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Serological testing reveals the hidden COVID-19 burden among healthcare workers experiencing a SARS-CoV-2 nosocomial outbreak.

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30

Abstract—word count 148

We describe the results of testing healthcare workers from a tertiary care hospital in Japan, which had experienced a COVID-19 outbreak during the first peak of the pandemic, for SARS-CoV-2 specific antibody seroconversion. Using two chemiluminescent immunoassays and a confirmatory surrogate virus neutralization test, serological testing unveiled that a surprising 42.2% (27/64) of overlooked COVID-19 diagnoses had occurred when case detection had relied solely on SARS-CoV-2 nucleic acid amplification testing. This undetected portion of the COVID-19 iceberg beneath the surface may potentially have led to silent transmissions and triggered the spread. A questionnaire-based risk assessment was further indicative of exposures to specific aerosol-generating procedures, i.e. non-invasive ventilation, having had conveyed the highest transmission risks and served as the origin of outbreak. Our observations are supportive of a multi-tiered testing approach, including the use of serological diagnostics, in order to accomplish exhaustive case detection along the whole COVID-19 spectrum.

Text—word count 2695

Introduction

When the COVID-19 pandemic landed in January 2020, Japan was no exception to the rest of the world, where access to diagnostic testing was limited. Shortages in testing resources during the first wave of the pandemic in spring 2020 had compromised timely case detection and forced healthcare workers (HCWs) to work in a deep diagnostic fog. The situation caused frontline healthcare facilities to suffer unexpected SARS-CoV-2 exposures followed by nosocomial outbreaks. However, even after a profound increase in molecular testing capacity and an apparent clearance of the fog, the SARS-CoV-2 virus continued to sneak through the shield of symptom-driven screening strategies (*1*). Infections free of symptoms, i.e. pre-symptomatic or asymptomatic infections, and thus left untested were hypothesized to constitute a major burden and contribute to transmission (*2*).

In support of the hypothesis, reports from later active screening studies have revealed a significant majority of SARS-CoV-2 infections to manifest atypical non-respiratory presentations, or even at times remain asymptomatic (*3*). Such minimally symptomatic individuals, never to be suspected of COVID-19, lack the opportunity to undergo SARS-CoV-2 nucleic acid amplification testing (NAT), and together with those

64 false-negative for NAT, continue to carry the risk of becoming a source of transmission.
65 COVID-19, being an unprecedentedly heterogenous pathology, constitute a spectrum of
66 disease resembling an "iceberg". Behind the most severe, devastating pneumonia patients
67 lies the large majority that are only mildly symptomatic or even remain asymptomatic (*4*).
68 Thus, NAT alone prone to overlooking the hidden burden, multi-tiered testing with the
69 use of variable diagnostic modalities shall aid in exhaustive case detection along the
70 whole spectrum.

71 The incidence as well as the origin of pauci-symptomatic or asymptomatic
72 individuals forming a significant portion of the COVID-19 iceberg remain to be fully
73 elucidated. In this study, 414 HCWs of a tertiary care hospital in Japan were tested for
74 SARS-CoV-2 specific antibody seroconversion after facing an outbreak during the first
75 wave of the pandemic in April-May 2020. The now-unveiled, overall perspective of the
76 COVID-19 iceberg highlights the shocking underestimation of true disease burden, and
77 holds an important lesson to be learned in minimizing nosocomial spreads and further
78 enhancing preparedness against future pandemics.

79

80 **Materials and Methods**

81 *Cohort and samples*

A total 414 HCWs of St. Marianna University School of Medicine, Yokohama City Seibu Hospital, Kanagawa, Japan, who gave consent to participating in the study were recruited. Sera were obtained from the entire cohort within three consecutive days, from June 30th to July 2nd 2020, when approximately two months had passed since experiencing the nosocomial outbreak during April-May 2020. Amongst the individuals with a known date of COVID-19 diagnosis, the interval between the date of diagnosis and the date of serum sampling ranged from 6–10 weeks.

Molecular testing

NAT for SARS-CoV-2 detection was performed using nasal swabs based on the RT-PCR protocol developed by the National Institute of Infectious Diseases, Japan. The method targets two sites of the nucleocapsid gene (*5*).

Serological testing

Two chemiluminescent immunoassays, the Abbott SARS-CoV-2 IgG and SARS-CoV-2 IgG II Quant (Abbott, Illinois, USA), designed to detect serum IgG antibodies targeting the nucleocapsid and the spike proteins of SARS-CoV-2, respectively, were performed in accordance with the manufacturer's instructions. A signal equal to or above a cutoff of

1.4 Index (S/C) and 50 AU/mL, respectively, was considered serologically positive. An orthogonal testing algorithm was adopted in order to idealize positive-predictivity and determine, with high specificity, the individuals who were truly sero-positive of SARS-CoV-2 specific antibodies (*3*). In this algorithm the individuals who initially tested positive for anti-nucleocapsid antibodies were tested with a second test targeting the SARS-CoV-2 spike antigen. Participants positive for both SARS-CoV-2 specific antibodies were finally confirmed of COVID-19 serological diagnosis by detecting neutralizing antibodies against SARS-CoV-2 using the Genscript SARS-CoV-2 sVNT (Genscript, Leiden, Netherlands), a competition ELISA-based surrogate virus neutralization assay. An inhibition rate (%inhibition) of 30% or above, which, according to the manufacturer's instructions, is predictive of a half-maximal plaque reduction neutralization titer of 20 or higher, was selected as cutoff to determine positivity for neutralizing antibodies.

COVID-19 case definition

Participants were defined as definitive COVID-19 patients, when either; (i) positive for NAT ("NAT-confirmed COVID-19"), or (ii) confirmed of positive serology by the orthogonal testing algorithm ("Serologically confirmed COVID-19").

118

119 *Questionnaire for procedural exposure risk assessment*

120 Participants completed a questionnaire which included demographic data, past medical
121 history, occupational exposure to aerosol-generating procedures performed on confirmed
122 COVID-19 patients, presence/absence of symptoms compatible with COVID-19, and
123 state of NAT diagnosis. The procedural exposures of interest in this study were
124 participation in (a) airway suctioning, (b) non-invasive ventilation (NIV), (c) bag mask
125 ventilation, (d) nebulizer administration, (e) sputum induction, (f) oxygen
126 supplementation as part of tracheostomy care, (g) endotracheal intubation/extubation, (h)
127 tracheostomy, (i) bronchoscopy, and (j) cardiopulmonary resuscitation.

128

129 *Statistical analysis*

130 The results of molecular or serological testing were described as frequencies and
131 percentages among the participants screened. To assess the differences among
132 demographic characteristics between NAT-confirmed and serologically confirmed
133 COVID-19 patients, the following demographic variables were compared by t-tests (for
134 the “Age” variable) or Fisher's exact test (for the other variables; “Male sex”, “Pre-
135 existing risk condition”, “Severity” and “Signs and symptoms”). Magnitude of

serological response against the nucleocapsid and spike antigens, and the %inhibition surrogate virus neutralizability were compared by Mann-Whitney's test according to symptom category; participants carrying respiratory and/or other systematic symptoms ("Symptomatic"), expressing no symptoms ("Asymptomatic") and complaining of isolated smell impairments ("Hyposmia/anosmia only"). Spearman's correlation coefficient was calculated for the various indices of serological response. For the procedural exposure risk assessment, risk ratio (RR), and risk difference (RD), per exposure were calculated as the ratio, or the absolute difference, between COVID-19 incidence among those exposed to the aerosol-generating procedures and the reference ("Not exposed") group. The association between exposures to aerosol-generating procedures and COVID-19 incidence was tested by Fisher's exact test. To evaluate the extent of harm attributable to each procedure regarding the actual increase of COVID-19 cases, the attributable fraction among the exposed (AFe) and the attributable number of events (AN) were calculated. AFe is the proportion of COVID-19 diagnoses in the exposed group that is attributable to the occupational exposure and was calculated per exposure as; AFe = (RR-1) / RR (*6*). AN is the absolute number of COVID-19 diagnoses attributable to the occupational exposure and was calculated per exposure as; AN = AFe × (number of COVID-19 diagnoses among the exposed). P-values less than 0.05 were

considered statistically significant.

Results

Antibody seroconversion elucidates the true burden of the nosocomial outbreak underestimated by symptom-driven NAT screening

Of the 414 eligible and consented HCWs, 186 of 414 (44.9%) had underwent NAT screening for SARS-CoV-2 during the active emergence of the hospital cluster infection during April-May 2020. At the time, the approach towards screening of at-risk HCWs for COVID-19 was symptom-driven, and thus the participants who had never underwent NAT were those less prioritized due to either their lacking typical manifestations, or occupational exposures to aerosol-generating procedures performed on suspected/confirmed COVID-19 patients. 37 (19.9% of those tested by NAT and 8.9% of the entire HCW cohort) tested positive for SARS-CoV-2.

Approximately two months after the nosocomial outbreak had subsided, sera were collected from the participants and tested under the orthogonal testing algorithm (Figure 1). NAT and serological testing results are summarized in Table 1. Combining the NAT-confirmed and serologically confirmed diagnoses, the total number of COVID-19 cases and the overall prevalence rate summed to 64 and 15.5% (64/414), respectively.

Symptom-driven NAT screening had overlooked 42.2% (27/64) of the definitive COVID-19 diagnoses. Of those serologically diagnosed, 23 of 27 (85.2%) had received negative NAT results, and 4 of 27 (14.8%) had been never suspected of COVID-19 and thus had not undergone NAT screening. After excluding those four individuals never having been tested by NAT, the sensitivity of NAT in COVID-19 case detection resulted to be as low as 61.7% (37/60).

Clinical presentation, mode of diagnosis, and magnitude of serological response among the COVID-19 HCW cohort

Demographic data from the COVID-19 cases within the HCW cohort of the present study is demonstrated in Table 2. The mean age was 35 (±12) years and 11 of 64 (17.2%) were male. Only 4 of 64 (6.3%) had known high-risk comorbidities (hypertension and/or diabetes) and 4.7% (3 of 64) reported chronic steroid use. Regarding the severity of disease, the majority of symptomatic COVID-19 cases were mild to moderate illnesses and only 1 of 64 (1.6%) required O_2 supplementation, with no case fatality reported. Typical respiratory symptoms were present in 31 of 64 (48.4%) of the COVID-19 cases and others presented with isolated hyposmia/anosmia (6 of 64, 9.4%) or less specific systemic symptoms; headache, abdominal symptoms and/or malaise (8 of

64, 12.5%). Notably, all six cases presenting with isolated hyposmia/anosmia were confirmed by NAT (6 of 6, 100%). To the contrary, asymptomatic cases (19 of 64, 29.7%) were mainly confirmed by serological testing (17 of 19, 89.5%).

Cross quantitative comparison of the elicited immune responses (Figure 2, panel A) showed that the magnitude of immune response targeting the two major nucleocapsid and spike antigens showed significant correlation within an individual (Spearman's $r = 0.666$, $p < 0.0001$). Further, compared with the levels of anti-nucleocapsid antibody titer (Spearman's $r = 0.560$, $p < 0.0001$), a stronger correlation was observed between anti-spike antibody titers and surrogate virus neutralizability (Spearman's $r = 0.857$, $p < 0.0001$) (Figure 2, panel B). Interestingly, compared with the other symptom categories, participants presenting with isolated hyposmia/anosmia elicited anti-nucleocapsid and anti-spike antibody responses of significantly lower magnitude, constituting an immunologically distinct subpopulation (Figure 3, panel A). Similarly, competition ELISA-based surrogate virus neutralization assay showed a trend towards lower neutralizability of the "hyposmia/anosmia only" subpopulation, though not reaching statistical significance (Figure 3, panel B).

Defining procedural exposure-related risks

Of the 414 eligible participants, 212 (51.2%) reported to have had participated in aerosol-generating procedures and thus had experienced SARS-CoV-2 exposures (Table 3). Amongst the variable types of aerosol-generating procedures, NIV (RR 3.10, p = 0.008) conveyed the highest risk of SARS-CoV-2 transmission to the exposed HCWs, followed by airway suctioning (RR 1.67, p = 0.040). Although sputum induction and cardiopulmonary resuscitation also seemed to convey substantial transmission risks to the exposed, the present study was underpowered to observe statistical significance in the risk increase related to these exposures.

Although the procedural risk inherent to airway suctioning seemed substantially lower compared with NIV, airway suctioning, being a commonly performed aerosol-generating procedure, was the exposure to which the highest number of excess COVID-19 cases were attributed (Table 3).

Discussions

The composite approach of combining NAT and serology-based diagnoses exhaustively detected definitive COVID-19 cases in the Japanese HCW cohort experiencing a nosocomial outbreak during April-May 2020. A surprising 42.2% of overlooked COVID-19 diagnoses had occurred when case detection had relied solely on

NAT, leading to undetected transmission, and triggering the outbreak. Keeping this iceberg phenomenon in mind, appropriate allocation of testing resources is needed in order to effectively detect contagious individuals, clarify the true burden of COVID-19, and eradicate transmission in nosocomial settings.

NAT-based case detection in Japan had been counted on as a promising strategy, capable of thoroughly tracking SARS-CoV-2 transmissions, and identifying and sizing cluster infections (*1*). It was not until June 2020, when the first national seroprevalence survey was performed, that the Japanese realized their 3–8 fold underestimation of the actual spread of the disease within the society (*3*). With the aim of enhancing case detection for effective quarantine, especially among the pre-symptomatic or asymptomatic affected individuals, testing recommendations since then have shifted from a symptom-driven approach towards a rather universal approach. Against expectations, however, having been the sole first-tier diagnostic against this emerging infection, it is now increasingly recognized that NAT-based SARS-CoV-2 pathogen detection faces serious limitations. COVID-19 illness being primarily a lower respiratory tract infection, the probability of pathogen detection from upper respiratory tract specimens decrease rapidly and nearly halves within approximately two weeks from onset (*7*). Previous reports have suggested that a substantial fraction, as high as up to 54%, of COVID-19

patients may present with undetectable viral loads and show false-negative RT-PCR results (*8–10*). Our observation recapitulates such findings, by demonstrating the sensitivity of NAT to have remained as low as 61.7%. Missed diagnoses having occurred not only in the pauci-symptomatic and the asymptomatic populations but also in acutely ill cases of high suspicion, indefinite molecular testing results already have left behind a significant burden of those in need of a diagnosis. A well-defined diagnostic complementary to NAT is still in serious need.

Since the host immune response lags behind viral invasion, the ability of antibody tests to detect an acute infection in its early phase is usually limited and considered inferior to NAT. However, in the case of COVID-19, NAT performance itself remains suboptimal and thus serological testing may well aid in early-phase case detection (*11,12*). While the present study targeted pre-exposed HCWs and was designed so as to establish delayed COVID-19 diagnoses, accumulating evidence further supports the clinical usefulness of serological testing in acute care and diagnosis. COVID-19 pneumonia with repeatedly false-negative NAT results, is not an uncommon clinical scenario, where serological testing, having an extended detectable window, may work complementarily and establish the diagnosis in the early phase of illness (*11*). Used in combination with NAT, serological testing has proven to enhance case detection and help

control the spread of SARS-CoV-2 when applied to carefully targeted, high-risk populations, such as in-hospital outbreaks resembling the HCW cohort of the present study (*12*). By the use of chemiluminescence immunoassays as applied in the present study, exerted sensitivities may rise nearly as high as 40% and 80% by day 7 and day 14 of illnesses, respectively (*13,14*). Therefore, the performance of well-designed platforms may potentially serve as comparable alternatives to NAT in the very acute phase (day 4–7) and may even outperform NAT in the later phases (beyond day 10). In addition, COVID-19 related long-lasting sequelae, such as anosmia or the multisystem inflammatory syndrome in children are widely accepted suitable indications for serological testing (*15,16*). Limitations in capacity for molecular testing, still an ongoing issue in resource-deprived settings, is another factor which makes serological diagnostics an attractive alternative for acute diagnosis purposes.

In addition, exhaustive case detection has here enabled precision of risk estimates innate to aerosol-generating procedures. Our observations are in support of the prevailing concerns on the risks that aerosol-generating NIV may create for HCWs and provide implications regarding the origin of nosocomial spreads (*17*). Notably, while the procedural risk inherent to airway suctioning seemed substantially lower compared with NIV, airway suctioning, being a commonly performed aerosol-generating procedure, was

the exposure to which the highest number of excess COVID-19 cases were attributed. The above findings warn frontline HCWs about the harms of undervaluing risks related to any specific procedural exposure and stress once again the importance of being equipped with appropriate protectives when confronting novel pathogens.

Cross-comparison of the participants' serological responses has highlighted the heterogeneity in width and magnitude of humoral immune responses among the affected. The observed higher detection rate of isolated hyposmia/anosmia patients by NAT, and the uniquely suppressed humoral immune response of the subpopulation may be reflecting confined viral replication and subsequent localized host immune reactions to the nasal airway. However, to draw conclusions on the relationships between viral tropism and serological responses of the host, data laying emphasis on individuals presenting with isolated hyposmia/anosmia are still lacking. Therefore, it remains a future consideration to refine pretest probabilities and to individualize diagnostic approaches based on case presentation (*18,19*).

In conclusion, by way of analyzing serological status against SARS-CoV-2, we unveiled the missed diagnoses within HCWs from a tertiary care hospital in Japan, which had experienced a COVID-19 outbreak during the first peak of the pandemic. Our observations here emphasize the efficiency of well-designed serological diagnostics in

298 the detection of COVID-19 cases and SARS-COV-2 transmissions, and indicate that the
299 true spread within the hospital was almost twice as extensive than previously estimated
300 using a symptom-based NAT surveillance. Multi-tiered diagnostics are key to tracing the
301 exact shape of the COVID-19 iceberg and without the consideration of the hidden but
302 significant portion of the iceberg beneath the surface, we face the risks of under-
303 estimating COVID-19 disease prevalence, over-estimating death rates, and
304 misinterpreting exposure-specific risks.
305

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Disclosures

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Ethical Statement

Analyses were conducted in accordance with the ethical standards noted in the 1964 Declaration of Helsinki and its later amendments. The research was approved by the institutional ethics committee (#2020-003). Consent for participation and publication was obtained from every participant.

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389

390 **Tables**

391 Table 1. Summary of testing results

		Serological testing		
		Positive	Negative	Total
NAT*	Positive			37
	Negative	23	126	149
	Not available	4	224	228

392

*Nucleic acid amplification test

393

394 Table 2. Participant demographics

Demographics	*COVID-19* (N = 64)	*%*	*NAT*-confirmed* (N = 37)	*%*	*Serologically-confirmed* (N = 27)	*%*	*p-value*
Age (years), mean ± SD†	35	± 12	36	± 12	33	± 13	0.184
Male sex	11	17.2	7	18.9	4	14.8	0.748
Pre-existing risk condition							
Comorbidity	4	6.3	2	5.4	2	7.4	1.000
Immunosuppressant use	3	4.7	2	5.4	1	3.7	1.000
Severity							
O_2 supplementation	1	1.6	1	2.7	0	0.0	1.000
Death	0	0.0	0	0.0	0	0.0	–
Signs and symptoms							
Respiratory	31	48.4	26	70.3	5	18.5	< 0.001‡
Hyposmia/anosmia	6	9.4	6	16.2	0	0.0	0.035‡
Other	8	12.5	3	8.1	5	18.5	0.266
None	19	29.7	2	5.4	17	63.0	< 0.001‡
Imaging abnormality	29	45.3	21	56.8	8	29.6	0.079

395 *Nucleic acid amplification test
†Standard deviation
‡$p < 0.05$ (t-test or Fisher's exact test)

396

397

398 Table 3. Risk of SARS-CoV-2 transmissibility during exposure to aerosol-generating

399 procedures

Procedural exposure status	*Total*	*%*	*COVID-19*	*%*	*RR*[*]	*RD*[†]	*AFe*[‡]	*AN*[§]	*p-value*
Not exposed	202	48.8	24	11.9	ref	ref	–	–	–
Exposed	212	51.2	40	18.9	1.59	0.07	0.37	14.81	0.057
Type of exposure									
Airway suctioning	202	48.8	40	19.8	1.67	0.08	0.40	16.00	0.040[**]
Non-invasive ventilation	19	4.6	7	36.8	3.10	0.25	0.68	4.74	0.008[**]
Bag mask ventilation	13	3.1	0	0.0	–	–	–	–	0.370
Nebulizer administration	8	1.9	1	12.5	1.05	0.01	0.05	0.05	1.000
Sputum induction	12	2.9	4	33.3	2.81	0.21	0.64	2.57	0.055
O_2 supplementation via tracheostomy	63	15.2	8	12.7	1.07	0.01	0.06	0.51	0.828
Endotracheal intubation/extubation	21	5.1	2	9.5	0.80	-0.02	-0.25	-0.50	1.000
Tracheostomy	3	0.7	0	0.0	–	–	–	–	1.000
Bronchoscopy	0	0.0	0	–	–	–	–	–	–
Cardiopulmonary resuscitation	13	3.1	3	23.1	1.94	0.11	0.49	1.46	0.214

[*]Risk ratio
[†]Risk difference
[‡]Attributable fraction among the exposed
[§]Attributable number of events
[**]$p < 0.05$ Fisher's exact test

400 **Figure Legends**

401 Figure 1. Enrollment, results of testing and algorithm for diagnosis. Of the 414 eligible
402 and consented participants, 186 had underwent NAT testing for SARS-CoV-2. A total 37
403 of 186 tested healthcare workers were positive for NAT. The orthogonal testing algorithm
404 led to the detection of 27 excess COVID-19 cases, diagnosed serologically. With NAT-
405 and serology-confirmed cases combined, the total number of COVID-19 diagnoses
406 summed to 64. NAT = Nucleic acid amplification test.

407

408 Figure 2. Quantitative assessment of serological responses and their mutual relationships.
409 A) Magnitude of serological response against the two major SARS-CoV-2 antigens.
410 Dotted lines indicate cutoff values. B) In comparison to the anti-nucleocapsid IgG titer,
411 the level of SARS-CoV-2 neutralizability, assessed by the surrogate virus neutralization
412 assay, was correlated to a stronger extent with the anti-spike IgG titer.

413

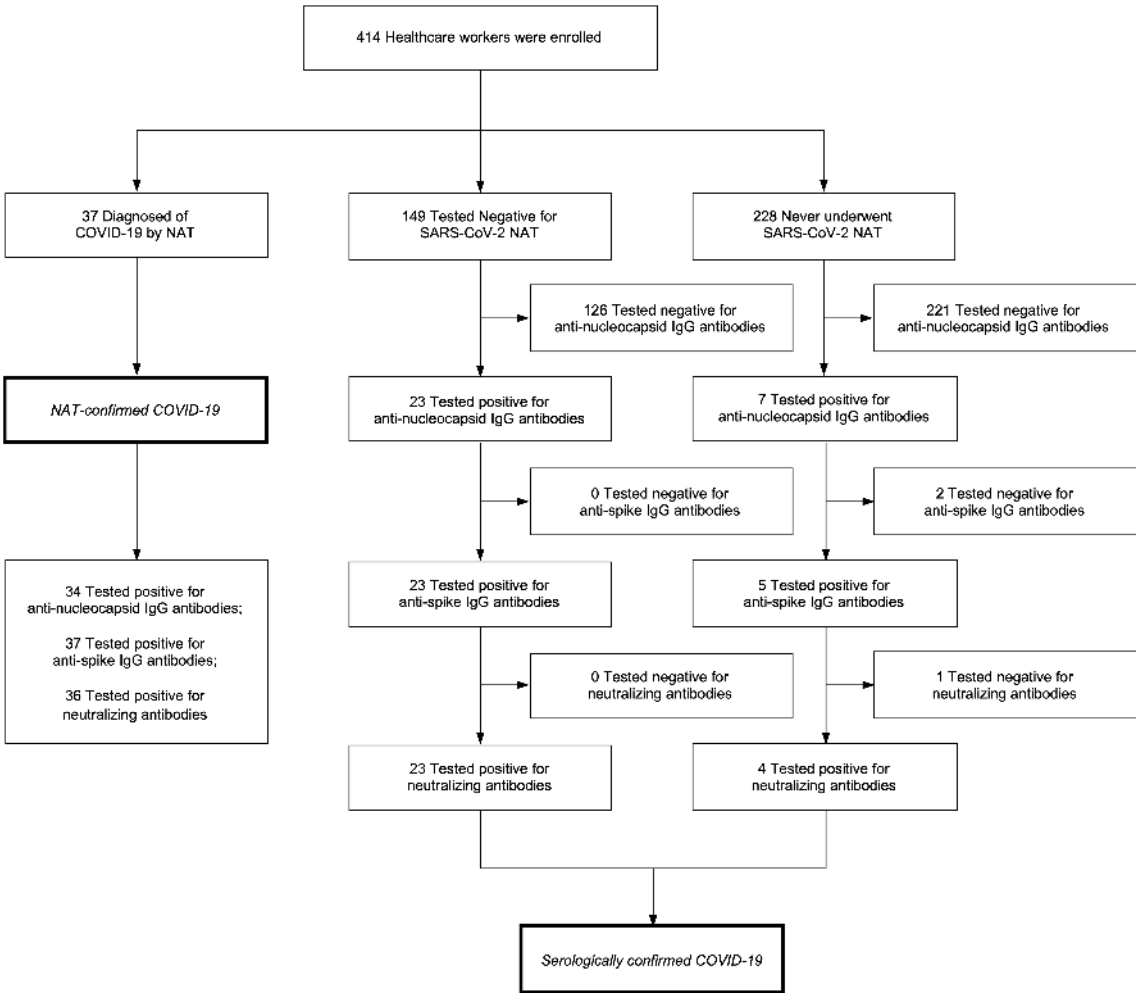
414 Figure 3. Serological status of SARS-CoV-2 affected healthcare workers by symptom
415 category. A) Healthcare workers with COVID-19 diagnosis that had manifested isolated
416 hyposmia/anosmia were characterized by diminished serological responses against the
417 two major SARS-CoV-2 antigens. B) The similar trend towards lower SARS-CoV-2

418 neutralizability of sera obtained from the 6 participants with isolated hyposmia/anosmia

419 did not reach statistical significance. $^{*}p < 0.05$, $^{**}p < 0.01$ Mann-Whitney's test.

420

421 Figure 1.



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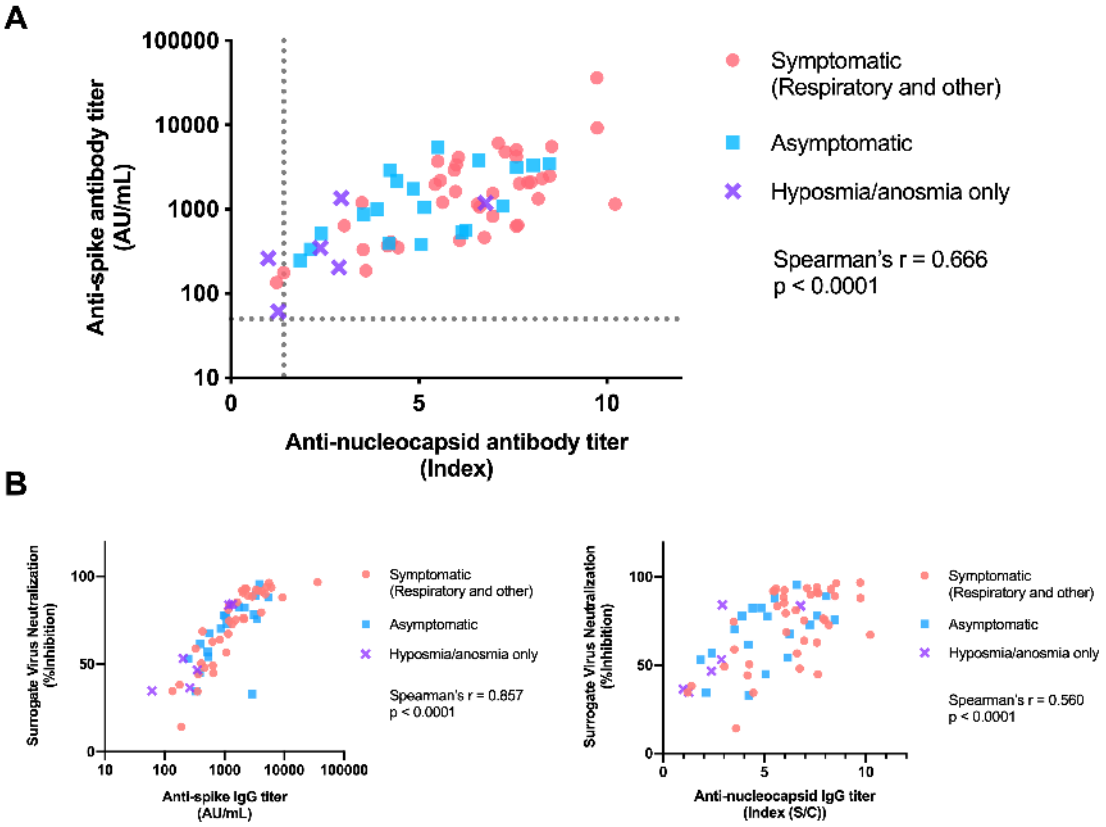
424 Figure 2.



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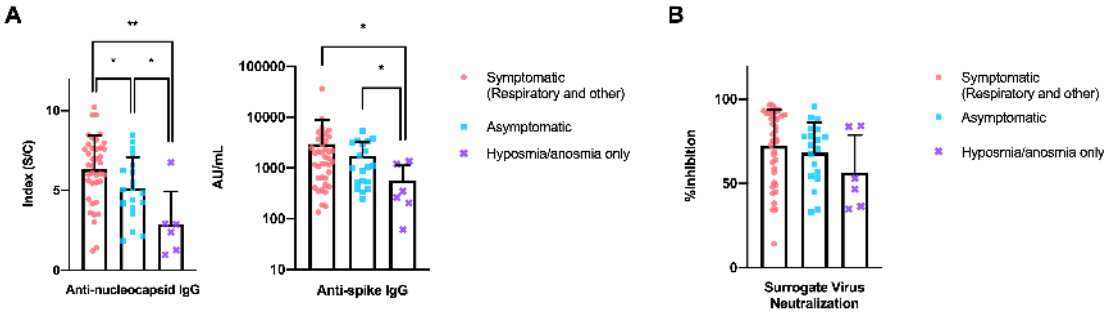
427 Figure 3.



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