1 **Multiplex Detection of Antibody Landscapes to SARS-CoV-2/Influenza/Common Human**

2 **Coronaviruses Following Vaccination or Infection with SARS-CoV-2 and Influenza**

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19   **Running title:** SARS-CoV-2/influenza/H-CoV Ab landscape

1 **ABSTRACT**

2 **Background:** SARS-CoV-2 and influenza viruses continue to co-circulate, representing two major public

3 health threats from respiratory infections with similar clinical presentations. SARS-CoV-2 and influenza

4 vaccines can also now be co-administered. However, data on antibody responses to SARS-CoV-2 and

5 influenza co-infection, and vaccine co-administration remains limited.

6 **Methods:** We developed a 41-plex antibody immunity assay that can simultaneously characterize antibody

7 landscapes to SARS-CoV-2/influenza/common human coronaviruses. We analyzed sera from 840

8 individuals (11-93 years), including sera from reverse transcription polymerase chain reaction (RT-PCR)

9 confirmed SARS-CoV-2 positive (n=218) and negative (n=120) cases, paired sera from SARS-CoV-2

10 vaccination (n=29) and infection (n=11), and paired sera from influenza vaccination (n=56) and RT-PCR

11 confirmed influenza infection (n=158) cases. Lastly, we analyzed sera collected from 377 individual that

12 exhibited acute respiratory illness (ARI) in 2020.

13 **Results:** This 41-plex assay has high sensitivity and specificity in detecting SARS-CoV-2 infections. It

14 differentiated SARS-CoV-2 vaccination (antibody responses only to spike protein) from infection (antibody

15 responses to both spike and nucleoprotein). No cross-reactive antibodies were detected to SARS-CoV-2

16 from influenza vaccination and infection, and vice versa, suggesting no interaction between SARS-CoV-2

17 and influenza antibody responses. However, cross-reactive antibodies were detected between spike proteins

18 of SARS-CoV-2 and common human coronaviruses that were removed by serum adsorption. Among 377

19 individual who exhibited ARI in 2020, 129 were influenza positive, none had serological evidence of SARS-

20 CoV-2/influenza co-infections.

21 **Conclusions:** Multiplex detection of antibody landscapes can provide in-depth analysis of the

22 antibody protective immunity to SARS-CoV-2 in the context of other respiratory viruses including

23 influenza.

24 **Key words:** SARS-CoV-2, influenza, common human coronaviruses, multiplex detection, antibody

25 landscape

26

3

1 INTRODUCTION

2   The current unprecedented coronavirus disease 2019 (COVID-19) global pandemic is caused by

3   severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2) [1] . As of March 2022, more than 450

4   million cases and 6 million deaths have been reported worldwide [2].

5   SARS-CoV-2 viruses belong to betacoronaviruses that also include SARS-CoV-1, common human

6   coronavirus OC43 and HKU1; alphacoronaviruses include common human coronavirus 229E and NL63.

7   The spike protein of SARS-CoV-2 is a homotrimeric class I fusion protein that can be cleaved into two

8   subunits: SARS-CoV-2-S1 contains receptor binding domain (RBD) that binds to angiotensin-converting

9   enzyme 2 (ACE2) [3], SARS-CoV-2-S2 mediates fusion of the viral envelope and host cell membranes [4].

10   Currently, all SARS-CoV-2 vaccines authorized in the United States (US) are based on spike protein targets.

11   Influenza viruses have caused 4 pandemics over the past century. Currently, influenza

12   A(H1N1)pdm09, A(H3N2), and influenza B viruses co-circulate in the human population causing seasonal

13   epidemics. Surface proteins of influenza A and B virus hemagglutinin (HA) and neuraminidase (NA) are

14   major targets of host humoral immune responses. Influenza HA is a homotrimer, expressed as precursor, then

15   further cleaved by host proteinases into globular HA1 (G HA1) and HA2 (HA stalk). Influenza vaccines are

16   evaluated and updated every season due to continuous virus antigenic drift.

17   SARS-CoV-2 and influenza viruses are anticipated to co-circulate in the foreseeable future,

18   representing two major public health threats from respiratory infections with similar clinical presentations.

19   SARS-CoV-2 and influenza co-infections can occur [5]. SARS-CoV-2 vaccines now can also be

20   administered together with influenza vaccines [6]. However, data on antibody immune responses to SARS-

21   CoV-2/influenza co-infection and vaccine co-administration is still limited.

22   Here, we developed a high throughput multiplex influenza/SARS-CoV-2/common human

23   coronavirus antibody detection assay that can measure antibody response landscapes to 40 antigens from

1 influenza, SARS-CoV-2, and 4 common human coronaviruses simultaneously, and analyzed antibody

2 landscape shifts following SARS-CoV-2 or influenza vaccination and infection.

3 **MATERIALS AND METHODS**

4 **Human sera**

5   Sera collected from 840 persons in the US were used in the study (Table 1). For SARS-CoV-2

6 infection and vaccination, archived anonymous sera from reverse transcription polymerase chain reaction

7 (RT-PCR) confirmed SARS-CoV-2 positive (n=218) and negative (n=120) persons were used as reference

8 sera in sensitivity and specificity analysis. In addition, paired sera from 11 SARS-CoV-2 infected persons

9 and 29 SARS-CoV-2 spike protein mRNA vaccine recipients from a prospective cohort [7] were also

10 analyzed (Table 1).

11   For influenza vaccination, pre- (S1) and post- (S2) vaccination sera collected through a CDC

12 contract from adults (19-49 yrs) who received quadrivalent inactivated influenza vaccines (IIV4) in 3

13 influenza seasons: 2016-17 (n=15), 2018-19 (n=21), and 2019-20 (n=20) were analyzed. Sera collected in

14 US Flu VE network from 377 persons exhibiting acute respiratory illness (ARI) during December 2019 to

15 March 2020 [8] and sera from 29 RT-PCR confirmed influenza A(H3N2) infections in 2018-19 season [9]

16 were also analyzed (Table 1).

17   The use of sera was approved by Centers for Disease Control and Prevention (CDC) Human Subjects

18 Research Determination. The study was reviewed by CDC and conducted consistent with applicable federal

19 law and CDC policy [10-14].

20 **Multiplex influenza/SARS-CoV-2/common human coronavirus antibody detection assay (MISHADA).**

21 The 41-plex MISHADA assay contains 40 antigens, including SARS-CoV-2 virus spike protein SARS-

22 CoV-2-S-RBD, SARS-CoV-2-S-Ec (Ectodomain), SARS-CoV-2-S1, SARS-CoV-2-S2, and nucleoprotein

23 (SARS-CoV-2-N); spike proteins from 4 common human coronaviruses (OC43, 229E, NL63 and HKU1);

1 influenza HA Ec, globular HA1 (G), and/or stalk from A(H1N1), A(H2N2), A(H3N2), A(H5N1), A(H7N9),

2 A(H9N2), A(H13N9) and influenza B viruses; NAs, influenza A nucleoprotein (NP), and a protein A control

3 (Table 2) [15-17]. Antigens were coupled to Bio-Plex Pro™ Magnetic COOH beads, incubated with 1:500-

4 diluted human sera, and detected by phycoerythrin-conjugated goat F(ab')2 anti-human pan Immunoglobulin

5 (Pan Ig), IgG, IgA, and IgM reporters [18]. Detailed method is described in the supplementary material.

6 **Enzyme linked immunosorbent assay (ELISA)**

7 ELISA was performed by the method described by Freeman et al, with minor modifications [19].

8 SARS-CoV-2-S-RBD or SARS-CoV-2-S-Ec were coated on microtiter plates, incubated with 2-fold diluted

9 sera, and detected by horseradish peroxidase (HRP)-labeled goat anti-human pan Ig. Detailed method is

10 described in the supplementary material. The ELISA titers were determined as the reciprocal of the highest

11 dilution of serum samples that achieved an optic density (OD) value of ≥ 0.4.

12 **SARS-CoV-2 Surrogate Virus Neutralization Test (sVNT).**

13 sVNT assay was performed following manufacturer's (GenScript USA Inc. NJ) instructions. Sera

14 were analyzed at 1:10 dilutions, incubated with HRP conjugated SARS-CoV-2-S-RBD, the mixture was then

15 transferred to plates coated with the human ACE2 protein, incubated, developed and read at $OD_{450\,nm}$.

16 Percent Inhibition (%) was calculated as: $(1 - OD_{sample}/OD_{negative\ control}) \times 100$.

17 **Serum antibody adsorption**

18 Serum adsorption (Ads) was performed with nickel magnetic beads conjugated with below

19 antigens: mock (human serum albumin, HSA-Ads), SARS-CoV-2-S-RBD (SARS-CoV-2-S-RBD-

20 Ads), a cocktail of 4 common human coronaviruses ectodomain spike proteins (H-CoV-S-Ec-Ads) or

21 a cocktail of 8 influenza HA antigens (Flu-Ads) (Table 2) [20, 21]. Sera were incubated with

22 antigen-coated beads to remove cross-reactive antibodies and analyzed pre- and post-adsorption.

23 Detailed method is described in the supplementary material.

1   **Statistical analysis.**

2   Assay sensitivity and specificity for each antigen target were measured based on Pan Ig antibody

3   responses. Various cut-off values were analyzed, the cutoff values with the highest j-index ([22] were used

4   as the positivity threshold (Supplementary Table 1).

5   Comparison of antibody responses were analyzed using two-tailed *t* tests. Statistical analyses

6   including Pearson correlation were performed using GraphPad Prism 8.

7   **RESULTS**

8   **Sensitivity and specificity of MISHADA assay in detecting SARS-CoV-2 antibodies from RT-PCR-**

9   **confirmed SARS-CoV-2 infections.**

10   We developed a 41-plex MISHADA assay that can simultaneously measure antibody landscapes to

11   SARS-CoV-2, influenza, and common human coronavirus antigens using less than 10μl of sera. The serum

12   dilution at 1:500 is well within the wide dynamic linear range [18, 23] for most antigens for pan Ig, IgG, IgA

13   and IgM antibodies (Supplementary Figure 1), therefore, 1:500 dilution of sera was used in the subsequent

14   analyses. For the same antigens, the median fluorescent intensity (MFI) in 41-plex and 1-plex were very well

15   correlated (Pearson correlation *r* values >0.99) (Supplementary Figure 2). The difference in MFI values

16   detected between 41-plex and 1-plex was less than 20 %.

17   To determine the sensitivity and specificity of MISHADA, ELISA and sVNT assays in detecting

18   antibodies from RT-PCR confirmed SARS-CoV-2 infection, we analyzed convalescent sera collected from

19   218 RT-PCR confirmed SARS-CoV-2 positive patients and baseline sera from 120 RT-PCR confirmed

20   SARS-CoV-2 negative persons (Table 1 and Supplementary Figure 3). In the MISHADA assay, when using

21   both SARS-CoV-2-S-RBD and SARS-CoV-2-S-Ec targets, it has 93.6% sensitivity and 98.3% specificity;

22   when using SARS-CoV-2-N protein alone, it has 93.1% sensitivity and 95.0% specificity, whereas when

1 using all 3 antigen targets (SARS-CoV-2-S-RBD, SARS-CoV-2-S-Ec and SARS-CoV-2-N), it achieved

2 sensitivity of 93.0% and specificity of 99.0% (Table 3).

3   Pearson correlation *r* values between ELISA titers and MFIs were 0.91 for SARS-CoV-2-S-RBD

4 and 0.88 for SARS-CoV-2-S-Ec (Supplementary Figure 4); *r* values between neutralizing antibodies (%

5 inhibition) detected by sVNT and MFI values were 0.67- 0.85 for SARS-CoV-2 antigens (Supplementary

6 Figure 5).

7 **Antibody landscape shifts to SARS-CoV-2/influenza/common human coronavirus antigens following**

8 **SARS-CoV-2 infection and vaccination.**

9   To characterize antibody landscape shifts following SARS-CoV-2 infection or vaccination, we

10 analyzed paired sera from 11 SARS-CoV-2 infected and 29 SARS-CoV-2 mRNA vaccinated persons [7].

11 SARS-CoV-2 vaccinees were either seronegative (SN-Vac group, n=19) or seropositive (SP-Vac, group

12 n=10) prior to vaccination (Table 1). SARS-CoV-2 infection induced antibody responses against all spike

13 antigens (SARS-CoV-2-S-RBD, SARS-CoV-2-S-Ec, SARS-CoV-2-S1, and SARS-CoV-2-S2) and

14 nucleoprotein (SARS-CoV-2-N), whereas SARS-CoV-2 mRNA-based vaccination induced antibody

15 responses only to spike protein, thus SARS-CoV-2-N response can be used to differentiate infection from

16 spike protein-based vaccination (Figure 1). When analyzed by antibody immunoglobin classes, SARS-CoV-

17 2 infection induced pan Ig, IgG, IgA, and IgM responses with high fold-rises to multiple antigens (SARS-

18 CoV-2-S-RBD, SARS-CoV-2-S-Ec, SARS-CoV-2-S1, SARS-CoV-2-S2, and SARS-CoV-2-N) whereas

19 vaccination mainly induced IgG antibodies targeting components of the spike protein (Figure 1 and 2). In

20 both vaccination and infection, IgG antibody responses were the most dominant and constituted the majority

21 of the Pan Ig responses compared to IgM and IgA (Figure 1 and 2). Primary SARS-CoV-2 infection here

22 also induced higher IgM antibody responses than vaccination (Figure 2F). Among the vaccinated groups, SP-

23 Vac group had lower fold-rise than SN-Vac group, likely due to the higher antibodies in pre-vaccination sera

24 (Figure 1 and 2), this was also confirmed by sVNT assay (Supplementary Figure 6).

1   Following SARS-CoV-2 infection and vaccination, antibody rises were also detected to spike

2   proteins of common human coronaviruses OC43 and HKU1 (Figure 2 A-B), suggesting cross-reactive

3   antibody responses with other betacoronaviruses. No cross-reactive antibody responses to spike proteins

4   from alphacoronaviruses (229E and NL63) and influenza antigens were detected (Figure 1-2, Supplementary

5   Figure 7).

6   **Antibody landscape shifts to SARS-CoV-2/influenza/common human coronavirus antigens following**

7   **influenza vaccination.**

8   To characterize antibody landscape shifts following influenza vaccination, we analyzed Pan Ig

9   antibody responses using pre- and post- IIV4 vaccination sera from 3 influenza seasons (2016-17, 2018-19

10   and 2019-20) (Table 1). Vaccination induced the strongest antibody responses to HAs of vaccine strains

11   [A(H1N1), A(H3N2), and influenza Bs]. For A(H1N1), vaccination also back-boosted MFI antibody

12   responses to HAs of all historic viruses tested. Whereas for A(H3N2), vaccination mostly back-boosted

13   antibody responses to HAs from more recent A(H3N2) viruses, but not to older A(H3N2) viruses such as

14   A/Hong Kong/8/1968 (Figure 3 A-C). IIV4 vaccinations also induced antibody responses to N1, N2 and

15   influenza A NP to varying degrees; however, HA stalk antibody responses were only detected to A(H1N1),

16   but not to A(H3N2) (Figure 3). Lastly, influenza vaccination did not induce any cross-reactive antibody rises

17   to SARS-CoV-2 and 4 common human coronavirus antigens (Figure 3 A-C).

18   **Antibody landscape shifts following influenza infection and investigation of serological evidence of**

19   **influenza/SARS-CoV-2 co-infection in persons with acute respiratory illness (ARI).**

20   During December 2019 and March 2020, the US Flu VE network collected sera and nasal swabs

21   from 377 outpatient participants exhibiting ARI [8]. Sera and nasal swabs were collected within 7 days from

22   symptom onset. Nasal swabs were tested for influenza infection by RT-PCR, patients that were RT-PCR

23   positive for influenza also provided convalescent sera (Table 1). Although all participants exhibited

24   respiratory illness, RT-PCR for SARS-CoV-2 was not performed at the time. To investigate whether there

9

1   were SARS-CoV-2/influenza co-infection among these participants with ARI during the early stage of the

2   COVID-19 pandemic in US, we analyzed these sera by the MISHADA assay.

3   Among the 377 participants who exhibited ARI, 44 were positive for influenza B/Victoria lineage

4   viruses, 85 were positive for A(H1N1)pdm09 viruses, 248 were negative for influenza. First, we analyzed

5   the antibody landscape shifts following influenza B and A(H1N1) infection. In influenza B/Victoria lineage

6   virus infected persons (n=44), antibody rises in MFIs were only detected to influenza B virus HAs (Figure

7   4A). In influenza A(H1N1)pdm09 virus infected persons (n=85), antibody rises in MFIs were detected to

8   HAs from all A(H1N1) viruses tested including A/South Carolina/1/1918, as well as N1 and influenza A NP

9   (Figure 4B). We also plotted MFIs from those influenza negative cases (n=248) in the antibody landscapes.

10   Interestingly, antibodies to HAs of B and A(H1N1)pdm09 viruses in influenza negative persons (S1) were

11   significantly higher than those in S1 sera from either influenza B or A(H1N1) infected persons ($p<0.05$,

12   Figure 4A-B), suggesting high HA antibodies may be associated with protection from influenza infections.

13   No cross-reactivity to SARS-CoV-2 or common human coronavirus antigens were detected from influenza

14   A(H1N1) or B infections.

15   Due to the low A(H3N2) activity in 2019-20, none of the participants were positive for influenza

16   A(H3N2), we therefore also analyzed paired sera from A(H3N2) infected adults in 2018-19 influenza season

17   (n=29). A(H3N2) infection induced antibody rises in MFIs to HAs from all A(H3N2) strains between 1968

18   and 2017, N2 and influenza A NP (Figure 4C). Furthermore, Influenza A(H3N2) infection did not induce any

19   cross-reactive antibody rises to any antigens from SARS-CoV-2 and common human coronaviruses.

20   Lastly, to investigate whether there were SARS-CoV-2 infection among the 377 participants with

21   ARI, we identified those that had MFI antibody values to SARS-CoV-2 antigens above the thresholds

22   defined in Table 3. These sera were collected before any SARS-CoV-2 vaccines were available, therefore

23   elevated antibody levels to either SARS-CoV-2 spike protein or nucleoprotein could be indicative of

24   infection. Many participants had high pre-existing antibodies to common human coronavirus spike proteins

1   (Figure 5A). When using SARS-CoV-2 spike antigen targets, 49 participants were positive to either SARS-

2   CoV-2-S-RBD or SARS-CoV-2-S-Ec, 3 were positive to both components of the spike protein (Figure 5B).

3   However, none was positive for both spike and nucleoprotein targets. To further verify the positivity, we

4   then analyzed the 49 sera by both ELISA and sVNT assays (Figure 5C). By ELISA, 10 persons were

5   positive to either SARS-CoV-2-S-RBD (n=1) or SARS-CoV-2-S-Ec (n=9), but none was positive to both.

6   None of the 49 persons was positive in the sVNT assay (Figure 5C).

7   **Cross-reactive antibodies to SARS-CoV-2 spike protein can be removed by serum adsorption with**

8   **spike proteins from common human coronaviruses**

9   To further elucidate the nature of the positive signals detected to SARS-CoV-2 spike proteins, we

10   first performed serum adsorption using cocktails of antigens (Table 2) with ten sera that had positive ELISA

11   titers (Table 5C). Following adsorption with spike proteins from 4 common human coronaviruses (H-CoV-S-

12   Ec-Ads), ELISA titers to SARS-CoV-2-S-Ec and MFIs to both spike protein SARS-CoV-2-S-Ec and SARS-

13   CoV-2-S2 were reduced to baseline levels (Figure 6A-B), suggesting these positives were likely due to cross-

14   reactive responses from past exposures to common human coronaviruses, rather than from SARS-CoV-2

15   infection. Pearson correlation analysis showed MFIs between SARS-CoV-2-S-Ec, SARS-CoV-2-S2 and H-

16   CoV-S-Ec correlated well (r: 0.45-0.80) (Figure 7). As positive controls, we then performed serum

17   adsorption of convalescent sera from 10 SARS-CoV-2 infected participants. High ELISA titers to SARS-

18   CoV-2-S-RBD in these sera were completely removed by adsorption with SARS-CoV-2-S-RBD, but not by

19   the cocktail of 4 spike proteins from common human coronaviruses (H-CoV-S-Ec-Ads) suggesting authentic

20   antibody responses to SARS-CoV-2 infection. Moreover, adsorption with a cocktail of 8 influenza HA

21   proteins (Flu-Ads) only reduced MFIs to influenza antigens (Figure 6D), but not to any SARS-CoV-2

22   antigens (Figure 6C-D), confirming no cross-reactivity between influenza and SARS-CoV-2 virus antigens.

23   **DISCUSSION**

This 41-plex MISHADA assay is a powerful tool that can provide in-depth analysis of antibody responses to multiple antigens that contribute to the protective immunity of SARS-CoV-2 and influenza. The assay can identify SARS-CoV-2, influenza, common human coronavirus infections and co-infections, and differentiate infection from vaccination.

Humans have complex immunity to influenza. The antibody immune profile of an individual is often shaped by the initial priming to influenza viruses in childhood, and subsequent exposure to influenza through vaccination and infection later in life. The back boost effect to antigenic-related viruses from the current influenza vaccination/infection is evident in the antibody landscape analysis. For influenza, the antibody landscape of an individual can impact one's susceptibility to infection, and immune response to vaccination [24, 25]. Compared to influenza, SARS-CoV-2 viruses were able to spread rapidly across the globe and caused an unprecedented global pandemic, in part, due to naïve population immunity. The seropositivity against SARS-CoV-2 was low (1.0%-6.9%) among the US population in early 2020 during the early stage of the pandemic [26]. Since then, more than 450 million COVID-19 cases have been confirmed globally [2] including 79 million cases in the US [27], massive vaccination campaigns were carried out in many countries. With increased vaccination/infection to SARS-CoV-2 in the population, repeated exposures to the emerging variants (e.g. Delta and Omicron), and the potential future new vaccine formulations, antibody profiles in the population to SARS-CoV-2 are becoming more complex [28]. Similar to influenza, antibody landscapes tailored to SARS-CoV-2 antigens may be needed to anticipate population susceptibility to emerging SARS-CoV-2 variants and to inform future vaccination strategies.

Using the antibody landscape analysis and serum adsorption, we demonstrated the presence of pre-existing, cross-reactive antibodies between the spike proteins of SARS-CoV-2 and common human coronaviruses. Vaccination and infection with SARS-CoV-2 also induced antibody rises to spike proteins from common human coronaviruses, mostly from betacoronaviruses (OC43 and HKU1), which are more closely related to SARS-CoV-2 than alphacoronaviruses (NL63 and 229E) [29]. Others also reported cross-reactive antibodies against spike proteins of common human coronaviruses following SARS-CoV-2 infection [30-33], which could be due to immunological imprinting, or shared epitopes. It was hypothesized that high

12

levels of antibodies against common human coronaviruses in children may have contributed to the mild symptoms often observed in this age group [34, 35]. Nonetheless, the protective potential of cross-reactive antibodies against SARS-CoV-2 infection is not well understood [36, 37]. Moreover, cross-reactive antibodies will also complicate the interpretation of serologic results, thus multiple antigen detection may be necessary to fully assess the antibody immunity to SARS-CoV-2 virus.

SARS-CoV-2 and influenza viruses continue to co-circulate, posing a threat to public health. Studies have reported that co-infection with influenza may enhance the SAS-CoV-2 infectivity and disease severity [38] [39, 40]. Among the participants who exhibited respiratory illness during the early stage of the pandemic, we did not identify SARS-CoV-2/influenza co-infections. In participants vaccinated or infected with SARS-CoV-2 or influenza, our analysis indicated that there were no interactions or cross-reactivity of antibody responses between these two respiratory viruses.

Our study has limitations: first, we were not able to obtain SARS-CoV-2/influenza coinfection sera in the current analysis, in part, due to the low influenza circulation since the onset of the COVID-19 pandemic; further studies are warranted. Second, given the timeframe of the sera collection, we only included SARS-CoV-2 antigens from Wuhan-Hu-1 virus. Additional antigens from Delta, Omicron and future emerging variants, can be included in the further analysis of SARS-CoV-2 antibody landscapes.

In summary, the multiplex detection of antibody landscapes against SARS-CoV-2/influenza/common human coronaviruses is a high throughput tool to investigate the antibody responses to these respiratory pathogens. Our results demonstrated no cross-reactivity between influenza and SARS-CoV-2 antibodies following infection and vaccination by either virus, providing scientific evidence to support the co-administration of SARS-CoV-2 and influenza vaccination [6]. As the COVID-19 pandemic is progressing through yet another flu season, it is important to gain better understanding of the humoral protective immunity to SARS-CoV-2 in the context of other respiratory illness, especially influenza and common human coronaviruses, to identify the most effective public health strategies for the control and prevention of these respiratory pathogens.

1 **NOTES**

9 **Disclaimer:**

10 The findings and conclusions in this report are those of the authors and do not necessarily represent the

11 official positions of CDC.

12 **Conflict of interest statement**

13 EM reports funding from the Centers for Disease Control and Prevention and Merck. JPK reports funding

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Table 1 Human sera collected in the Unites States from SARS-CoV-2 and influenza negative persons, SARS-CoV-2 vaccination or infections, influenza vaccination or infections used in the study.

Sera panels with known SARS-CoV-2 or influenza status			Numbers of persons	Age range (median)	Timing of the collection	S1 (days post ARI or PCR) - range (median)	S2 (days post ARI or PCR) - range (median)	Days between S1 and S2 - range (median)	S2 (days post influenza vaccination - range (median)	S2 (days post 2nd dose COVID-19 vaccination) - range (median)	Paired sera	Total serum sample number
Reference sera panels with RT-PCR confirmed SARS-CoV-2 status for sensitivity and specificity analysis		SARS-CoV-2 RT-PCR positive	218	3-89 (47)[a]	Mar 2020-Aug 2020[b]	N/A	6-71 (24)[c]	N/A	N/A	N/A[d]	N	218
		SARS-CoV-2 RT-PCR negative	120	19-76 (48)	Oct 2018-Sep 2020	N/A	N/A	N/A	N/A	N/A	N	120
SARS-CoV-2	Infection (RT-PCR positive)	SARS-CoV-2 infected persons	11	N/A	Aug 2020-Mar 2021	-148-0 (-53)*	20-52 (37)	42-196 (85)	N/A	N/A	Y	22
	Vaccination	SARS-CoV-2 seronegative before vaccination (SN-Vac)	19	N/A	Oct 2020-Mar 2021	N/A	N/A	39-128 (79)	N/A	11-44 (19)	Y	38
		SARS-CoV-2 seropositive before vaccination (SP-Vac)	10	N/A	Nov 2020-Mar 2021	N/A	N/A	18-115 (65)	N/A	7-33 (22)	Y	20
Influenza	Vaccination	IIV4[e] recipients 2016-17	15	18-47 (35)	Aug 2016-Oct 2016	N/A	N/A	N/A	21-21 (21)	N/A	Y	30
		IIV4 recipients 2018-19	21	18-47 (25)	Sep 2018 -Oct 2018	N/A	N/A	N/A	21-24 (21)	N/A	Y	42
		IIV4 recipients 2019-20	20	22-48 (31)	Oct 2019 -Nov 2019	N/A	N/A	N/A	21-26 (22)	N/A	Y	40
	Infection (RT-PCR positive)	Acute/convalescent 2018-19 A(H3N2) infection	29	19-85 (58)	Feb 2019-May 2019	0-7 (3)	21-49 (26)	16-42 (22)	N/A	N/A	Y	58
		Acute/convalescent 2019-20 A(H1N1) infection	85	11-80 (49)	Dec 2019-Mar 2020	0-7 (2)	19-45 (24)	17-43 (21)	N/A	N/A	Y	170
		influenza B infection	44	19-80 (33)	Dec 2019-Mar 2020	1-7 (3)	21-55 (27)	18-53 (25)	N/A	N/A	Y	88
	Negative (RT-PCR negative)	2019-20 Influenza negative	248	16-93 (48)	Dec 2019-Mar 2020	0-7 (4)	N/A	N/A	N/A	N/A	N	248
Total number of persons			840									1094

a Age range (median) was calculated based on available information (n=207).
b Sample collection date range.
c Days post ARI or RT-PCR days are expressed as range (median) calculated based on available information (n=155).
d Sera were collected before SARS-CoV-2 vaccines became available
e IIV4: inactivated quadrivalent influenza vaccination.
*: sera were collected prior to SARS-CoV-2 RT-PCR from a prospective cohort [7].
N/A: not applicable or not available.

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Table 2 Antigens from SARS-CoV-2 virus, influenza viruses and common human coronaviruses included in the 41-plex MISHADA assay.

										41-plex		Adsorption
Viruses	#	Antigen	Virus strain	Type (subtype or lineage)	Influenza HA group	Egg or Cell-origin	Ecto HA/G HA1/HA stalk[2] (influenza)	Resource	GISAID/GenBank Accession No.	S-CoV-2-S-RBD-Ads	H-CoV-S-Ec Ads	Flu-Ads
SARS-CoV-2	1	SARS-CoV-2-S-RBD (spike receptor binding domain)	Wuhan-Hu-1	Betacoronavirus	N/A[1]	N/A	N/A	Sino Biological US Inc	YP_009724390.1	S-CoV-2-S-RBD		
	2	SARS-CoV-2-S-Ec (spike ectodomain)	Wuhan-Hu-1	Betacoronavirus	N/A	N/A	N/A	Sino Biological US Inc	YP_009724390.1			
	3	SARS-CoV-2-S1 (spike S1)	Wuhan-Hu-1	Betacoronavirus	N/A	N/A	N/A	Sino Biological US Inc	YP_009724390.1			
	4	SARS-CoV-2-S2 (spike S2)	Wuhan-Hu-1	Betacoronavirus	N/A	N/A	N/A	Sino Biological US Inc	YP_009724390.1			
	5	SARS-CoV-2-N (nucleoprotein)	Wuhan-Hu-1	Betacoronavirus	N/A	N/A	N/A	Sino Biological US Inc	YP_009724390.1			
Human coronaviruses	6	H-CoV-OC43-S-Ec (spike ectodomain)	Human coronavirus OC43	Betacoronavirus	N/A	N/A	N/A	Sino Biological US Inc	AVR40344.1		H-CoV-OC43-S-Ec	
	7	H-CoV-229E-S-Ec (spike ectodomain)	Human coronavirus 229E	Alphacoronavirus	N/A	N/A	N/A	Sino Biological US Inc	APT69883.1		H-CoV-229E-S-Ec	
	8	H-CoV-NL63-S-Ec (spike ectodomain)	Human coronavirus NL63	Alphacoronavirus	N/A	N/A	N/A	Sino Biological US Inc	APF29071.1		H-CoV-NL63-S-Ec	
	9	H-CoV-HKU1-S-Ec (spike ectodomain)	Human coronavirus HKU1	Betacoronavirus	N/A	N/A	N/A	Sino Biological US Inc	Q0ZME7.1		H-CoV-HKU1-S-	
Influenza viruses	10	H1.SC.18 Ec	A/South Carolina/1/18	A(H1N1)	1	lung biopsy	Ecto HA	CDC	EPI5571			
	11	H1.USS.77 Ec	A/USSR/90/77	A(H1N1)	1	Egg	Ecto HA	CDC	EPI390455			
	12	H1.TW.86 Ec	A/Taiwan/01/86	A(H1N1)	1	Cell	Ecto HA	CDC	EPI318034			
	13	H1.NC.99 Ec	A/New Caledonia/20/99	A(H1N1)	1	Unknown	Ecto HA	CDC	EPI18473			
	14	H1.CA.09 G	A/California/7/2009	A(H1N1)	1	Cell	G HA1	CDC	EPI177294			
	15	H1.MI.15 G	A/Michigan/45/2015	A(H1N1)	1	Egg	G HA1	CDC	EPI685579			H1.MI.15 Ec
	17	H1.BR.18 Ec	A/Brisbane/02/2018	A(H1N1)	1	Cell	Ecto HA	CDC	EPI1212884			H1.BR.18 Ec
	18	H1.HI.19 Ec	A/Hawaii/70/2019	A(H1N1)	1	Cell	Ecto HA	CDC	EPI1617983			H1.HI.19 Ec
	19	H3.HK.68 Ec	A/Hong Kong/8/68	A(H3N2)	2	Unknown	Ecto HA	CDC	EPI240947			
	20	H3.BK.79 Ec	A/Bangkok/1/79	A(H3N2)	2	Egg	Ecto HA	CDC	EPI377537			
	21	H3.BJ.92 G	A/Beijing/32/92	A(H3N2)	2	Cell	Ecto HA	CDC	EPI365898			
	22	H3.Pan.99 Ec	A/Panama/2007/99	A(H3N2)	2	Unknown	Ecto HA	CDC	EPI105036			
	23	H3.Per.09 G	A/Perth/16/2009	A(H3N2)	2	Cell	G HA1	IRR	EPI182941			
	24	H3.Tx.12 G	A/Texas/50/2012	A(H3N2)	2	Cell	Ecto HA	CDC	EPI398417			
	25	H3.MD.14 G[3]	A/Maryland/26/2014	A(H3N2)	2	Cell	G HA1	CDC	EPI550983			
	26	H3.Sin.16 G	A/Singapore/INFIMH-	A(H3N2)	2	Cell	G HA1	CDC	EPI780183			H3.Sin.16 Ec
	28	H3.KS.17 Ec	A/Kansas/14/2017	A(H3N2)	2	Cell	Ecto HA	CDC	EPI1146345			H3.KS.17 Ec
	29	H3.KS.17 G	A/Kansas/14/2017	A(H3N2)	2	Cell	G HA1	CDC	EPI1146345			
												H3.HK.19 Ec[3]
	30	B-V-WA.19 G	B/Washington/02/2019	Victoria lineage	N/A	Clinical	G HA1	CDC	EPI1368874			B-V-WA.19 G
	31	B-Y-Phu.13 G	B/Phuket/3073/2013	Yamagata lineage	N/A	Cell	G HA1	CDC	EPI529345			B-Y-Phu.13 G
	16	H1. HA Stalk	A/Michigan/45/2015	A(H1N1)	1	Egg	HA Stalk	CDC	EPI685579			
	27	H3. HA Stalk	A/Singapore/INFIMH-	A(H3N2)	2	Cell	HA Stalk	CDC	EPI780183			
	32	N1.CA.09 (neuraminidase)	A/California/7/2009	A(H1N1)	N/A	Cell	Ecto HA	CDC	EPI221062			
	33	N2.NA.HK.14 (neuraminidase)	A/Hong Kong/4801/2014	A(H3N2)	N/A	Cell	Ecto HA	CDC	EPI539577			
	34	N9.NA.SH.13 (neuraminidase)	A/Shanghai/2/2013	A(H7N9)	N/A	Egg	Ecto HA	CDC	EPI439500			
	35	NP (nucleoprotein)	A/Brisbane/10/2007	A(H3N2)	N/A	Cell	N/A	IRR	EPI353307			
	36	H2.Jap.57 G	A/Japan/305/57	A(H2N2)	1	Unknown	G HA1	IRR	EPI128485			
	37	H5.Ind.05 G	A/Indonesia/05/2005	A(H5N1)	1	Egg	G HA1	CDC	EPI376537			
	38	H7.SH.13 G	A/Shanghai/2/2013	A(H7N9)	2	Egg	G HA1	CDC	EPI439502			
	39	H9.HK.09 G	A/Hong Kong/33982/2009	A(H9N2)	1	Cell	G HA1	IRR	EPI470900			
	40	H13.DE.04 G	A/shorebird/DE/68/2004	A(H13N9)	1	Egg	G HA1	IRR	EPI744939			
	41	PA	Protein A	N/A	N/A	N/A	N/A	Fisher Scientific	N/A			

1. N/A not applicable.
2. Ecto HA (Ec): ectodomain influenza hemagglutinin, G HA1: globular influenza hemagglutinin HA1.
3. H3.HK.19 Ec: A(H3N2) A/Hong Kong/45/2019 HA ectodomain

1 **Table 3 Sensitivity and specificity of MISHADA, ELISA, and sVNT assays in detecting SARS-CoV-2 antibodies from RT-PCR confirmed SARS-**

2 **CoV-2 infection**

Assay	Antigen	Cutoff threshold[a]	Sensitivity %	Specificity %	j-index
MISHADA (Pan Ig)	S-CoV-2-S-RBD	2000	94.5	95	0.895
	S-CoV-2-S-Ec	1500	94.5	98.3	0.928
	S-CoV-2-S-RBD and S-CoV2-S-Ec	2000 and 1500	93.6	98.3	0.919
	S-CoV-2-N	1000	93.1	95	0.881
	S-CoV-2-S-RBD and S-CoV-2-S-Ec and S-CoV-2-N	2000 (RBD) + 1500 (Ec)+1000 (N)	93.0	99.0	0.920
ELISA (Pan Ig)	S-CoV-2-S-RBD	50	97.2	99.2	0.964
	S-CoV-2-S-Ec	100	95	97.5	0.925
	S-CoV-2-S-RBD and S-CoV-2-S-Ec	50 (RBD) and 100 (Ec)	94.5	99.2	0.937
sVNT	N/A	20%	91.3	98.3	0.896

4 [a] Cutoff MFI value, ELISA titer and inhibition (%) that achieved the highest j-index in MISHADA, ELISA and sVNT, respectively

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FIGURE LEGENDS

Figure 1. Antibody landscape shifts following SARS-CoV-2 infection and vaccination. Baseline (S1) and convalescent (S2) serum samples collected from SARS-CoV-2 infected persons (Inf, n=11); pre (S1) and post (S2) vaccination sera from SARS-CoV-2 vaccine recipients that were seronegative prior to vaccination (SN-Vac, n=19), and SARS-CoV-2 vaccine recipients that were seropositive prior to vaccination (SP-Vac, n=10) were tested in MISHADA. **A:** pan Ig antibody; **B:** IgG, **C:** IgA, and **D:** IgM antibody responses. Mean MFIs and 95% confidence interval are shown.

Figure 2. Antibody MFI and fold rise to SARS-CoV-2 and H-CoV following SARS-CoV-2 infection and vaccination. Baseline (S1) and convalescent (S2) sera from SARS-CoV-2 infected persons (Inf, n=11); pre (S1) and post (S2) vaccination sera from SARS-CoV-2 vaccine recipients who were seronegative prior to vaccination (SN-Vac, n=19), and SARS-CoV-2 vaccine recipients who were seropositive prior to vaccination (SP-Vac, n=10) were tested in MISHADA. **A:** MFI of Pan Ig antibody to SARS-CoV-2; **B:** MFI of Pan Ig antibody to H-CoV-S-Ec; **C:** Fold rise of pan Ig antibody to SARS-CoV-2 and H-CoV-S-Ec; **D**: Fold rise of IgG antibody to SARS-CoV-2 and H-CoV-S-Ec; **E** Fold rise of IgA antibody to SARS-CoV-2 and H-CoV-S-Ec; **F**: Fold rise of IgM antibody to SARS-CoV-2 and H-CoV-S-Ec.

Figure 3. Antibody landscape shifts following inactivated quadrivalent influenza vaccination (IIV4). Pre (S1) and post (S2) vaccination sera from IIV4 recipients in 3 influenza seasons during 2016-2020 were tested for pan Ig antibody responses in MISHADA. **A.** 2016-2017 (n=15); **B.** 2018-2019 (n=21); **C.** 2019-2020 (n=20). Antigens representing influenza IIV4 vaccine components in each season are indicated by arrows. **D.** The comparison of MFIs between S1 and S2 for representative antigens each season; H1, H3 and B HAs are the corresponding HAs in the vaccine strain for respective seasons. Mean MFIs and 95% confidence interval are shown.

Figure 4. Antibody landscape shifts following influenza natural infections. Serum panels included 248 acute (S1) sera from the persons that were RT-PCR negative for influenza; acute (S1) and convalescent (S2) sera from 44 RT-PCR confirmed influenza B or 85 A(H1N1) virus infected persons from December 2019 to April 2020; and 29 paired sera from RT-PCR confirmed influenza A(H3N2) virus infected persons from 2018-19 influenza season. All samples were tested for pan Ig antibody responses in MISHADA. **A.** influenza B (n=44); **B.** A(H1N1) (n=85); **C.** A(H3N2) (n=29). Arrow indicates HA of infected virus strain or the most closely related HA to the infected strain. **D.** MFIs between S1 and S2 for representative antigens. Mean MFIs and 95% confidence interval are shown. *: $p<0.05$ when comparing antibody MFIs to B/Victoria lineage antigen in the influenza negative persons vs influenza B infected persons in (A); and when comparing antibody MFIs to H1.HI.19.Ec (representing HA of infecting virus) in influenza negative persons versus A(H1N1) infected persons in (B).

Figure 5. Antibodies to SARS-CoV-2 in persons exhibiting acute respiratory illness (ARI) during December 2019 – March 2020. Sera were collected from 377 persons with ARI, including 248 persons who were RT-PCR negative for influenza (S1) and 44 RT-PCR confirmed influenza B (S2) or 85 A(H1N1) (S2) virus infected persons from December 2019 to March 2020. All sera were tested for pan Ig antibody responses in MISHADA. **A.** Scatter plots of Pan Ig antibodies to SARS-CoV-2 and H-CoV antigens in sera from all 377 persons with ARI. **B.** Positivity in MISHADA as determined by the cutoff values in Table 3. **C.** Positivity in ELISA and sVNT assays: sera from 49 persons that were positive for SARS-CoV-2-S-RBD and/or SARS-CoV-2-S-Ec in pan Ig MISHADA were tested by ELISA and sVNT. Positivity was determined by the cutoff values in Table 3.

Figure 6. Cross-reactive pan Ig antibodies against SARS-CoV-S-Ec and SARS-CoV-S2 were removed by serum adsorption with spike proteins from 4 common human coronaviruses. Sera from 10 persons exhibiting ARI with positive MISHADA MFI and ELISA titers to SARS-CoV-2-S-RBD or Ec were adsorbed under 4 conditions: not treated (control), mock-Ads (control), SARS-CoV-2-S-RBD-Ads or H-CoV-S-Ads (adsorbed with a cocktail of spike proteins from 4 common human coronaviruses), then tested by ELISA (**A**) and MISHADA(**B**). Convalescent sera from 10 RT-PCR confirmed COVID-19 patients were adsorbed under 5 conditions: not treated (control), mock-Ads (control), SARS-CoV-2-S-RBD-Ads, H-CoV-S-Ec-Ads, or Flu-Ads (adsorbed with a cocktail of 8 HAs from influenza A and B viruses), then tested by ELISA in (**C**) and MISHADA in (**D**).

Figure 7. Correlations between antibody MFI responses to SARS-CoV-2 and 4 human common coronavirus virus antigens. Total 506 serum samples including 248 acute only from influenza RT-PCR negative persons, acute and convalescent sera collected from 44 RT-PCR confirmed influenza B or 85 A(H1N1) virus infected persons from December 2019 to April 2020 were tested for pan Ig responses in MISHADA. Pearson correlation coefficient *r* values between antibody MFI responses to SARS-CoV-2-S-RBD, SARS-CoV-2-S-Ec, SARS-CoV-2-S1, SARS-CoV-2-S2 and antibody MFIs to spike proteins from H-CoV-OC43-S-Ec, H-CoV-229E-S-Ec, H-CoV-NL63-S-Ec, H-CoV-HKU1-S-Ec were analyzed.
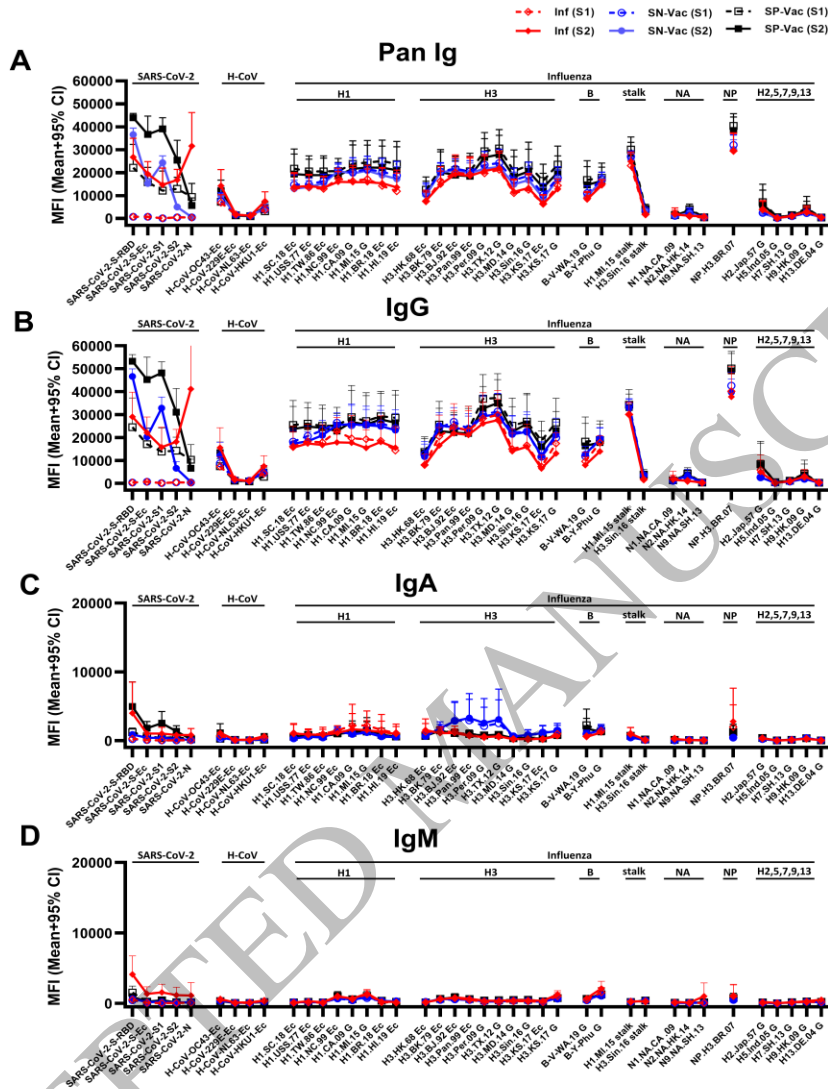
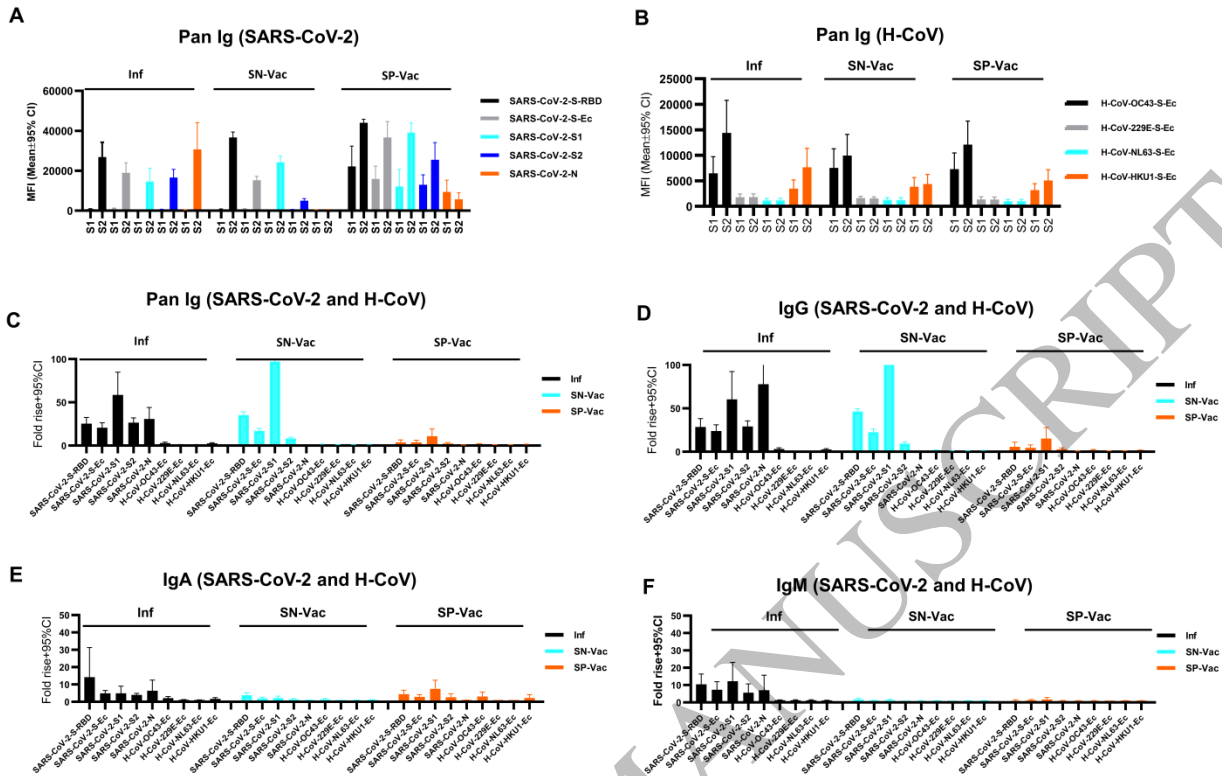21

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Figure 1
124x165 mm (.42 x DPI)
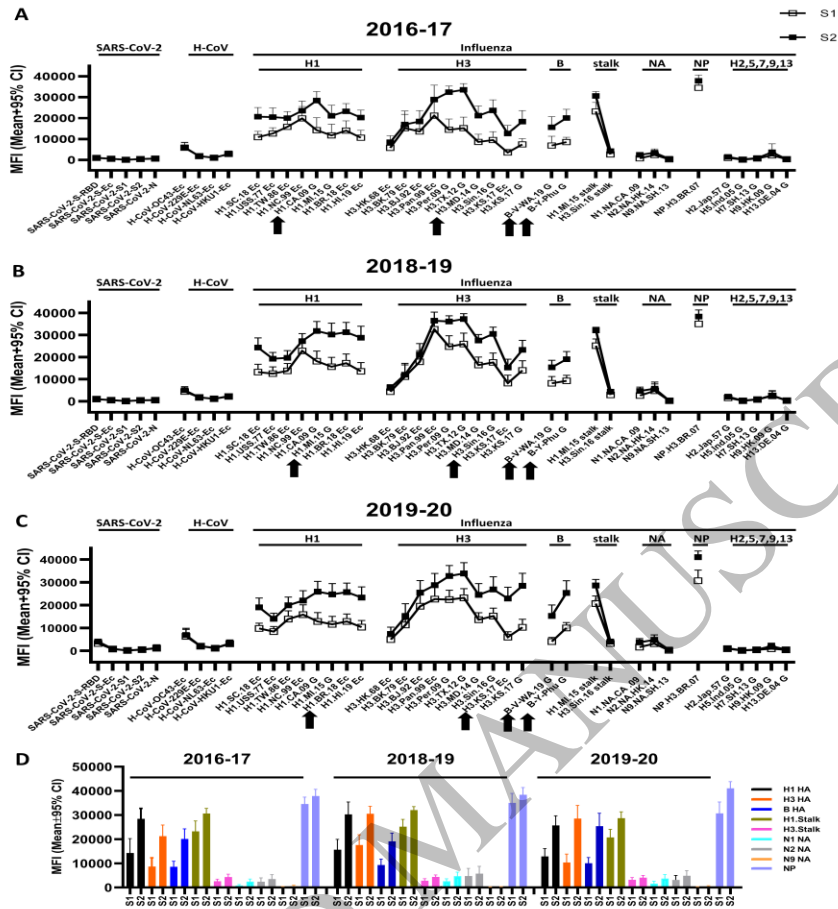
Figure 2
229x148 mm (.42 x DPI)

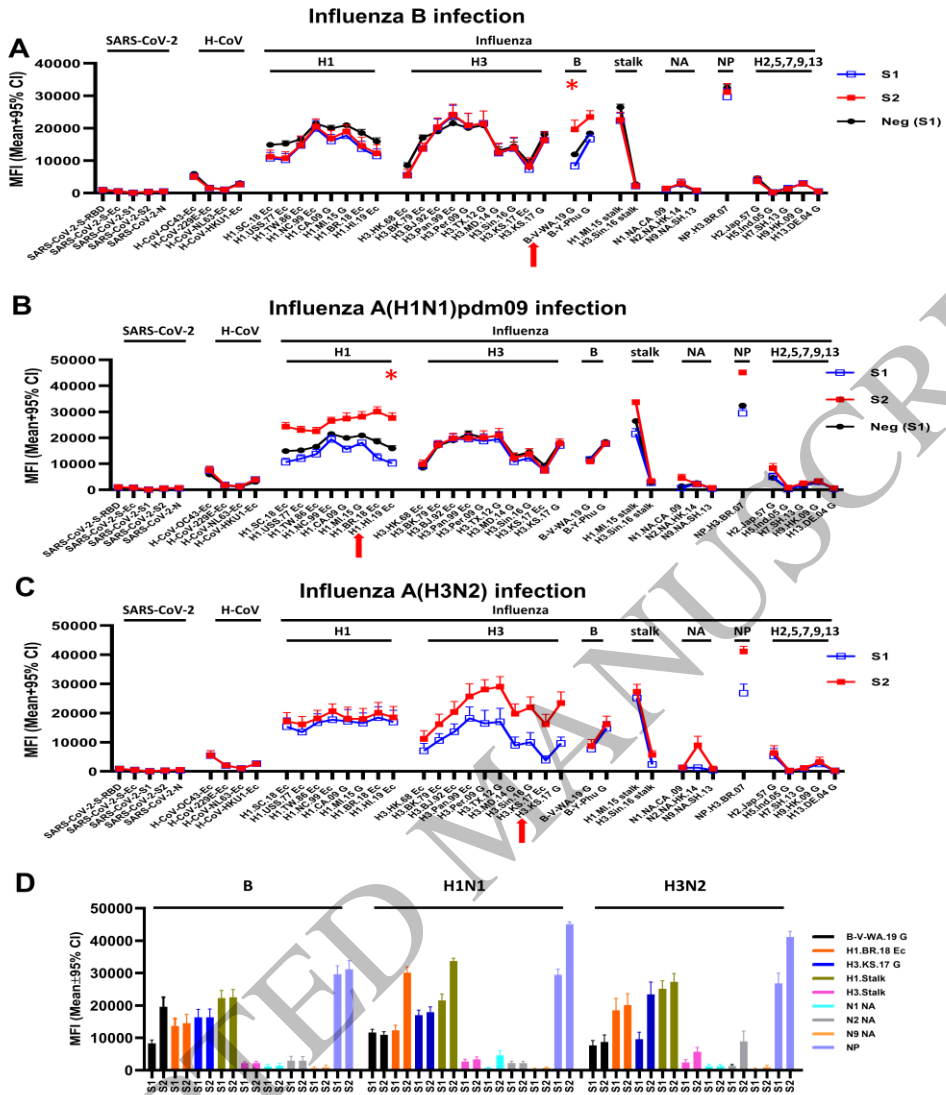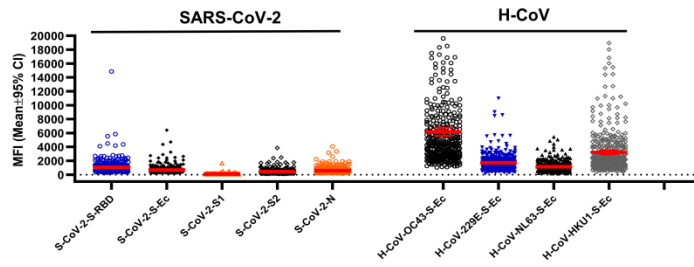**Figure 3**
130x165 mm (.42 x DPI)

Figure 4
129x165 mm (.42 x DPI)

A

B. Positivity in MISHADA

Assay	Total tested N	Targets	Total # positive N (%)	Antigens used to determine positivity	Positivity threshold	#Positivity N (%)	Positive for influenza
MISHADA	377	S	49 (12.9%)	SARS-CoV-2-S-RBD	2000 (RBD)	32 (8.5%)	9/32
				SARS-CoV-2-S-Ec	1500 (Ec)	20 (5.3%)	8/20
				SARS-CoV-2-S-RBD and SARS-CoV-2-S-Ec	2000 (RBD) and 1500 (Ec)	3 (0.8%)	1/3
		N	39 (10.3%)	SARS-CoV-2 N	1000 (N)	39 (10.3%)	19/39
		S and N	0 (0%)	SARS-CoV-2-S-RBD and SARS-CoV-2-S-Ec and SARS-CoV-2-N	2000 (RBD)+1500 (Ec)+1000 (N)	0 (0%)	0

C. Positivity in ELISA and sVNT assays

Confirmative Assay	Total tested N	Antigen	Positivity threshold	Positivity N (%)
ELISA	49	S-CoV-2-S-RBD	50	1 (2.0%)
		S-CoV-2-S-Ec	100	9 (18.4%)
		S-CoV-2-S-RBD and S-CoV-2-S-Ec	50 (RBD) and 100 (Ec)	0 (0%)
sVNT	49	NA	20% inhibition	0 (0%)

1
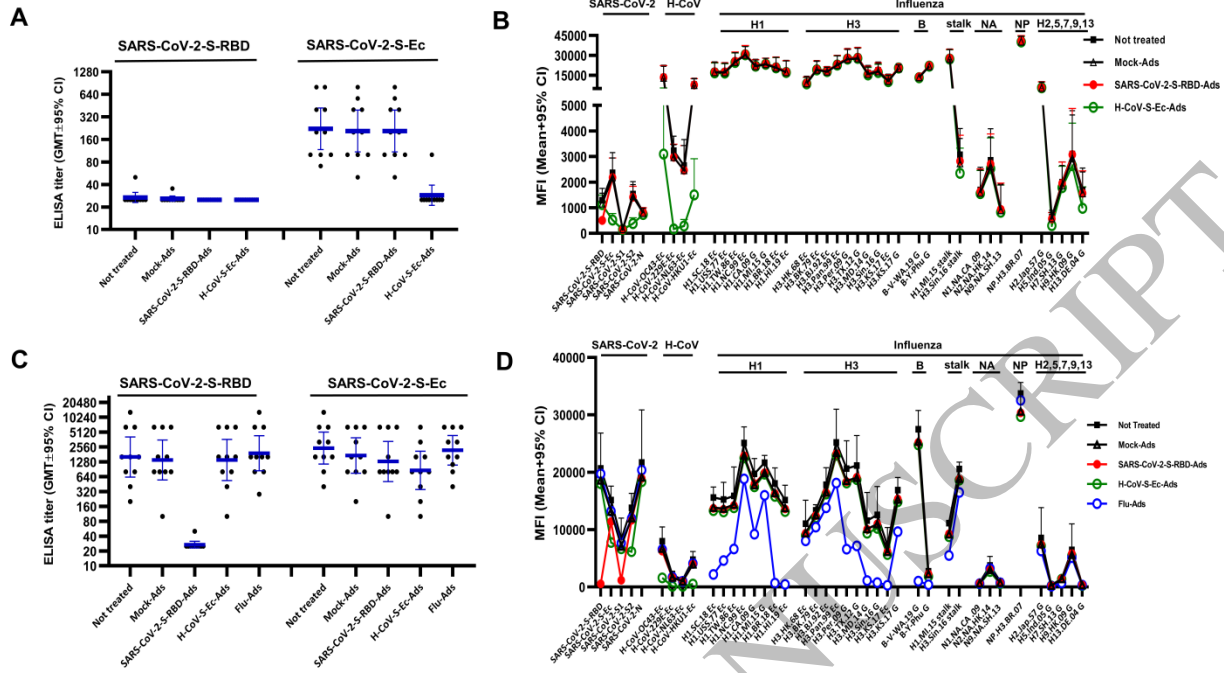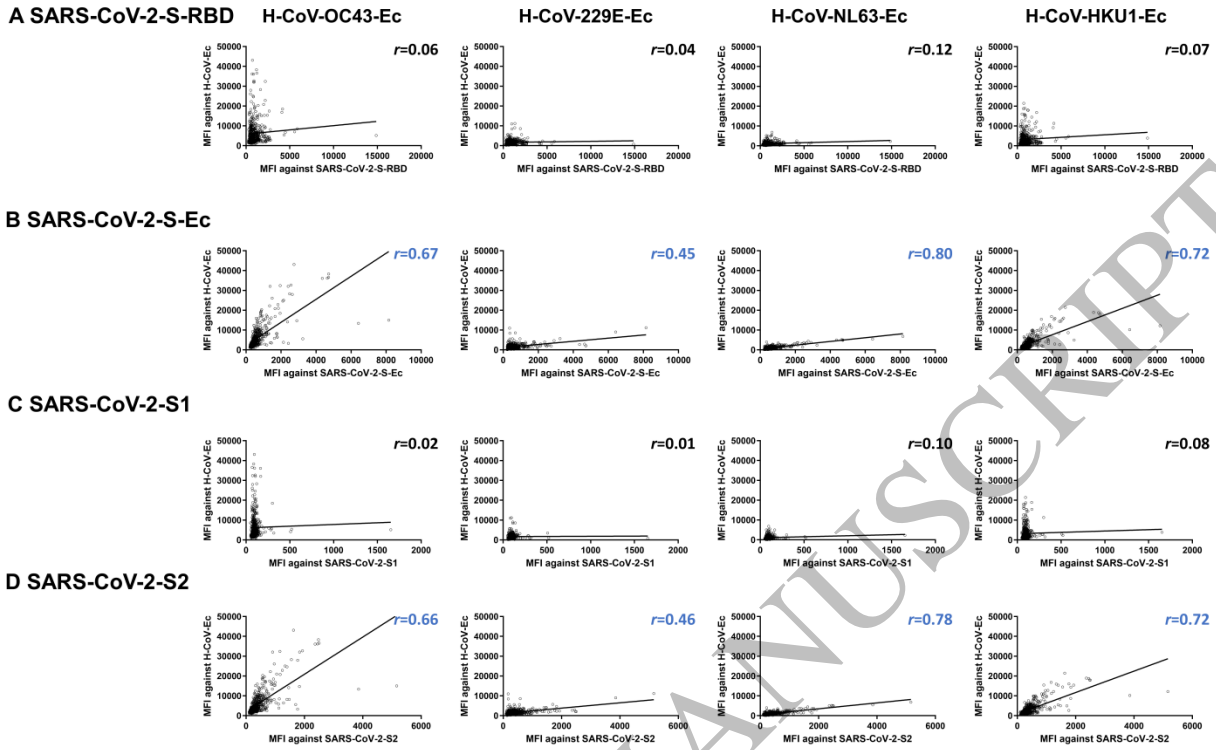
Figure 5
229x135 mm (.42 x DPI)

4

Figure 6
229x129 mm (.42 x DPI)

Figure 7
229x144 mm (.42 x DPI)