal HCoVs can display cross-reactivity with SARS-CoV-2 spike protein. Acute and convalescent serum samples from HCoV PCR-positive subjects were tested in a β-CoV MMIA. Subjects are grouped together based on HCoV PCR confirmation, **(A)** OC43 (n= 16), **(B)** HKU1 (n= 6), **(C)** NL63 (n= 13) and **(D)** 229E (n= 10), A dashed line indicates the 3-fold change in the mean MFI of a mock antigen-coupled microsphere. MFI values represent the MEAN of two independent experiments performed in technical duplicates. MFI, median fluorescence intensity.

290   *Assay threshold cutoffs for SARS-CoV-2 spike protein reactive antibodies*

291   To control for pre-existing HKU1 and OC43 spike protein reactive antibody cross-

292   reactivity with SARS-CoV-2 spike protein, rather than using a cutoff of three times the mean MFI

293   obtained for mock antigen, antibody threshold cutoffs were established with HCoV PCR-positive

294   convalescent sera (Figure 5A-B). A conventional 99.7% probability, mean and three standard

295   deviations higher, threshold cutoff was employed to distinguish positive and negative IgG

296   antibodies. The mean IgG reactivity to SARS-CoV-2 spike protein was 1569 MFI with a 99.7%

297   probability threshold cutoff of 4911 MFI. The SARS-CoV-2 RBD protein had a notably higher

298   background IgG antibody reactivity and threshold cutoff, 2846 MFI and 7951 MFI, respectively.

299   Given the inherently less-specific nature of IgM, a 99.9% probability threshold cutoff was

300   preferred. The 99.9% probability threshold cutoffs for SARS-CoV-2 spike protein and RBD

301   protein reactive IgM were 846 MFI and 15352 MFI. The remaining archival sera (n= 84),

302   representing HCoV PCR-positive acute sera, rhinovirus PCR-positive acute/convalescent sera,

303   and acute/convalescent sera from 'no pathogen detected' subjects, did not react with SARS-

304   CoV-2 spike protein or RBD protein above the established threshold cutoffs for either antigen

305   (Figure 5C-D). Although only 17%/7% of ARIC human subjects were OC43/HKU1 PCR positive,

306   we observed 97.6% OC43 and 89.2% HKU1 IgG, and none were IgM positive (Figure 5C-D).

307   Interestingly, the three OC43 spike IgM positive serum samples were collected from subjects
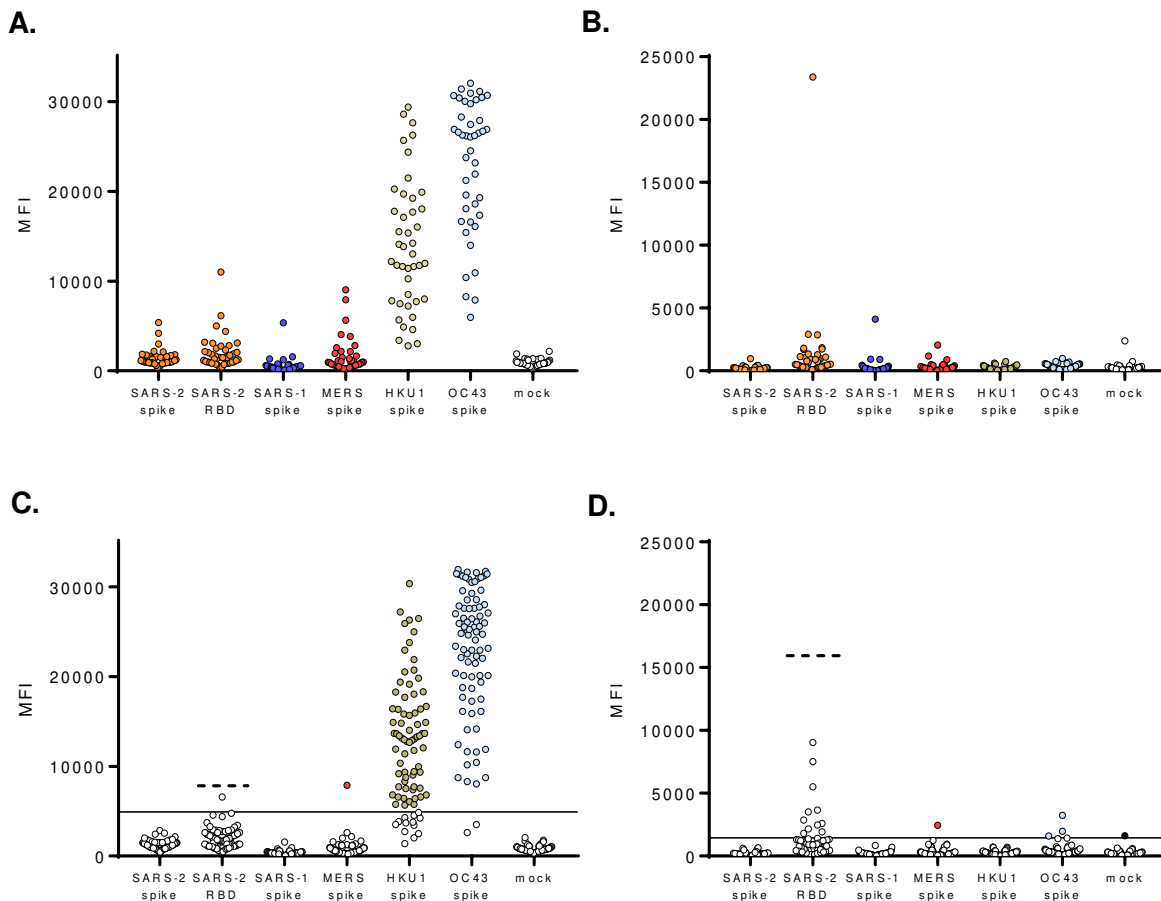
308   who had no pathogen detected by PCR.

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Figure 5. Archival sera generated threshold cutoffs for IgG and IgM antibodies confer specificity for SARS-CoV-2. Convalescent serum samples (n= 43) from HCoV PCR-positive subjects were tested in the β-CoV MMIA with **(A)** IgG antibody and **(B)** IgM antibody. **(C-D)** Archival sera (n= 84), acute serum samples from HCoV PCR-positive subjects, acute/convalescent serum samples from rhinovirus PCR-positive subjects and acute/convalescent serum samples from 'no pathogen detected' subjects were tested for **(C)** IgG and **(D)** IgM antibody reactivity tested against the established threshold cutoffs. A solid line indicates the threshold cutoff for positivity with SARS-CoV-2 spike protein and a dashed line indicates the threshold cutoff for SARS-CoV-2 RBD. Colored dots in **(C-D)** indicate samples with MFI above the SARS-CoV-2 spike protein threshold cutoff for positive antibody. MFI is the average of sera diluted 1:400, adjusted with PBS controls and tested across technical duplicate plates. IgG data is a representation of three independent screenings.

342   *Multiplex microsphere-based immunoassay performance*

343   Sera from persons with SARS-CoV-2 infection were screened for IgG and IgM antibody

344   reactivity with betacoronavirus spike proteins in our MMIA. As SARS-CoV-2 seroconversion was

345   detected by 10 dpi in NHPs, MMIA SARS-CoV-2 spike protein IgG sensitivity for human

346   serology was evaluated in confirmed subjects ≥ 10 days post-symptom onset (dpso).

347   Additionally, a combination of archival human serum samples, and PCR negative MTF

348   hospitalized subjects and outpatients with serum samples collected < 30 dpso were included in

349   a negative agreement analysis. Subjects that were PCR confirmed as SARS-CoV-2 negative

350   displayed no IgG with SARS-CoV-2 spike protein, whereas one subject had IgM reactivity to

351   SARS-CoV-2 spike protein above the 99.9% cutoff MFI value. A receiver operating

352   characteristic (ROC) curve analysis was then performed to further establish a more

353   conservative threshold value (1446 MFI cutoff point) for SARS-CoV-2 positive IgM (Figure 6A-

354   B). Further, HKU1 and OC43 IgG antibodies were observed in SARS-CoV-2 PCR negative

355   subjects (Figure 6A). SARS-CoV-2 reactive IgG antibodies were only detected above the 99.7%

356   threshold cutoff in the PCR positive subjects enrolled at military hospitals or the Javits Center

357   field hospital; cross-reactive antibodies to SARS-CoV-1 and MERS-CoV were observed in

358   SARS-CoV-2 PCR positive and IgG positive subjects (Figure 6B).

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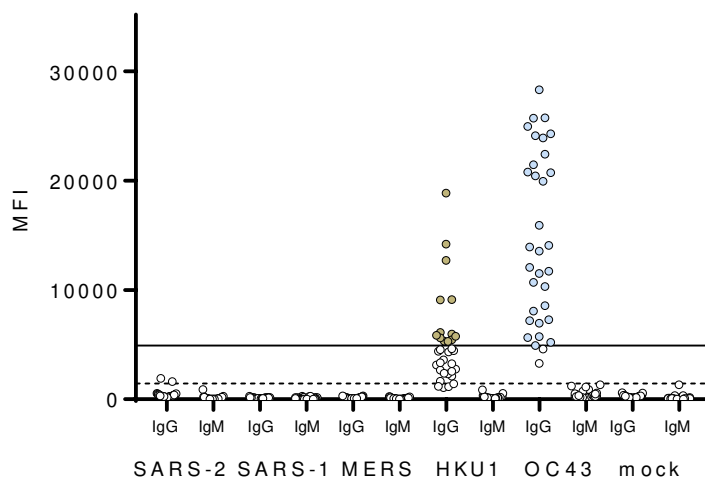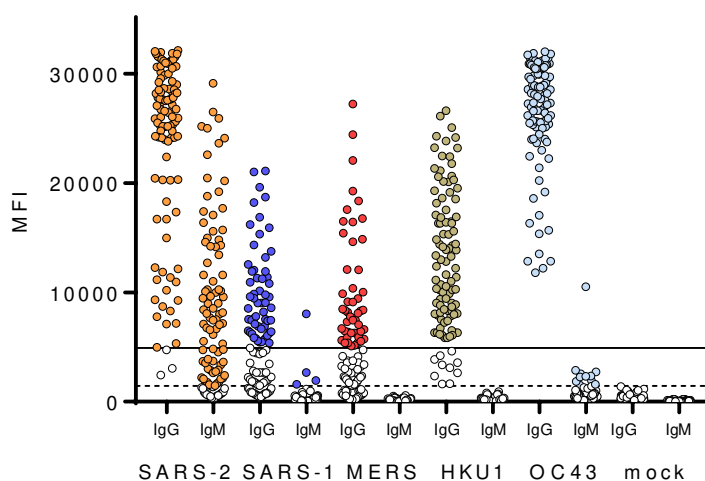
Figure 6. A multiplex antibody test can detect SARS-CoV-2 specific and cross-reactive antibodies. Serum samples from **(A)** SARS-CoV-2 PCR negative subjects **(B)** SARS-CoV-2 PCR-positive subjects collected ≥ 10 dpso were tested by β-CoV MMIA. Serum were diluted 1:400 and tested in duplicate plates. MFI, median fluorescence intensities, are the average of PBS-subtracted technical duplicates. A solid line indicates the IgG threshold cutoff and a dashed line indicates the IgM threshold cutoff. Colored dots indicate positive serum samples. SARS-2, SARS-COV-2; SARS-1, SARS-CoV-1; MERS, MERS-CoV; HKU1, HCoV-HKU1; OC43, HCoV-OC43.

394   Performance assessments of this β-CoV MMIA for SARS-CoV-2 spike protein IgG and

395   IgM detection are included in Table 1. SARS-CoV-2 spike protein reactive IgG antibody

396   detection was calculated with 95% confidence intervals (CI) as follows, sensitivity = 98.06%

397   (94.45% – 99.60% CI), specificity= 100% (96.82% – 100.00% CI). Assuming a US disease

398   prevalence of 1.0%, the positive predictive value (PPV) = 100.00%, and negative predictive

399   value (NPV) = 99.98% (99.94% - 99.99% CI). SARS-CoV-2 spike protein reactive IgM detection

400   sensitivity was lower than IgG, with performance analysis conducted with serum samples

401   collected ≥ 7 days post-symptom onset, sensitivity= 78.10% (68.97% to 85.58%), specificity=

402   100% (96.95% - 100.00% CI) and PPV= 100.00% (Table 1).

403   When comparing spike protein and RBD, notably, utility of SARS-CoV-2 RBD for IgG

404   detection had a reduced sensitivity (87.10%, 80.78% - 91.94% CI) (Table 2). To assess MMIA

405   precision, three positive and one negative samples were tested over five independent

406   experiments, with at least two distinct in-house antigen-coupled bead lots and serum sample

407   freeze-thaws. Coefficient of variations (CV) were calculated for all three positive samples and

408   remained <20% (Figure 7). Although the negative sample had a > 20%, the MFI never went

409   above the threshold cutoff for positive IgG across five independent tests.

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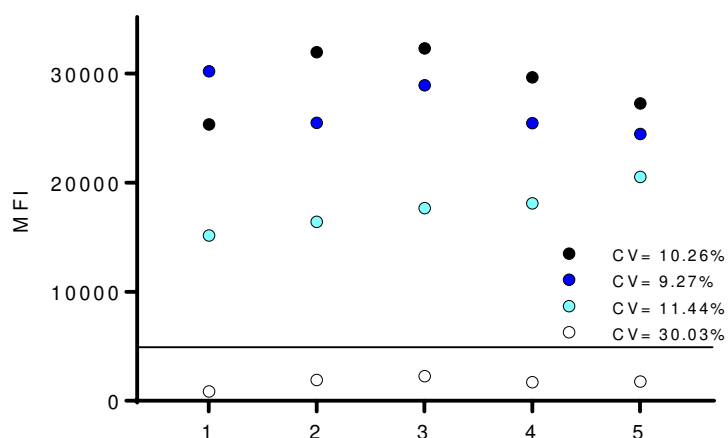
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Figure 7. Positive and negative results are reproducible over independent MMIA tests.

Selected positive(s) and negative serum samples were tested across independent experiments. CV, coefficient of variation, percentages are indicated on the graphs for each sample. A solid line indicates the threshold cutoff for positive IgG.

DISCUSSION

439

440   In this study, we have demonstrated that use of a multiplex microsphere-based

441   immunoassay (MMIA) built using Luminex xMAP-based technology in which individual

442   microspheres are bound to pre-fusion stabilized S glycoprotein trimers of SARS-CoV-2, SARS-

443   CoV-1, MERS-CoV, and each of the two seasonal betacoronaviruses enables highly sensitive

444   and specific detection of SARS-CoV-2 IgG antibodies. In contrast to commercial ELISA and

445   lateral flow assays for SARS-CoV-2 IgG, which typically have a sensitivity in the range of 65-

446   70% up to 14 days after symptom onset (7), the MMIA has a sensitivity of 98% at just 10 days

447   after symptom onset in PCR-confirmed cases of SARS-CoV-2 infection. Use of this highly

448   sensitive assay will allow for improved assessments of the kinetics of humoral responses to

449   SARS-CoV-2 infection.

450   We hypothesize that the high sensitivity and specificity of the MMIA assay is due both to

451   the physics of the Luminex xMAP-based platform, which enables a high dynamic range of

452   measurement, as well as to the use of a multiplexing system. Multiplex microsphere-based

453   immunoassays have been shown to be more sensitive than standard ELISA for SARS-CoV-2

454   antibody detection (20) and several other virus infections, including Lassa virus, Ebola virus,

455   and simian immunodeficiency virus (8-10). Additionally, and perhaps more importantly,

456   simultaneously incubating serum against spike proteins of seasonal HCoVs may enable the

457   establishment of a lower threshold of positivity for detection of SARS-CoV-2 antibodies. Given

458   the presence of cross-reactive antibodies, assays that only test for antibodies against SARS-

459   CoV-2 may have to utilize a high signal threshold as a cut-off for positivity to reduce false

460   positive rates of detection. By incubating serum against multiple coronavirus spike proteins, the

461   MMIA platform may allow preferential binding of cross-reactive antibodies to the antigens of the

462   coronavirus against which they were initially induced, enabling a lower, and potentially more

463   sensitive, cut-off for the detection of SARS-CoV-2 specific antibodies.

464   Now nine months into the COVID-19 pandemic, this β-CoV MMIA adds to an established

465   body of antibody tests and serology results. Early in the pandemic, SARS-CoV-2 IgG

466   seroconversion was surprisingly detected early after exposure and sometimes in parallel with

467   IgM seroconversion (21-23). The temporal window to capture SARS-CoV-2 IgM is shorter than

468   IgG, and with IgG seroconversion occurring 10 dpi in NHP SARS-CoV-2 disease models, and

469   detectable as early as 7 dpso in subjects, there appears little benefit for continued SARS-CoV-2

470   IgM detection (Table 3). As we placed no upper limit on the dpso of the first serum collection

471   included in performance analysis, IgM sensitivity is lower than IgG driven by outpatient

472   enrollments in the EPICC, IDCRP-085 study on average 28 dpso that were IgG positive, but IgM

473   negative. Although less sensitive and less specific than IgG detection, the benefit of IgM

474   detection may lay in its ability to place a temporal window on SARS-CoV-2 exposure in

475   asymptomatic IgG positive individuals, particularly useful for cross-sectional studies of

476   seroprevalence.

477   Conservation of epitopes present in the prefusion stabilized native-like trimeric S

478   glycoprotein oligomers may be the major factor in the observed cross reactions between the

479   CoV S glycoproteins. Since the RBD protein is only a domain within the S1 subunit of the S

480   glycoprotein and lacks potentially conserved protein residues with seasonal HCoV S

481   glycoproteins, utility in antigen-based immunoassays confers specificity for SARS-CoV-2 and is

482   thus employed in several antibody tests (24-27). We noted that in the present β-CoV MMIA, the

483   commercially sourced RBD protein, which shares an equivalent number of protein residues with

484   the expressed RBD protein used in a microsphere-based immunoassay developed by the

485   Ragon Institute of MGH, MIT and Harvard (20), had a higher threshold cutoff that limited IgG

486   detection sensitivity compared to the spike protein. This reactivity to RBD may be driven by

487   artificial epitopes, exposure of epitopes otherwise inaccessible within the context of the native-

488   like S glycoprotein trimer or a product of microsphere coupling.

489   Immunoassay detection of IgG antibodies that can bind to RBD has been used as a

490   surrogate for neutralization tests which require cell-culture, pseudoviruses, or biosafety-

491   containment and wild-type SARS-CoV-2 (25, 28, 29). SARS-CoV-2 neutralizing antibodies

492   target the S glycoprotein S1 subunit, particularly the RBD and N-terminal domains, and

493   sterically interfere with human ACE-2 receptor interaction (30-34). Yet, SARS-CoV-2 and

494   MERS-CoV neutralizing monoclonal antibodies have been identified that binds epitopes that do

495   not interfere with receptor engagement (35, 36). Furthermore, non-neutralizing antibody-

496   mediated protection has been observed in other virus infections, including HIV and Ebola virus

497   (37, 38). Given the relatively poor performance of this RBD protein in this β-CoV MMIA, and our

498   ability to capture the full-breadth of the humoral response, e.g., RBD-binding, neutralizing and

499   non-neutralizing, to SARS-CoV-2 infection with the native-like spike protein trimer (39), future

500   studies will exclude the monomeric RBD antigen from β-CoV MMIA strategy.

501   The MMIA approach provided additional antibody detection data that is suggestive of

502   SARS-CoV-2 IgG cross-reactivity with SARS-CoV-1 and MERS-CoV. Conserved cross-

503   neutralizing epitopes between MERS-CoV, SARS-CoV-1 and SARS-CoV-2 S glycoproteins

504   have been identified (35, 40). Whether SARS-CoV-2 induced polyclonal IgG antibody responses

505   to SARS-CoV-1 and MERS-CoV spike proteins are retained after affinity maturation, or are

506   cross-neutralizing requires further investigation. Future studies with this MMIA will incorporate

507   spike proteins from the seasonal alphacoronaviruses, HCoV-NL63 and HCoV-229E, to improve

508   upon specificity for SARS-CoV-2 antibody detection. We hypothesize that the inclusion of NL63

509   and 229E spike proteins will provide additional off-target control of cross-reactive antibodies to

510   SARS-CoV-2 spike protein, decreasing the threshold cutoff for positive SARS-CoV-2 and

511   enabling improved detection of waning and low positive SARS-CoV-2 IgG antibody. To attempt

512   to achieve similar success at detecting SARS-CoV-2 antibodies and seroprevalence four

513   months after diagnosis (41), next steps in MMIA development will be focused on a re-calibration

514   of threshold cutoffs using archived sera in an MMIA that is limited to spike proteins from SARS-

515   CoV-2, HCoV-HKU1, HCoV-OC43, HCoV-NL63 and HCoV-229E. Importantly, when utilized in

516   select subject cohorts from prospective, longitudinal observational studies in which serum

517   samples are obtained before SARS-CoV-2 infection, this MMIA approach has the potential to

518   measure antibody cross reactions with seasonal HCoVs, and investigate whether HCoV-

519   induced antibodies confer any protection against COVID-19.

520

521   **CONCLUSION**

522   In summary, we have presented the development of a multiplex microsphere-based

523   immunoassay for SARS-CoV-2 serology that includes envelope spike glycoproteins from

524   zoonotic, SARS-CoV-1 and MERS-CoV, and seasonal endemic betacoronaviruses, HCoV-

525   HKU1 and HCoV-OC43. Performance assessment of this immunoassay with serum samples

526   from a pre-2019 archived sera bank and SARS-CoV-2 PCR positive subjects who sought

527   medical treatment at military hospitals demonstrated 100% specificity for SARS-CoV-2 IgG

528   antibody detection and 98% sensitivity with samples collected as early as 10 days after the

529   onset of symptoms. Through this multiplex approach we are able to measure the potential cross

530   reactions of HCoV-induced antibodies to SARS-CoV-2 spike glycoproteins. Application of this

531   multiplex approach to prospective observational studies will enable the direct examination of

532   whether pre-existing HCoV-induced antibodies affect COVID-19 clinical outcomes, i.e.

533   asymptomatic presentation and symptomatic severity.

534

535   **MATERIALS AND METHODS**

536   *Recombinant protein antigens and microsphere coupling*

537   Prefusion stabilized SARS-CoV-2 S-2P glycoprotein ectodomain trimers (hereafter

538   referred to as spike protein) and SARS-CoV-2 RBD were purchased from LakePharma, Inc.

539   (Hopkinton, MA USA). This SARS-CoV-2 spike protein shares an equivalent ectodomain with

540   the NIH Vaccine Research Center designed SARS-CoV-2 S-2P protein, and the Mount Sinai

541   SARS-CoV-2 S-2P protein used in ELISA-based serology (26, 27, 42-44). Differences between

542   LakePharna, Inc. and VRC or Mount Sinai spike protein constructs are highlighted in the C-

543   terminus tags and selection of mammalian cell-line for expression.

544   Design and expression of prefusion stabilized HCoV-HKU1, HCoV-OC43, SARS-CoV-1

545   and MERS-CoV spike proteins have been previously described (15, 42). A mock antigen,

546   consisting of cell culture supernatant from untransfected HEK cells was collected via

547   centrifugation then filtered through a 0.22 µM PES filter to remove debris. Mock antigen-coupled

548   beads are included in each microtiter well to control for non-specific/artificial antisera binding;

549   samples that react with the mock antigen above an established 3-fold cutoff are retested. Spike

550   proteins were coupled to carboxylated magnetic MagPlex microspheres (Bio-Rad, Hercules,

551   CA) at a protein to microsphere ratio of 15 µg:100 µL, and antigen-coupled microspheres were

552   resuspended in a final volume of 650 µL following manufacturer's protocol (Bio-Rad) for amine

553   coupling.

554   *Non-human primate sera*

555   Archived sera were used from rhesus macaques inoculated with a total dose of 2.6×10^6

556   TCID50 of SARS-CoV-2 via a combination of intranasal, intratracheal, oral and ocular

557   inoculation routes (16). Serum samples were collected at dpi 0 (baseline), 1, 3, 5, 7, 10, 12, 14,

558   17 and 21. To purify serum IgG antibody, 250 µL of serum from each of four experimentally

559   infected NHPs collected 21 dpi were pooled then subjected to thermal inactivated for 30 minutes

560   at 60 °C. During inactivation, 2 mL of Protein G agarose, 50% suspension (Sigma-Aldrich, St.

561   Louis, Missouri, USA) was added to a chromatography column and the buffer was allowed to

562   flow through. The bead bed was then washed three times with 10 mL of PBS. Inactivated

563   pooled sera were diluted 1:5 in PBS then added to the column. Flow-through was collected,

564   then re-added to the column; this process was repeated for a total of three passes through the

565   column. The bead bed was again washed three times with 10 mL of PBS. Finally, IgG was

566   eluted from the Protein G agarose using a 0.1M Glycine elution buffer, pH 2.5 then returned to

567   neutral pH using 1M Tris-HCl, pH 8.0. Eluted IgG was concentrated using an Amicon Ultra

568   Centrifugal Filter unit (Merck Millipore, Burlington, Massachusetts, USA), and the buffer was

569   exchanged to a 1X PBS buffer containing 25% glycerol.

570   SARS-CoV-2 IgG and IgM antibody seroconversion was determined as the first dpi

571   where a 4-fold increase in the median fluorescence intensity (MFI) was measured compared to

572   the baseline sera collection. Between the 1:250 and 1:1000 dilutions, some NHP IgG antibody

573   reactivities were no longer saturating the upper level of the MMIA. We chose further sera

574   screening at a 1:400 dilution, retaining the ability of the MMIA to detect positives at near MMIA

575   saturation, i.e. >20,000 MFI, while still within the linear region of detection.

576   *Participant enrollment and sera collection*

577   SARS-CoV-2 negative human serum specimens utilized were from archived sera

578   collected between 2012 – 2018 in the Infectious Disease Clinical Research Program (IDCRP)

579   Acute Respiratory Infection Consortium Natural History Study (ARIC, IDCRP-045) (45). ARIC

580   sera predate the COVID-19 pandemic and were collected from subjects who had

581   nasopharyngeal swabs tested by nucleic acid amplification methods for virus etiologies of acute

582   respiratory infections; samples collected from individuals with rhinovirus and the seasonal

583   human coronaviruses HCoV-OC43, -HKU1, -229E and -NL63 were used (46). In addition,

584   serum samples were collected since the emergence of SARS-CoV-2 under the IDCRP

585   Epidemiology, Immunology, and Clinical Characteristics of Emerging Infectious Diseases with

586   Pandemic Potential (EPICC, IDCRP-085) protocol; a prospective, longitudinal study to analyze

587   COVID-19 disease. Subjects were enrolled at five hospitals across the continental U.S.,

588   including Walter Reed National Military Medical Center (WRNMMC, Bethesda, MD), Brooke

589   Army Medical Center (BAMC, San Antonio, TX), Naval Medical Center San Diego (NMCSD,

590   San Diego, CA), Madigan Army Medical Center (MAMC, Tacoma, WA) and Fort Belvoir

591   Community Hospital (FBCH, Fort Belvoir, VA). Subjects of all race and gender seeking

592   treatment for acute illness at these military hospitals were offered enrollment into the EPICC,

593   IDCRP-085 protocol. Study enrollment included subjects with laboratory-confirmed SARS-CoV-

594   2 infection by nucleic acid amplification test, subjects with compatible illness in whom SARS-

595   CoV-2 infection is initially suspected but PCR confirmed as SARS-CoV-2 negative, and

596   asymptomatic subjects at risk of SARS-CoV-2 due to high risk exposure. In this study, 422

597   sera samples from 204 individual subjects were tested. The earliest serum samples collected ≥

598   10 dpso from subjects with longitudinal samples were included in positive performance

599   agreement. Additionally, serum samples from 35 subjects undergoing treatment at the COVID-

600   19 field hospital at the Jacob K. Javits Convention Center (New York, NY) under the COVID-19

601   Antibody Prevalence in Military Personnel Deployed to New York (COVID-19 NYC) protocol

602   were included in the assessment of assay performance. EPICC, IDCRP-085 and COVID-19

603   NYC protocols were approved by the Uniformed Services University Institutional Review Board.

604   *Multiplex microsphere-based immunoassay screening procedures*

605   Serum samples were collected from venipuncture in serum separator tubes, processed

606   and stored at -80 °C in 250 µL aliquots until use. For each 96-well plate, a multiplex master mix

607   of antigen-coupled microspheres was made by diluting 100 µL of each antigen-coupled

608   microsphere working stock into 10 mL (1:100) 1XPBS without calcium and magnesium (Corning

609   Inc., Corning, NY) (all mentions of PBS refer to solutions without calcium and magnesium), and

610   100 µL of this master mix were added to each well so that each well contained 1 µL (~23 ng) of

611   each antigen-coupled microsphere per well. Wells were washed with 1XPBS + 0.05% Tween20

612   + 0.02% sodium azide two times. One hundred microliters of each serum sample was added to

613   each well. Serum samples were initially diluted within a class II type A2 biological safety cabinet

614   (BSC) then subjected to thermal inactivation for 30 min at 60 °C, further serum dilutions are

615   noted in each respective figure legend. Human serum samples (1.25 µL) were diluted 1:400 in

616   PBS and tested in technical duplicate A and B plates. Controls on each duplicate plate included

617   a PBS blank (wells: A1, B1, G12, H12) and positive (C1, F12) and negative (D1, E12) non-

618   human primate serum. As testing progressed, PCR and serology confirmed human

619   positive/negative samples replaced non-human primate serum samples as the qualified controls

620   for inter- and intra-plate variation.

621   Samples were incubated at room temperature for 45 minutes with agitation (900 rpm),

622   and plates were washed three times. Secondary antibody (goat anti-human IgG cross-absorbed

623   biotin-conjugated or goat anti-human IgM cross-absorbed biotin-conjugated; Thermo Fisher

624   Scientific, Waltham, MA) was diluted 1:5000 in 1XPBS + 0.05% Tween20 (PBST) and 100 µL of

625   each secondary was added to each well and incubated for 45 minutes with agitation, and plates

626   were washed three times. Streptavidin-phycoerythrin (Bio-Rad) was diluted 1:1000 in PBST and

627   100 µL was then added to each well and incubated for 30 minutes with agitation, and plates

628   were washed three times. Lastly, 100 µL of PBST was added to each well and plates were

629   resuspended by agitation for 5 minutes. Plates were read on Bio-Plex 200 multiplexing systems

630   (Bio-Rad) with PMT voltage setting to the High RP1 target and 100 bead count requirements.

631   The MFI for the four PBS blank wells on each plate were subtracted from the MFI of each

632   sample well and MFI values for samples are reported as the PBS adjusted average from

633   duplicate plates.

634   *Threshold cutoffs for SARS-CoV-2 antibody*

635   To establish threshold cutoffs for SARS-CoV-2 spike protein-specific antibody reactivity,

636   we tested 127 archival acute and convalescent human serum samples from ARIC. Acute and

637   convalescent serum samples were collected within approximately three and twenty-eight days

638   of symptom onset, respectively. A cut-off of three times the mean MFI obtained using a mock

639   antigen preparation coupled microsphere was initially used to determine positivity of cross-

640   reactive antibodies in archival serum samples. As cross-reactive antibodies were found to occur

641   in 4 out of 45 serum samples from archival HCoV PCR-positive individuals, we then established

642   a cut-off of three standard deviations above the mean (99.7% probability) MFI of these archival

643   HCoV convalescent serum samples (n= 43) to establish a positivity threshold for detection of

644   SARS-CoV-2 spike protein reactive IgG and IgM antibodies. The remaining 84 archival serum

645   samples were tested against this MFI threshold cutoff for SARS-CoV-2 reactivity. The 127

646   archival ARIC serum samples were tested in technical duplicates in three independent

647   experiments to establish threshold cutoffs and specificity for SARS-CoV-2.

648   *Enzyme-linked immunosorbent assay*

649   Flat bottom 96-well microtiter plates (Corning) were coated with 300 ng of SARS-CoV-2

650   spike protein per well diluted in 100 µl of ELISA coating buffer (1XPBS, 5.3g Na_2CO_3, 4.2g

651    $NaHCO_3$, pH 9.6) and incubated overnight at 4 °C. The next day, spike protein was removed

652   and 125 µl of 5% BSA blocking buffer were added to each well and incubated for 1 hour at 37

653   °C. SARS-CoV-2 spike protein coated and blocked plates were then wasted plate three times

654   with 200 µl PBST. Serum samples were subjected to thermal inactivation after being initially

655   diluted in a BSC. Inactivated serum samples were then serially diluted 2-fold in PBS. One

656   hundred microliters of each dilution was added in duplicate to the antigen coated plate, sealed,

657   and incubated at 37°C for 1 hour. Plates were then washed three times with 200 µl PBST. One

658   hundred microliters of secondary antibody, anti-human (H&L) HRP conjugated, diluted 1:5000 in

659   PBS to each well was added to each well and plates were incubated at 37°C for 1 hour. Plates

660   were then washed three times with 200 µl PBST. Eighty-five microliters ABST Substrate

661   Solution (Thermo Fisher Scientific) was added to each well and plates were agitated (900 rpm)

662   at room temperature for 30 minutes, then analyzed at 650 nm absorbance on a plate reader

663   (Molecular Devices, San Jose, CA).

664   *Statistical analysis*

665   Figures were generated and statistical analyses were performed in GraphPad Prism

666   version 7.0. The positive predictive value and negative predictive value were calculated with

667   MedCalc statistical software. ROC analysis was conducted using R version 4.0.2.

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788 **TABLES**

789 **Table 1. MMIA SARS-CoV-2 spike protein performance**

	SARS-CoV-2 PCR Status/Archival Sera		
		Positive[1]	Negative	Total
SARS-CoV-2 MMIA IgG Antibody Test[2]	Positive	152	0	152
	Negative	3	117	120
	Total	155	117	272
	Sensitivity	98.1%		
	Specificity		100%	
SARS-CoV-2 MMIA IgM Antibody Test[3]	Positive	82	0	82
	Negative	23	117	140
	Total	105	117	222
	Sensitivity	78.1%		
	Specificity		100%	

790 [1]84 archival serum samples and 33 serum samples from PCR negative study enrollees are

791 included as SARS-CoV-2 negative.

792 [2]IgG antibody test included serum samples from n= 155 PCR positive subjects including 82

793 EPICC outpatients, 38 EPICC hospitalized subjects and 35 COVID-19 NYC hospitalized

794 subjects

795 [3]IgM antibody test included serum samples from n= 105 PCR positive subjects including 41

796 EPICC outpatients, 29 EPICC hospitalized subjects and 35 COVID-19 NYC hospitalized

797 subjects

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800

801   **Table 2. MMIA SARS-CoV-2 RBD performance**

		SARS-CoV-2 PCR Status/Archival Sera		
		Positive	Negative	Total
SARS-CoV-2 MMIA IgG Antibody Test	Positive	135	0	135
	Negative	20	117	137
	Total	155	117	266
	Sensitivity	87.1%		
	Specificity		100%	

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803   **Table 3. IgG and IgM seropositivity within 28 days post-symptom onset (dpso)**

dspo	IgG+	IgG+/IgM+
7 – 14	80.0% (12/15)	73.3% (11/15)
15 – 28	100% (31/31)	93.5% (29/31)

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816 **DECLARATIONS**

817 These research protocols, IDCRP-085, IDCRP-045 and COVID-19 NYC, were approved by the

818 USU IRB.

819

820 **CONFLICT OF INTEREST**

821 None of the authors have any conflicts of interest of relevance to disclose.

822

823 **DISCLAIMER**

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825 not necessarily reflect the views, opinions, or policies of the Uniformed Services University

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