Since January 2020 Elsevier has created a COVID-19 resource centre with free information in English and Mandarin on the novel coronavirus COVID-19. The COVID-19 resource centre is hosted on Elsevier Connect, the company's public news and information website.

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Letters to the Editor

Early observations on the impact of a healthcare worker COVID-19 vaccination programme at a major UK tertiary centre

Dear Editor,

The pandemic of severe-acute-respiratory-syndrome-coronavirus-2 (SARS-CoV-2) has precipitated the most extensive global vaccine development programme in history and has resulted in several vaccines receiving emergency use authorisation.[1] Here, we report preliminary observations on the impact of a healthcare worker (HCW) COVID-19 vaccination programme at University Hospitals Birmingham (UHB) NHS Foundation Trust, one of the UK's largest hospital Trusts. The vast majority of vaccinated staff participating in this programme have so far received one dose of the Pfizer-BioNTech (BNT162b) vaccine.

An occupational health database of all COVID-19 positive HCWs was interrogated against an informatics search of all vaccinated HCWs. The UHB testing programme detected SARS-CoV-2 RNA from nasopharyngeal swabs using a Panther Hologic real time polymerase chain reaction platform. All HCWs are encouraged to undertake lateral flow COVID-19 tests (Innova Medical Group, Inc.); positive tests were confirmed by PCR. The Trust started its COVID-19 vaccination programme on 12/12/20, using the BNT162b vaccine. Multivariate logistic and weighted Cox regression models were used to estimate probabilitys.[2]

Between 28/03/20–21/03/21, UHB had 13,544 patient cases of COVID-19. Since 01/04/20, UHB have tested 32,717 staff for COVID-19, with 2721 (8.3%) HCWs testing positive. Up to 23/02/21, the Trust has delivered one dose of vaccine to 25,335 HCWs out of a possible 30,000 workforce. Between 28/01/21–21/03/21, 51% (88/174; 61 patient facing HCWs) of PCR positive UHB HCWs had been vaccinated, with 38% (66/174; 53 patient facing HCWs) having had their vaccination at least 10 days previously. Similarly, 52% percent of HCWs that reported a positive lateral flow test (57/109; 45 patient facing HCWs) had been vaccinated, with 41% (45/109; 40 patient facing HCWs) having had their vaccination >10 days previously. During this period, UHB had eight staff outbreaks with 52 HCWs being positive for COVID-19. Only three of these staff had not been vaccinated. Of all our positive HCW cases across UHB during this time period, 121 (70%) were symptomatic with mild illness. There have been no reports of vaccinated UHB HCWs requiring hospital admission for COVID-19 or COVID-19 related deaths in our vaccinated staff to date.

Of the 33,460 HCW positive samples; 12,701 were mapped to a HCW who was vaccinated and 5468 to a HCW who was not vaccinated. Of the vaccinated staff, there were 178 positive and 3947 negative samples; for the unvaccinated staff, those figures were 330 and 2057, respectively. A multivariate logistic regression model found that being vaccinated was associated with a decreased probability of testing positive ($p = 1.40 \times 10^{-10}$, odds ratio 2.35, 95% CI: 1.81–3.05). The model also found that the probability of testing positive decreases as the gap between vaccination and testing increases ($p = 0.00607$). A weighted cox regression demonstrated that vaccination was associated with a significantly lower hazard of testing positive during the time period in question ($p < 0.0001$). This model gave a generalised concordance probability of 0.24 (95% CI: 0.20, 0.28), meaning that a HCW who had been vaccinated had only a 24% probability of testing positive before an equivalent unvaccinated HCW (Fig. 1).

Chodick et al., (2021) detailed the effectiveness of the first dose of BNT162b2 in Israel.[3] They demonstrated an effectiveness of 51% of BNT162b2 vaccine against SARS-CoV-2 infection 13–24 days after immunisation with the first dose.[3] This is similar to the data presented here, where 51% of HCWs testing positive for COVID-19 during a seven week period were those who had previously received one dose of BNT162b2. However, we saw a lower proportion of vaccinated positive HCWs who had the BNT162b2 (38%) >10days previously. Hunter et al., (2021) used Monte Carlo modelling to analyse the Israel data.[4] They found after initial injection, case numbers increased to day 8 before declining to low levels by day 21.[4] Estimating vaccine effectiveness, they concluded this was 0 at day 14 but then rose to about 90% at day 21 before levelling off.[4] They concluded the cause of the initial surge in infection risk may be related to people being less cautious about maintaining protective behaviours as soon as they have the injection.[4] This could also be a plausible reason why we see a significant numbers of our vaccinated HCWs being positive for SARS-CoV-2. Our data set is unique as we are looking specifically at HCWs. In our population, HCWs would be exposed to higher viral loads and infectious cases. As a result, they are more likely to get infections from SARS-CoV-2 than the general population, as per the work of Shields et al., (2020).[5] It is unsurprising we see a high proportion of vaccinated HCWs testing positive. We saw the same phenomenon of vaccinated HCWs being positive in the lateral flow antigen tests. Mahese (2020) reported that the lateral flow antigen tests will identify infected individuals with the highest viral loads.[6] We saw a significant proportion of our vaccinated infected HCWs positive via lateral flow, implying these HCWs could have higher viral loads. There is debate whether vaccinated individuals transmit SARS-CoV-2, however the fact a proportion of our vaccinated HCWs are positive on lateral flow tests suggestive of high viral loads, implies they will be a vector for transmission. This is further supported by the fact that 70% of our positive HCWs reported symptoms, thus increasing likelihood of transmission.

It is an important message, not just for HCW but for the wider public, that one dose of the vaccination will not prevent individuals from getting COVID-19 and potentially transmitting it. Will a second vaccination reduce HCWs having a high viral load? The clinical trial data showed the first dose of BNT162b2 having 52% efficacy (like our HCW data) and 2 doses having 95% efficacy.[7] This

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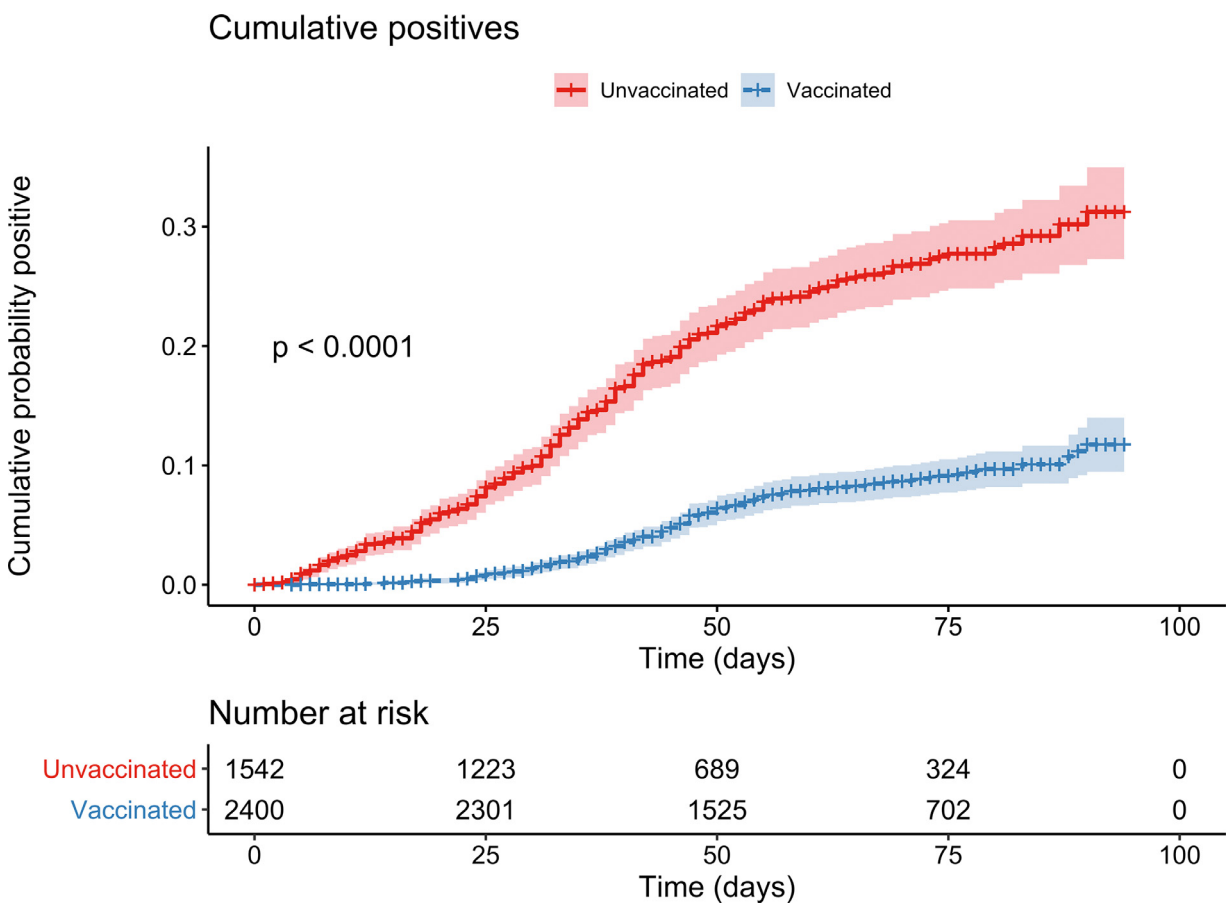

Fig. 1. Displaying the cumulative probability, of vaccinated and non-vaccinated HCWs, testing positive for COVID-19.
Key: The Table within the Figure shows the number of vaccinated and unvaccinated HCWs observed at each time point.

data supports HCWs having a second dose as a priority, to prevent spread in our vulnerable settings.

We conclude that it is imperative staff remain vigilant once they are vaccinated as we are still detecting a significant number of staff acquiring COVID-19, with the potential for onwards transmission to patients and other HCWs. We have also shown that the probability of our staff getting COVID-19 is reducing with a single dose of the vaccine and this is likely to decrease further once HCWs receive their second dose.

Ethics approval and consent to participate

Not applicable.

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Patient and public involvement

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Transparency declaration

The author affirms the manuscript is an honest accurate and transparent account of the study being reported; that no important aspects of the study have been omitted; and that any discrepancies from the study as planned have been explained.

Declaration of Competing Interests

None.

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The 501Y.V2 SARS-CoV-2 variant has an intermediate viral load between the 501Y.V1 and the historical variants in nasopharyngeal samples from newly diagnosed COVID-19 patients

Dear Editor,

In this study, we compared the relative VL of the 501Y.V2, with others SARS-CoV-2 variants: the 501Y.V1 and the historical SARS-CoV-2 variants collected from three hospital laboratories in Paris (Pitié-Salpêtrière, Bichat-Claude Bernard and Saint-Antoine/Trousseau hospitals). A total of 643 RT-PCR SARS-CoV-2 positive nasopharyngeal samples collected at diagnosis were screened to assess SARS-CoV-2 viral lineages with the TaqPath™ COVID-19 RT-PCR (ThermoFisher, Waltham, USA) and the VirSNiP SARS-CoV-2 Spike E484K (TIB Molbiol, Berlin, Germany). The TaqPath COVID-19 test amplified three target genes of the virus (ORF1ab, N, and S). The 501Y.V1 presents a specific deletion in the S gene, *69-70del*, which results in a failure of its detection by this assay. Then, a Sanger sequencing amplifying the RBD region was realised to distinct between the 501Y.V2 and a new variant, B1.1.248, (N501Y and E484K mutations). The relative VL (copies/ml) was assessed by linear regression with a standard curve established from a SARS-CoV-2 positive nasopharyngeal sample quantified by Droplet-Digital™ PCR (Bio-Rad). An ANOVA with a multiple comparison test was performed with the STATVIEW software.

We analysed the results from 643 SARS-CoV-2 infected patients: 332 historical SARS-CoV-2, 249 501Y.V1 and 62 501Y.V2 which presented similar median age at 57 years [38-76] and sex ratio with 53% of female. For the N gene, the 501Y.V2 presented a relative VL two times higher (median 2.32e+7 copies/ml [8.52e+5-2.40e+8]) than the historical variants (median 1.05e+7 [1.81e+5-1.41e+8]) ($p<0.0001$). Moreover, the 501Y.V1 (median 1.12e+8 [1.34e+6-1.19e+9]) presented a relative VL ten times higher than the historical ($p<0.0001$) (Fig. 1A). For the ORF1ab gene, the 501Y.V2 presented also a relative VL (median: 2.69e+7 [6.47e+5-2.40e+8]) two times higher than the historical variants (median: 1.18e+7 [1.77e+5-1.60e+8]) ($p<0.005$). Unlike the N gene, no statistical difference was found between the 501Y.V2 and the 501Y.V1 (median: 3.80e+7 [6.14e+5-4.85e+8]) which also presented a relative VL two times higher than the historical variants ($p<0.0001$) (Fig. 1B).

Our results showed significant differences of VL between these three SARS-CoV-2 variants. Indeed, we found that both new 501Y.V1 and 501Y.V2 variants have statistically higher nasopharyngeal relative VL at diagnosis than the historical lineages.

In other respiratory diseases like SARS and Flue, it has been shown that the level of VL influences outcomes of the disease. In Flue, a higher VL was observed for H5N1-infected patients compared to H3N2 and H1N1-infected patients and was associated with a strongest severity of the disease.[5] In the SARS-CoV a high VL was associated with the severity of symptoms and was shown to be a good indicator of the respiratory failure and death.[6]

A similar pattern was observed with the SARS-CoV-2 virus. A higher VL at the first SARS-CoV-2 RT-PCR testing could be associated with a longer viral persistence of the virus, the contagious condition of patient and can be used as a predictive indicator of the severity of the disease.[4,7] Further studies are required to confirm potential higher infectiousness and higher severity of the disease caused by these two emerging variants.

However, our study reinforces the hypothesis that the 501Y.V1 variant is more infectious than the historical SARS-CoV-2[3,4] and thorough this hypothesis for the 501Y.V2 which begins to spread in France.

Our study is limited by the fact that we were focused on the relative VL at one time, which could be influenced by the variation of the nasopharyngeal swab technique or by the timeline of

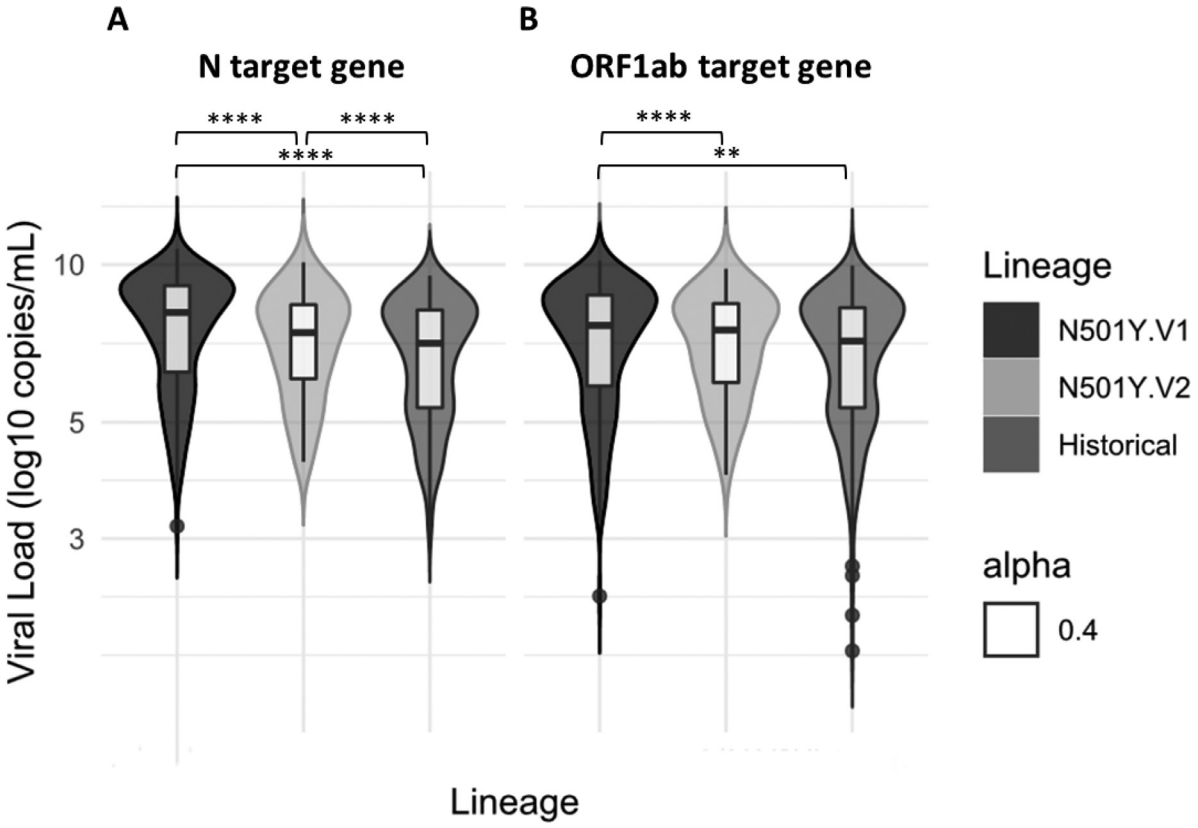

Fig. 1. 501Y.V2 and 501Y.V1 are associated with a higher viral load than historical SARS-CoV-2.
The graph presents the median and the minimal to maximal of the relative VL in log copies/ml of the three groups for the N gene (A) and for the ORF1ab gene (B). The median of the relative VL was higher for the 501Y.V2 and the 501Y.V1 than the historical SARS-CoV-2. **p<0.005; ****p<0.0001.

the epidemic. With the aim to limit this fluctuation, we only collected sample for the first presentation test and with the same SARS-CoV-2 assay at the same period (December 2020 to February 2021). Another limitation is the small number of positive cases for the 501Y.V2 compared to the two other strains principally due to the only few positive cases in Paris area at that time. However, given the co-circulation of these two variants in France and their high transmissibility, it is important to strengthen the SARS-CoV-2 genomic surveillance to follow the evolution of their respective prevalence.

The two new variants seem to present a higher VL compared to the historical SARS-CoV-2 which might arise from the fact that they share the same N501Y mutation probably leading to a better infectivity.[8] However, the 501Y.V2 seems to be associated with a lower VL compared to the 501Y.V1 for the N gene, a difference which fades for the ORF1ab gene. This could be due to their other specific mutations. Considering the 501Y.V2, it does not seem, contrary to the 501Y.V1, that the Spike RBD has higher affinity for ACE2 as compared to the reference Spike. If indeed the transmission is enhanced, this could be due to mutation outside the direct ACE2–Spike interface.[9] Moreover, it has been shown that an active viral replication was associated with the transcription of subgenomic viral RNA and that this active replication occurs during the first days after the onset of symptoms.[10] As we collected only the first presentation test sample, which suggest the presence of an active viral replication, the difference between the N and the ORF1ab genes could be explained by the presence of these subgenomic viral RNA.

Our study brings evidence of increased infectivity for the 501Y.V1 and new proofs for the little-known 501Y.V2 variant infectivity that could explain their propagation velocity worldwide.

Declaration of Competing Interest

Authors declare that they have no conflict of interest.

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Can we do better? A guide to pandemics – some Dos and Don'ts for the next one

We read with interest the study by Aitken et al.[1] on using the Global Health Security Index to assess a country's ability to respond to a pandemic. Although multiple national pandemic plans exist, clearly many of them (particularly in Western countries) have not worked well during the COVID-19 pandemic. Based on recent shared experiences and lessons learned from Southeast Asian countries,[2,3] we make some recommendations for a possibly more effective pandemic response.

Pandemics are defined as the global spread of an infection or disease across multiple countries and populations. As we saw with the 2003 severe acute respiratory syndrome coronavirus (SARS-CoV) outbreaks, and more recently with Ebola virus (West Africa), Middle East Respiratory Syndrome coronavirus (MERS-CoV) and Zika virus (South America), some outbreaks do not develop into true pandemics,[4–6] unlike influenza A(H1N1)pdm09 and coronavirus disease 2019 (COVID-19). [7]

So what are the main indicators for a pandemic threat of transmissible virus? These have been listed below in the form of a list of WARNING SIGNS (for policy makers, healthcare managers, clinicians):

(i) An exponentially increasing number of cases within a district, region or whole country, with no signs of attenuation over multiple generations of infections;
(ii) An initial crude case fatality rate (CFR)[8] of at least 5–10% (as CFRs are always higher at the beginning of a pandemic), showing potential for any illness to overwhelm local healthcare services;
(iii) Evidence for cases spreading overseas involving multiple countries in at least 3 continents (North and/or South America, Europe, Africa, Asia and/or Australasia);
(iv) Evidence for exponential increase of cases in other countries, demonstrating sustained transmission across multiple populations.

If a pandemic threat is taken seriously, then all interventions and actions need to be initiated quickly and comprehensively.[9] If the threat turns out to be minor and localised, it is much easier to relax strict lockdowns and other interventions without significant harm to the economy, education, or psychosocial health. The following lists requirements for INTER-PANDEMIC PREPARATION (for policy makers, healthcare managers):

(A) *The rapid development of diagnostic testing capability.* This capability should not be centralised, but devolved across the country's existing diagnostic laboratory network, with central support as needed. Non-conventional approaches should be explored whenever possible, to pre-empt and mitigate acute supply chain disruptions that may be associated with surges in demand.
(B) *The creation of additional capacity and resources in hospitals.* These should include negative pressure isolation rooms, intensive care beds, diagnostic laboratories and infection control personnel. This should also include large-scale isolation and quarantine facilities, which can be mothballed or repurposed between pandemic threats but activated within a few days if needed.

(C) *A plan for purpose built 'Nightingale' hospitals and designated community isolation/quarantine facilities.* These may include specific hotels adapted for quarantine, conference centres and other community facilities near hospitals, ports or airports that can accept large numbers of locally infected and/or returning travellers.
(D) *The maintenance (at least 6 months) of a stockpile of personal protective equipment (PPE).* This should be sufficient to supply all hospitals and clinics, with supplies being distributed for routine use during inter-pandemic periods, prior to expiry dates.
(E) *The capacity to set up mobile and fixed-point (e.g. 'drive-thru') community sampling stations within a few days.* Such sampling stations should be designed to be easy to set up near potentially vulnerable populations, such as areas of deprivation, prisons, high population densities, high concentration of older people and immigrant workers, etc.
(F) *Development and maintenance of a national test-track-trace team.* These should be supplied with real-time data using suitable and secure mobile phone apps, security dongles, or other tracking devices, and run by local public health teams that can report back to a central command.
(G) *Supportive government funding.* Substantial pandemic budget should be established to support businesses, maintain essential food supplies and other services such as water and sanitation, electricity, gas and other power sources – including WIFI, software and hardware support for home-schooling and university education during the pandemic during any national lockdowns.

Once a PANDEMIC has been declared, the above capabilities can be activated, with those below (for policy makers, healthcare managers, clinicians):

(A) *Rapid implementation of tiered, legally-enforceable social distancing, isolation and quarantine measures:* including the closure of international borders, schools and universities, bars and restaurants, non-essential shopping outlets; emergency powers to initiate curfews and stay-at-home orders, and other restrictions as required.[10] These measures should be initiated immediately once the pathogen is identified within the local population, without waiting for it to spread further.
(B) *Refocusing/repurposing of existing public health and epidemiological modelling teams:* sharing real-time data and estimating important epidemiological parameters (incubation period, serial interval, basic reproductive number), applying a variety of modelling approaches to guide policy.
(C) *Refocusing/repurposing existing laboratory-based surveillance systems:* including rapidly diagnostic PCR testing and viral sequencing to identify emerging variants of potential clinical and public health impact, which will also inform and aid appropriate infection control measures.
(D) *Refocusing/repurposing of existing anti-microbial therapies and vaccine development programmes:* using existing basic and clinical trial research infrastructure already in place to deal with other disease-related therapies, with dedicated government funding and support.
(E) *Clear and concise messaging to the public from the government throughout the pandemic:* allowing people to plan their livelihoods during any pandemic restrictions. Expert committees, including virologists, epidemiologists, clinicians, public health and infection control members, should be set-up to inform and review government public messaging and decision-making – including the combating of 'fake information'.

The above list leads us onto a list of *WHAT NOT TO DO* when preparing for or when dealing with a current pandemic (for policy makers):

(1) Do not underfund inter-pandemic preparedness.
(2) Do not underestimate the threat.
(3) Do not underfund intra-pandemic resourcing.
(4) Do not delay population level interventions.
(5) Do not relax the interventions too early.
(6) Do not underfund, downgrade, close or centralise diagnostic laboratories.

This guide is not intended to be comprehensive or prescriptive, but highlights important points for future pandemic planning. Funding and resource allocation to support these pandemic-related activities will be decided by individual jurisdictions, depending on available resources and priorities.

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Risk stratification of cardiac sequelae detected using cardiac magnetic resonance in late convalescence at the six-month follow-up of recovered COVID-19 patients

Dear Editor,

Follow-up studies in COVID-19 survivors have found persistent symptoms(fatigue, dyspnea, muscle pain et, al.), impaired pulmonary function, abnormal chest CT images in COVID-19 survivors even after 110 days and 6 month of follow-up.[1,2] Several studies also have reported that cardiac involvement, including myocardial edema, fibrosis, and cardiac dysfunction, detected by using multi-parameter cardiac magnetic resonance (CMR) techniques were identified in recovered COVID-19 patients during early convalescence.[3–8] However, whether COVID-19 has a continuous influence on the cardiovascular system in late convalescence is unknown. Therefore, we used traditional CMR sequences to evaluate cardiac abnormalities in late convalescence comprehensively, including cardiac function, myocardial deformation, and myocardial tissue characteristics, and explore its related risk factors.

34 recovered COVID-19 patients at Chengdu Public Health Clinical Medical Centre were prospectively enrolled and followed-up from Jan 1 to Oct 20, 2020. Diagnosis and discharging of COVID-19 patients were based on guidelines of the Chinese Center for Disease Control and Prevention.[9] Six months after discharging from hospital, gadolinium enhanced CMR scan (1.5T, Signa HDxt; GE Medical systems, USA) was performed and 20 healthy controls were enrolled too. All the patients and healthy controls signed informed consent and the institutional ethics board of our institutes approved this study (No. 2020.43). Electrocardiography, echocardiography, laboratory test, and clinical characters at admission were collected. Cardiac abnormalities were defined as a combination of elevated myocardial enzyme and injury marker, abnormal echocardiographic and electrocardiographic results. Patients was divided into two subgroups, subgroup with/without cardiac abnormalities at admission.

Biventricular function, myocardial deformation, myocardial edema and fibrosis were evaluated with postprocessing software Cvi42 (Circle Cardiovascular Imaging, Calgary, Canada). Cardiac dysfunction was a combination of left ventricular ejection fraction (LVEF) less than 50%, right ventricular ejection fraction (RVEF) less than 45%, and LV deformation dysfunction. CMR abnormalities was a combination of myocardial edema, fibrosis, and cardiac dysfunction.

At admission, 23 (67.65%) patients had cardiac abnormalities, 7 (20.59%) patients had elevated myocardial enzyme, 2 (5.88%) patients had elevated myocardial injury maker, 3 (8.82%) and 20 (58.82%) patients reported abnormal echocardiographic and electrocardiographic results. None of these 34 patients reported cardiovascular-related symptoms or signs during follow-up.

Cardiac function and LV myocardial deformation indexes were compared between the subgroups of patients and controls. All of the 34 recovered COVID-19 patients had normal LVEF (>50%). Nevertheless, right ventricular (RV) systolic dysfunction (RVEF<45%) was found in 5 (14.71%) patients. Meanwhile, right ventricular peak ejection rate (RVPER) in recovered COVID-19 patients with cardiac abnormalities at admission was significantly reduced compared to the controls (Fig. 1A). The other biventricular function indexes were not significantly different among subgroups of patients with/without cardiac abnormalities at admission and controls (all $p > 0.05$). Although similar to healthy controls, the global peak systolic velocity circumferential (PSVC) in patients with cardiac abnormalities at admission was lower than patients without cardiac abnormalities ($p = 0.011$) (Fig. 1A). A similar phenomenon was found in the basal ($p = 0.003$) and middle PSVC ($p = 0.013$) (Fig. 1A). Additionally, basal PSCV in patients with cardiac abnormalities at admission was significantly reduced compared to healthy controls ($p = 0.021$) (Fig. 1A). ROC analysis showed that basal PSVC could differentiate subclinical left ventricular dysfunction with good sensitivity and high specificity ($p = 0.004$). The area under the curve was 0.822, and the cut-off was 34.455 for basal PSVC (sensitivity: 0.909 and specificity: 0.652). According to the cut-off value, 16 (47.06%) patients had reduced basal myocardial deformation.

Except for dysfunction of segmental LV deformation and RV systolic function, myocardial abnormality was also identified. Edema was found in 10 (29.41%) and fibrosis in 2 (5.80%) patients. 70% of edema and all the fibrosis was in subgroup of patients with cardiac abnormalities at admission (Fig. 1B).

Finally, 19 (55.88%) patients developed cardiac dysfunction and 22 (64.71%) patients had abnormal CMR findings in our study. Further comparisons were performed to evaluate the characteristics of patients with abnormal CMR finding (Table 1). Patients with LV deformation dysfunction were older and had a higher percentage of elevated lactic dehydrogenase (LDH), elevated creatine kinase, echocardiographic abnormalities, and cardiac abnormalities at admission (all $p < 0.05$). For patients with right ventricle dysfunction/cardiac dysfunction/CMR abnormalities, a higher percentage of elevated LDH was identified (all $p < 0.05$). Severe or critically severe COVID-19 was more prevalent in patients with myocardial edema (40.00% vs 8.00%, $p = 0.048$).

To evaluate risk factors for CMR abnormalities, variables with $p < 0.1$ in Table 1 were included in univariable logistic regression. Referring to dysfunction of LV deformation, elevated LDH (OR:10.20, 95%CI: 1.07 to 97.41, $p = 0.044$), presence of echocardiographic abnormalities (OR:6.81, 95%CI: 1.41 to 32.83, $p = 0.017$), and presence of cardiac abnormalities at admission (OR:18.75, 95%CI: 2.02 to 173.9, $p = 0.010$) were risk factors. Patients with elevated LDH at admission also had a higher risk for RV systolic dysfunction (OR: 9.38, 95% CI: 1.17 to 74.84, $p = 0.035$). Severe or critically severe COVID-19 was a risk factor for myocardial edema. We also found that the presence of cardiac abnormalities at admission (OR: 6.30, 95% CI: 1.30 to 30.53, $p = 0.022$) was a risk factor for the presence of CMR abnormalities and cardiac dysfunction (OR: 6.10, 95% CI: 1.24 to 30.09, $p = 0.027$) at six months of follow-up.

In this recovered COVID-19 cohort, we screened for cardiac sequela in the late convalescence using CMR. We found that cardiac involvement, including RV systolic dysfunction, segmental LV deformation decrease, myocardial edema and fibrosis were not uncommon even after six months of recovery. Findings in our study was consistent with and a supplement to previous researches in early convalescence.[3–8] *Abnormal findings* in COVID-19 survivors after *110 days and* 6 months imply continuous inflammation, which may be the reason for the lasting cardiac involvement in our patients.[1,2] What's more, our study also identified that elevated LDH,

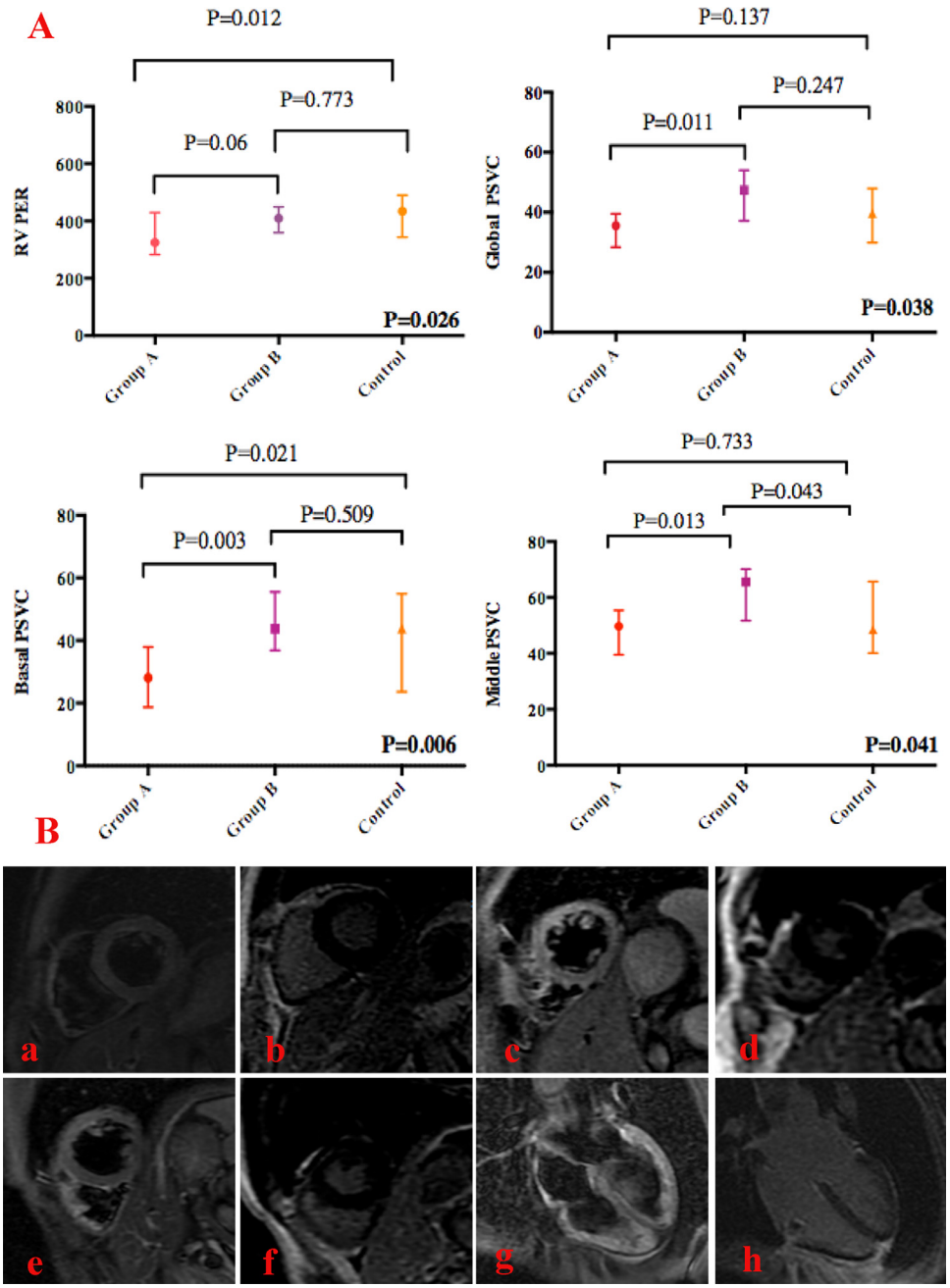

Fig. 1. A. Comparison of RVPER and PSVC between subgroups of recovered COVID-19 patients and controls; Group *A* represents patients with cardiac abnormalities at admission; Group *B* represents patients without cardiac abnormalities. Right ventricular peak filling rate(RVPFR); Peak systolic velocity circumferential(PSVC). B. Myocardial edema and fibrosis of recovered COVID-19 patients in late convalescence; a,b. A common type COVID-19 without cardiac abnormalities. No edema or LGE was identified. c,d. A mild type COVID-19 patient with mid mitral valvular regurgitation at admission on echocardiography, but turned to normal on the day of CMR. Edema was found in the anterior, anterolateral, and anteroseptal walls, and there was no evidence of LGE. e,f. A severe type COVID-19 patient with ICU admission and elevated LDH at admission and the day of CMR, other tests were normal. Edema was identified in anterior, anterolateral wall of basal and middle segment, and intra-wall LGE was identified. g,h. A mild type COVID-19 patient with mild tricuspid valvular regurgitation at admission and the day of CMR on echocardiography. CMR showed myocardial edema and sub-epicardial LGE at the lateral wall.

the presence of echocardiographic abnormalities, the presence of cardiac abnormalities at admission, and the severity of COVID-19 were risk factors for cardiac sequelae in COVID-19 survivors in the late convalescent stage. This was consistent with previous study that focused on risk stratification of cardiac involvement in COVID-19 patients during hospitalization.[10] This implies that physicians should pay more attention to these high risk patients during follow-up, and CMR could be performed to screen out cardiovascular involvement in late convalescence.

Declaration of Competing Interest

The authors report no conflicts of interest.

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A cross-sectional seroepidemiology study of seven major enteroviruses causing HFMD in Guangdong, China

Recent article in this Journal has reported a cohort of study on the seroepidemiology of EV-A71, CV-A16, CV-A6[1] and CV-A10[2] in infants and children. In the past two decades, HFMD has occurred frequently worldwide, especially in the Asia-Pacific region, where it has become an important public health problem.[1,2] Enterovirus A71 (EV-A71), coxsackievirus (CV)-A 16, CV-A6, and CV-A10 are the main pathogens causing HFMD outbreaks and epidemics.[1,2] However, CV-A2, CV-A4 and CV-A5, in the EV-A group, have emerged as active pathogens implicated in several HFMD and herpangina epidemics worldwide.[3–9]

In 2008, an EV epidemic caused HFMD and herpangina in children in Taiwan, China. CV-A2, the most prevalent among the

Abbreviations: HFMD, hand, foot, and mouth disease; EV, enterovirus; EV-A71, Enterovirus 71; CV-A2, Coxsackievirus A2; CV-A4, Coxsackievirus A4; CV-A5, Coxsackievirus A5; CV-A6, Coxsackievirus A6; CV-A16, Coxsackievirus A16; CV-A10, Coxsackievirus A10; NTAb, neutralizing antibodies; CPE, cytopathic effect; TCID, tissue culture infective dose.

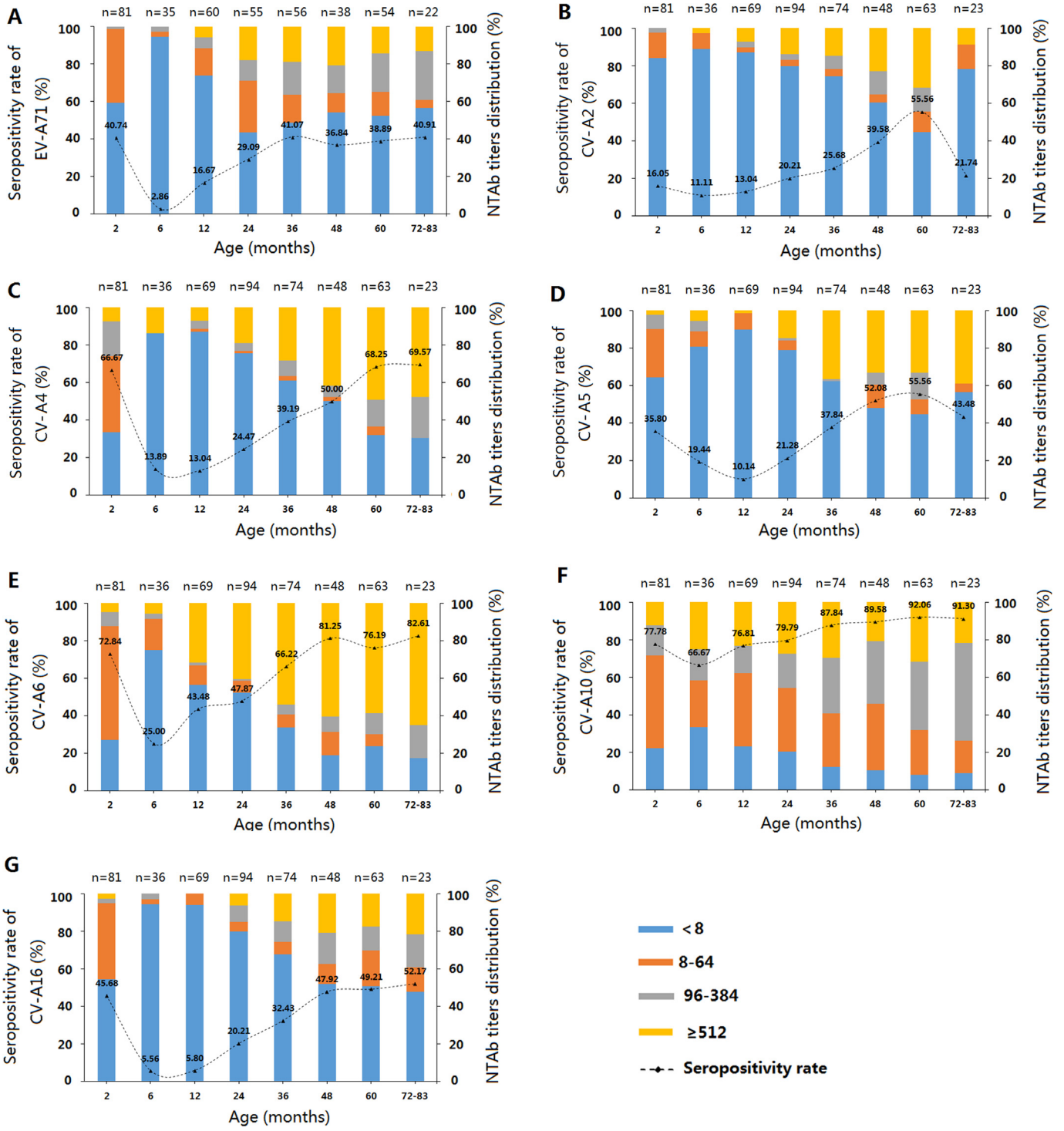

Fig. 1. The seropositivity rates and NTAbs titre distribution of EV-A71, CV-A2, CV-A4, CV-A5, CV-A6, CV-A10and CV-A16. NTAbs against EV-A71were measured only in participants from the unvaccinated group in this study. NTAb titres were classified into four groups: NTAbs titer <8 were determined as negative and shown in blue; NTAbs=8–64 indicate low titre and are shown in orange; NTAbs=96–384 indicate medium titre and are shown in grey; NTAbs≥512 indicate high titre and are shown in yellow. Dotted curves indicate the seropositivity rates. "n" refers to the number of the serum samples.

identifiable viruses (104/160), was the main cause of herpangina (98/161).[3] In 2015, CV-A2 (40/120) was the most frequent EV found in children with herpangina in Thailand.[4] Similar report of CV-A2 prevalence have also been reported in mainland China from 2016 to 2017.[5] CV-A4 was reported to have caused illness in Singapore in 2008,[6] Greece in 2009–2010[7] and mainland China in 2011.[8] Sparse HFMDs caused by CV-A5 have also been reported in Japan[9] and mainland China.[5] However, the seroepidemiologic characteristics of CV-A2, CV-A4, and CV-A5 are unknown. This is the first study detailing the seroepidemiology of CV-A2, CV-A4 and CV-A5 in infants and young children; compared with those of EV-A71, CV-A16, CV-A6 and CV-A10; to understand the epidemic status of them.

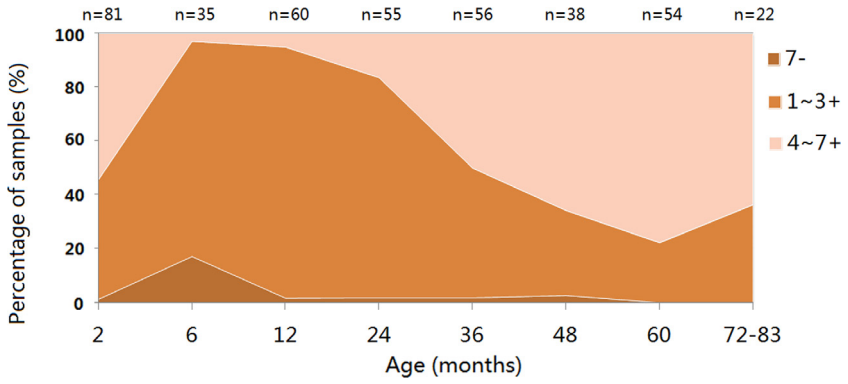

Fig. 2. Co-existence of NTAbs against different EV types. The unvaccinated participants with EV-A71 vaccine (401) were analysed for the co-existence of NTAbs against the seven types of EVs. The samples were divided into three groups: all EVs negative (7-), one to three EVs positive (1 ~ 3+), and four to seven EVs positive (4 ~ 7+). "n" refers to the number of the serum samples.

A total of 488 healthy participants aged 2–83 months were enrolled in a EV seroepidemiological survey in Yangchun City, Guangdong Province, China in 2018. Serum was collected to test for neutralizing antibodies (NTAbs) against EV-A71, CV-A2, CV-A4, CV-A5, CV-A6, CV-A10 and CV-A16. There were 255 males and 233 females, while eight age groups were divided from 2 months old to72–83 months. Altogether, 87 participants had been vaccinated with inactivated EV-A71 vaccines and 401 participants did not. Titers of NTAbs in serum samples were measured by cytopathogenic effect (CPE) method on Rhabdomyosarcoma cells using EV-A71 (523), CV-A2 (R1580-XY-2017), CV-A4 (R3179-XY-2017), CV-A5 (R3487-XY-2017), CV-A6 (WH-15), CV-A10 (WH-97) and CV-A16(731–3) strains.

The results of NTAbs against the seven EVs of different age groups are shown in Fig. 1. The changes in the trends of CV-A2, CV-A4 and CV-A5 NTAbs in newborns seemed to be consistent with those of EV-A71, CV-A16 and CV-A6, where the maternally derived NTAbs dropped to the lowest level (11.11%, 13.04% and 10.14%) between 6 and 12 months of age. With the increase in age (months) and the number of natural infections, the seropositivity rate gradually increases. In the 60-month-old group, the seropositivity rates of CV-A2, CV-A4 and CV-A5 (55.56% to 68.25%) were lower than those of CV-A6 and CV-A10 (76.19% and 92.06%), but higher than those of EV-A71 and CV-A16 (40.91% and 52.17%, respectively), suggesting that these three EVs were also widely circulating in this area.

Interestingly, the seropositivity rates of CV-A2 in the 2-month-old groups (16.05%) were significantly lower than those of other EVs ($\chi 2$ ranged from 8.23 to 61.96, $P<0.01$), and there was significant decrease in the 72–83-month-old group compared with the 60-month-old group ($\chi 2=7.74$, $P<0.01$).While the proportion of moderate and high levels in NTAb titre distribution were relatively low (2.47% in 2-month-old groups, 8.70% in 72–83-month-old groups). The reason for low CV-A2 NTAbs of maternally derived and pre-schooler was that CV-A2 antibody persistence might be relatively weaker. While it could not be ruled out that most of them might be not infected in CV-A2 epidemic and needs to be further researched. The same trend in CV-A5 seropositivity rate was also observed in 72–83-month-old group, but there was no significant decrease compared with 60-month-old group ($\chi 2=0.99$, $P>0.05$).

In addition, we found that CV-A10 still maintained a high seropositivity rate (66.67%) and much more distribution of moderate and high NTAb titre (41.67%) in 6-month-old group, with no significant decrease compared with the 2-month-old group ($\chi 2=1.61$, $P>0.05$ and $\chi 2=2.00$, $P>0.05$, respectively). Some report showed that the seropositivity rate of CV-A10 was very low (14.6%) in the infant group aged 6–11 months[2] and in the <1-year-old group (17.8%).[10] Therefore, we speculate that the abnormal increase in the seropositivity rate of CV-A10 in the 6-and 12-month-old groups in this study might be due to infection with CV-A10 at a younger age.

The unvaccinated participants with EV-A71 vaccine (401) were analysed for the co-existence of NTAbs against the seven types of EVs. As shown in Fig. 2, of the 81 children in the 2-month-old group, only 1 participant (1.23%) were negative for all seven EVs (7-), and 80 (54.32%) were positive for at least four EVs. In the 6-month-old groups, most participants (80.00%) co-existence statuses were "1 to 3+" . Most children aged 72–83-month-old had NTAbs against 4 to 7 EVs (64.63%), with no statistically significant difference between 60 and 72–83-month-old ($\chi 2=1.61$, $P>0.05$). These results suggest that these seven EVs NTAbs are often co-existence in infants and children in China, due to alternate infections or co-infections with different EVs. The possible gene recombination caused by co-infections of multiple EVs may cause serious and fatal diseases, which need to be focused on.

Moreover, EV-A71 vaccination had no impact on CV-A2, CV-A4, CV-A5, CV-A6, CV-A10 and CV-A16 infections ($\chi 2$ ranged from 0.04 to 2.80, $P>0.05$). No sex effect on the seropositivity rates was found in this population ($\chi 2$ ranged from 0 to 0.44, $P>0.05$).

In 2015, the China Food and Drug Administration approved the first inactivated EV-71 whole virus vaccine to prevent severe HFMD. With the application of the EV-A71 vaccine, the infection by and incidence of EV-A71 were suppressed. This may indirectly lead to an increase in the proportion of other EV infections. In order to control HFMD and other severe diseases caused by EV infections in the paediatric population in the post-EV-A71 period, pathogen surveillance and related basic research on emerging active pathogens of HFMD should be strengthened to prepare for possible changes in the pathogen spectrum of HFMD.

Ethical standard

The study was approved by the institutional review board of Guangdong Provincial Centre of Disease Control and Prevention, and was conducted in accordance with the Declaration of Helsinki, Good Clinical Practice, and Chinese regulatory requirements.

Declaration of Competing Interest

We have no conflict of interest to declare.

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Secondary bacterial infection in immunocompetent patients with refractory cryptococcal meningitis: A curse or a blessing? ☆

Dear Editor,

We read with interest the paper by Macsween et al., who stated that lumbar drainage is useful for control of high intracranial pressure (ICP) in patients with cryptococcal meningitis (CM).[1] High ICP is a hallmark of CM and is associated with increased mortality. Thus, it requires special management strategies, such as lumbar drainage and Ommaya reservoir (OmR) implantation. However, both lumbar drainage and OmR implantation can result in secondary bacterial infections. Although the infection rates of lumbar drainage and OmR implantation are relatively low, the consequence of secondary infection in patients with CM may be lethal.[2] We herein describe three immunocompetent patients with refractory CM who developed secondary bacterial infections related to lumbar drainage and OmR implantation. Interestingly, the indicators of fungal infection in these patients decreased or turned negative after the secondary bacterial infections developed, and all patients fully recovered.

Patient 1 was a 48-year-old man with a chief complaint of subacute headache. Brain magnetic resonance imaging (MRI) showed diffuse leptomeningeal enhancement (Fig. 1A). A lumbar puncture was performed at admission. The cerebrospinal fluid (CSF) opening pressure was >330 mmH_2O, glucose concentration was 0.1 mmol/L, protein concentration was 461 mg/L, and white blood cell (WBC) count was 35 cells/mm^3. The diagnosis of CM was made on day 8 based on a positive India ink stain of the CSF and positive cryptococcal antigen of both the CSF and plasma. To control the increased ICP, OmR implantation was performed on day 10 (Fig. 1B). The initial treatment was voriconazole. On day 40, voriconazole was switched to liposomal amphotericin B (L-AmB) plus flucytosine. Unfortunately, the patient's symptoms persisted, quantification of cryptococcal antigen remained high (>100 μg/L), and CSF cultures for *Cryptococcus. neoformans* kept positive. On day 102, a secondary bacterial infection was detected with positive CSF cultures for *Serratia marcescens*. Brain MRI showed retrograde infection of the OmR. The patient's prognosis seemed rather poor. However, after development of the secondary bacterial infection, the CSF cultures for *C. neoformans* turned negative. After another 6 months of treatment with fluconazole and meropenem, the patient was clinically normal and showed persistent negative mycological findings (Fig. 1C).

Patient 2 was a 41-year-old man with a progressively increasing headache. Brain MRI findings were unremarkable (Fig. 1D). Initial CSF analysis showed an opening pressure of >330 mmH_2O, glucose concentration of 1.2 mmol/L, protein concentration of 1028 mg/L, and WBC count of 210 cells/mm^3. The diagnosis of CM was made based on a positive CSF culture on day 7. Antifungal treatment with

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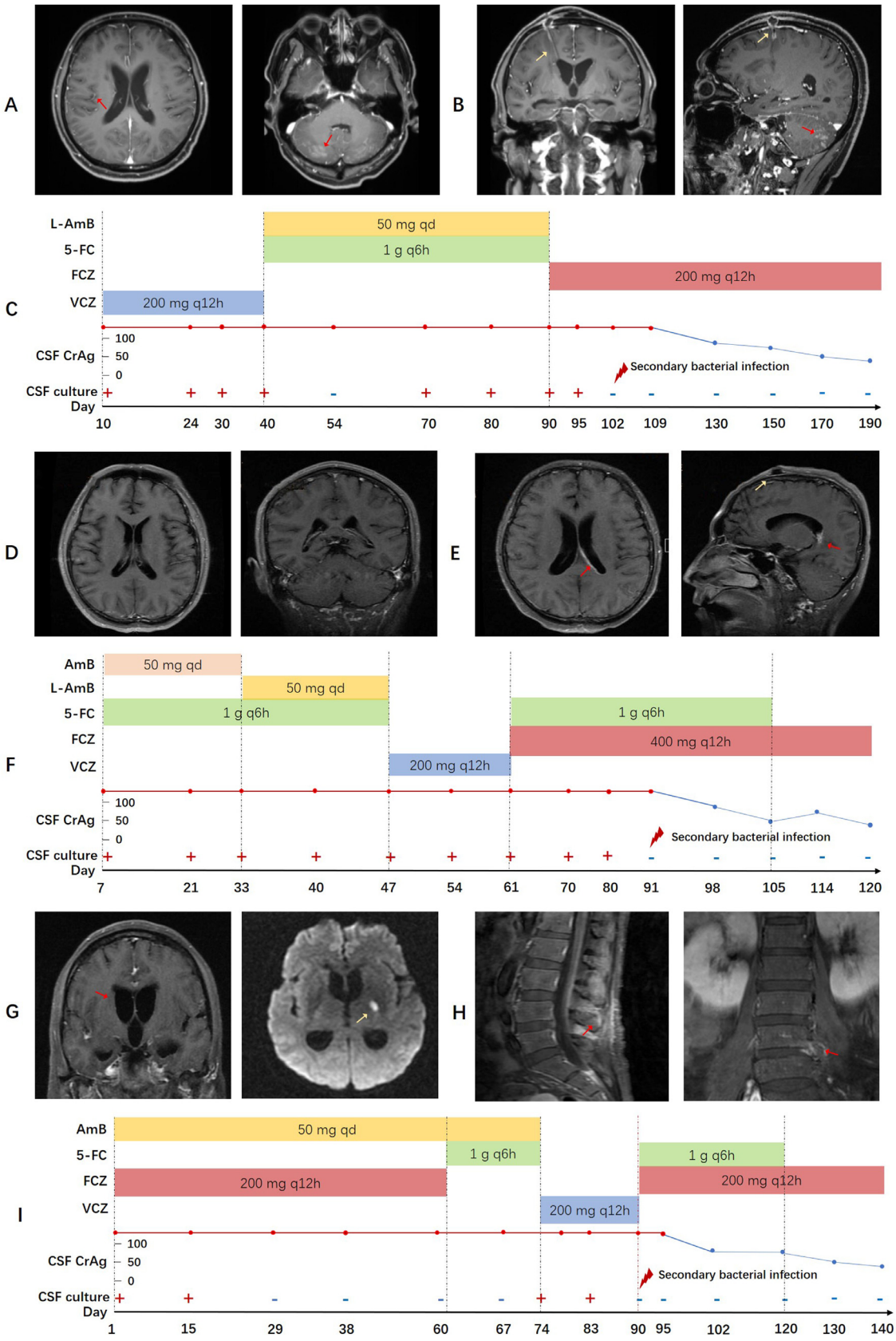

Fig. 1. Magnetic resonance imaging scanning and treatment timeline of the patients. A. Axial T1 post-gadolinium, red bars show leptomeningeal enhancement. B. Coronal and sagittal T1 post-gadolinium, red bar shows leptomeningeal enhancement, yellow bars show OmR implantation. C. Treatment timeline of patient 1. D. Axial and coronal T1 post-gadolinium. E. Axial and sagittal T1 post-gadolinium, red bars show retrograde infection of the OmR, yellow bar shows OmR implantation. F. Treatment timeline of patient 2. G. Axial and coronal scanning, red bar shows hydrocephalus, yellow bar shows infarction. H. Sagittal and coronal T1 post-gadolinium, red bars show paravertebral infection. I. Treatment timeline of patient 3. L-AmB, liposomal amphotericin B. 5-FC, flucytosine. FCZ, fluconazole. VCZ, voriconazole. CSF, cerebrospinal fluid. CrAg, cryptococcal antigen. OmR, Ommaya reservoir.

AmB plus flucytosine was started immediately. To control the increased ICP, lumbar drainage was performed on day 30 and OmR implantation was performed on day 37. Because of adverse effects and poor efficacy, AmB was switched to L-AmB on day 33, L-AmB was switched to voriconazole on day 47, and voriconazole was switched to fluconazole plus flucytosine on day 61. Despite these treatments, the patient's symptoms persisted, quantification of cryptococcal antigen remained high (>100 μg/L), and CSF cultures for *C. neoformans* remained positive. On day 91, a secondary bacterial infection was detected with positive CSF cultures for *Staphylococcus*. Brain MRI showed retrograde infection of the OmR (Fig. 1E). Linezolid and vancomycin were administered to treat *Staphylococcus* infection. Similar to patient 1, CSF cultures for *C. neoformans* turned negative and the quantification of cryptococcal antigen decreased after development of the secondary bacterial infection (Fig. 1F). After another 12 months of treatment with fluconazole, the patient recovered completely.

Patient 3 was a 48-year-old woman with initial symptoms of headache, vomiting, and fever. Brain MRI showed hydrocephalus, acute infarction, and diffuse leptomeningeal enhancement (Fig. 1G). Initial CSF analysis showed an opening pressure of >330 mmH_2O, glucose concentration of 0.45 mmol/L, protein concentration of 950 mg/L, and WBC count of 130 cells/mm^3. The diagnosis of CM was made based on a positive CSF culture and a positive India ink stain on day 1. She was treated with AmB plus fluconazole and subsequent placement of lumbar drainage to manage the increased ICP. CSF cultures turned negative on day 42; however, quantification of cryptococcal antigen remained high (>100 μg/L), and the daily drainage volume did not decrease (>200 mL/d). Because of the poor treatment efficacy, AmB was switched to voriconazole on day 74. On day 90, a secondary bacterial infection was detected with blood and CSF cultures for *Escherichia coli*. The patient developed low back pain, chills, and fever (40.2 °C). Additionally, lumbar MRI showed paravertebral infection (Fig. 1H). Similar to the two above-described patients, both the quantification of cryptococcal antigen and daily drainage volume decreased after development of the secondary bacterial infection (Fig. 1I). Meropenem and ceftazidime were used to treat the *E. coli* infection, and after another 12 months of treatment with fluconazole, the patient recovered fully.

Several reports have described dual infection of the central nervous system by *C. neoformans* with other pathogens, including *Mycobacterium tuberculosis* (8 cases), *Toxoplasma gondii* (3 cases), and *Streptococcus pneumoniae* (2 cases).[3,4] All of these patients were immunocompromised, and the majority had AIDS. However, among immunocompetent patients with CM, coinfection with other pathogens is rare. To our knowledge, only one case of an immunocompetent patient with CM (*C. gattii* infection) coinfected with *Listeria monocytogenes* has been reported[5].

We have herein presented three cases of refractory CM with secondary bacterial infections. All patients were immunocompetent and recovered completely. Our experiences from these cases indicate that secondary bacterial infections may inhibit the primary fungal infection and improve the treatment of refractory CM. There are three possible reasons for this phenomenon. First, bacterial infection activates the innate immune response, promotes the release of cytokines, and enhances adaptive immunity, resulting in accelerated *C. neoformans* clearance. As shown in Table 1, increases in the interleukin-6 concentration and WBC count after a secondary bacterial infection were observed in these patients. Second, bacterial infection and inflammation can undermine the integrity of the blood-brain barrier, which may improve the permeability of the blood-brain barrier to antifungal agents, such as AmB, and thus enhance the efficacy of antifungal treatment[6]. Third, bacteria can suppress fungal growth through either competition for a limited supply of nutrients in CSF or through the production of antibiotics, enzymes, and volatile substances.[7] Thus, determination of whether the secondary bacterial infection is a curse or a blessing for primary fungal infection requires further study.

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Rapid spread and high impact of the variant of concern P.1 in the largest city of Brazil

Dear Editor,,

A recent report in this journal has suggested a global prevalence of the variant P.1.[1] As the SARS-CoV-2 cases continue to emerge globally, variants of concern (VOC) and variants of interest (VOI) have been described and are continuously being monitored.[2] The variants share important points of mutations at the receptor-binding domain (RBD) of the Spike protein, which might increase the transmissibility of COVID-19 and promote escape from neutralizing antibodies.[2]

Table 1
Laboratory findings before and after secondary bacterial infection.

Laboratory findings	Case 1 Before	After	Case 2 Before	After	Case 3 Before	After
Blood						
White blood cells ($\times 10^9$/L)	0.73	2.09	7.71	15.54	3.27	7.62
C-reaction protein (mg/L)	3	19	0.172	0.087	0.05	8.85
Procalcitonin (ng/ml)	–	–	0.112	0.137	4.06	43.73
Interleukin-6 (pg/ml)	–	–	14.5	92.53	17.8	317.1
Cerebrospinal fluid						
White blood cells ($\times 10^6$/L)	28	1400	10	28	19	1559
Protein (mg/L)	700	1100	1822.3	1124.8	1869.5	1900
Glucose (mmol/L)	3.33	1.66	0.3	0.6	2.0	0.2
Chloride (mmol/L)	118	120	109.4	112.4	110.2	115.4
IgA	–	–	3.82	2.53	6.88	9.18
IgG	–	–	30.6	21.7	82.5	93.5
IgM	–	–	1.93	0.696	2.5	3.13

Brazil is currently the epicenter of COVID-19, with more than 13 million confirmed cases until early April 2021.[3] Two variants, VOC P.1 and VOI P.2, evolved from lineage B.1.1.28, have taken over the scene since late 2020 in the country.[4,5] The VOC P.1 was first detected in January 2021 in Japanese travelers returning from Manaus and was responsible for the second wave in the Amazonas in late November 2020.[5] In October 2020, the VOI P.2 was reported in Rio de Janeiro and was estimated to have emerged in late August 2020.[5,6] Both variants have been also associated with reinfection cases.[6]

In a recent investigation (data not published) of the circulating variants in São Paulo city, during the first week of March, 64.4% of samples were identified as P.1. The investigation of lineages not only contributes to epidemiological surveillance but also provides a better comprehension of the spread and circulation of variants, allowing the association to clinical outcomes and response to vaccines.[7] In this sense, we aimed to investigate the spread of P.1 and P.2 variants in samples from hospitalized patients (HP) and healthcare workers (HCW) attended in a university hospital in São Paulo city. This study was conducted in compliance with institutional guidelines, approved by the Ethics Committee of São Paulo Federal University (CEP/UNIFESP n. 29407720.4.0000.5505).

From March 1st to March 15th, 427 nasopharyngeal samples were collected from 245 HP and 125 from HCW outpatients (25.5% and 23.2% of positivity rate, respectively). We then selected 60 samples with RT-PCR Ct values ≤30 (38 samples from HP, and 22 from HCW) for whole genome sequencing (WGS). All HCW presented only mild symptoms and did not need hospitalization.

Of the 60 selected samples, 52 WGS were generated (30 from HP and 22 from HCW) following the sequencing protocol using the Illumina MiSeq platform and the analysis pipeline described by Resende et al.[8] The SARS-CoV-2 lineages were classified according to the proposed Phylogenetic Assignment of Named Global Outbreak LINeages (PANGOLIN) nomenclature.[9] The generated WGS has been deposited at the EpiCoV database on GISAID (https://www.gisaid.org/) under accession numbers EPI_ISL_1464630 to EPI_ISL_1464677.

Of the 52 sequenced samples, 44 (84.4%) were identified as VOC P.1; 5 (9.2%) as VOI P.2; 1 (1.9%) as B.1.1.7, and 2 (3.8%) B.1.1.28 (Fig. 1). The most notable variants circulating in the second wave, including B.1.1.7 (detected first in the United Kingdom) and B.1.1.351 (detected first in South Africa), and P.1, are related to an increase in transmissibility.[2,10] Interestingly, the P.1 variant was first identified in Manaus, State of Amazonas, about 3800 km apart from São Paulo.[5] It is evident that the P.1 variant prevailed during the first two weeks of March, showing a regular distribution among HP and HCW with no difference in terms of age, sex, vaccination, and outcome (Table 1). From the first to the second weeks of March, we observed a higher frequency of P.1 (78.6% and 91.7%, respectively). In this survey, only one sample from an HP was identified as VOC B.1.1.7. The other two samples were identified as B.1.1.28, a widely spread lineage during the first wave in Brazil.

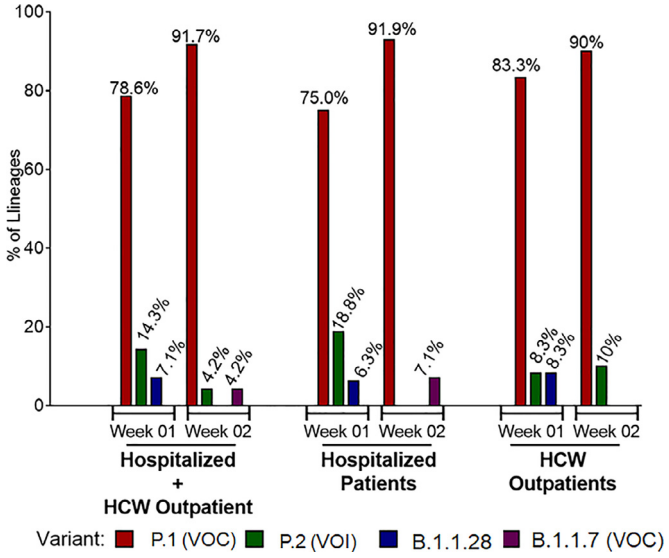

Fig. 1. Frequency of the VOC P.1 and other SARS-CoV-2 lineages among the first two weeks of March 2021 in São Paulo.

Table 1
Characteristics of individuals identified with the VOC P.1.

	Hospitalized Patients	HCW Outpatients
P.1	**25**	**19**
Age (years)	66 (33–86)	29 (19–64)
Sex		
Male	14	5
Female	11	14
Vaccinated	1	10
Death	10	–
Discharged	15	19
Other variants	5	3
Age (years)	53 (36–71)	23 (20–31)
Sex		
Male	2	–
Female	3	3
Vaccinated	–	3
Death	2	–
Discharged	3	3

There is a broad discussion about whether the available vaccines against SARS-CoV-2 will be less effective at preventing infec-

tion with the emerging variants.[10] In this work, 14 samples (26.9%) of the 52 WGS samples were from individuals that had received at least one dose of vaccine, ChAdOx1-S/nCoV-19 ($n = 2$) or SINOVAC ($n = 26$). Although they were vaccinated, they could not be considered immunized, regarding the days after vaccination.

Among the hospitalized patients, 19 (63%) were admitted to the intensive care unit, from which nine were discharged and ten died. Comparing the RT-PCR Ct values of all attended patients since the first wave, we did not observe any difference in the Ct mean values with those of P.1 (data not shown). May 2020 registered the peak of the number of positive cases with a Ct mean of 23.6. Now, as of April 2021, we are facing a rise in the number of cases. However, the Ct mean was 24.9, which may indicate that the spread of P.1 does not contribute to an actual increase in the viral load.

There is still a need for more epidemiologic surveys to assure the role of the VOCs in transmission and escape to neutralizing antibodies. Our findings emphasize that the P.1 variant has spread widely throughout the country. Despite all actions of interventions such as the use of masks, physical distancing, flight travel reductions, and the currently established lockdown in São Paulo, the frequency rates of P.1 increased significantly in two weeks, evidencing its fast spread.

Disclosure statement

No potential conflict of interest was reported by the authors.

Declaration of Competing Interest

None.

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Concerns about the clinical usefulness of saliva specimens for the diagnosis of COVID-19

Dear Editor,

Recently, saliva, the main source of the human-to-human transmission of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), has been proposed as an alternative material for the diagnosis of coronavirus disease 2019 (COVID-19).[1] A large number of studies have demonstrated that saliva samples are more sensitive than nasopharyngeal (NP) swab specimens for the detection of SARS-CoV-2, and it has also been well documented that saliva is a suitable alternative to an NP swab. However, studies comparing saliva samples to NP swabs have reported conflicting results.[2] In some studies demonstrating the usefulness of saliva specimens, the study population was limited to asymptomatic or mild COVID-19 cases.[3,4]

To understand the performance of saliva samples in more detail, we evaluated the diagnostic concordance of saliva specimens derived from COVID-19 patients with molecular diagnostic tools and an automated SARS-CoV-2 antigen test, both widely used in diagnostic laboratories. Thirty-five Japanese patients, who were ad-

mitted to Saitama Medical University Hospital in Japan from June 5 to July 31, 2020, were enrolled in this prospective study after being diagnosed with COVID-19. Their definitive diagnosis was confirmed with reverse transcription quantitative PCR (RT-qPCR) using NP swab specimens in accordance with the nationally recommended protocol[5]. After hospitalization, all participants were instructed to collect saliva samples repeatedly in a sterile sputum container before lunch after not eating or drinking for 30 min until approximately 2 mL of saliva was obtained. They were required to produce one sample every 3 days for up to 2 weeks. All participants were informed that they could withdraw participation at any time and sampling ended at discharge.

The saliva specimens were subjected to the following three procedures: (1) RNA extraction for a nucleic acid amplification test for SARS-CoV-2; (2) direct reverse transcription loop-mediated isothermal amplification (RT-LAMP) without RNA extraction; and (3) a LUMIPULSE® SARS-CoV-2 Ag test kit (Fujirebio, Tokyo, Japan) with the chemiluminescence enzyme immunoassay method, as described previously.[6] For RNA extraction, the samples were diluted 1:1 with 1 × phosphate-buffered saline (PBS) and homogenized by vortexing for 15 s. Subsequently, RNA extraction was performed with a QIAamp Viral RNA Mini Kit (Qiagen, Hilden, Germany) following the manufacturer's instructions. RNA was finally eluted in 60 μL of the provided AVE buffer. RNA was used as a template for the RT-qPCR and RT-LAMP methods.

Conventional RT-qPCR for the specific amplification of the N2 gene of SARS-CoV-2 was performed using TaqMan-based real-time PCR.[5] High-speed RT-qPCR amplification using GeneSoC® was also performed using SpeedSTAR HS DNA Polymerase (Takara Bio, Inc., Shiga, Japan) with the same primers and probe used for the conventional RT-qPCR.[7] Amplification of SARS-CoV-2 RNA with RT-LAMP was performed with a Loopamp® 2019-SARS-CoV-2 Detection Reagent Kit (Eiken Chemical, Tokyo, Japan) at 62.5 °C for 35 min according to the manufacturer's instructions.[8] For direct RT-LAMP without RNA extraction, the specimen was pretreated with a Viral RNA Extraction Kit (Eiken Chemical). The LUMIPULSE® SASR-CoV-2 Ag test was performed on a LUMIPULSE® G1200 (Fujirebio) according to the manufacturer's instructions, and the amount of SARS-CoV-2 antigen was obtained based on the quantitative intensity of the reaction signal. Briefly, each saliva sample was mixed 1:1 with 1 × PBS and centrifuged at 20,000 × g for 10 min. The supernatant was used for subsequent analysis on the LUMIPULSE® G1200 automated system.

A total of 85 saliva specimens were collected from 35 hospitalized COVID-19 patients. The median age was 37 years (interquartile range [IQR]: 29–55), and 12 patients were female. At the time of sampling, 8 patients were receiving oxygen and 4 patients were asymptomatic carriers. Finally, 1 of the 8 patients receiving oxygen progressed to severe disease requiring invasive mechanical ventilation. Of the 85 saliva specimens, 48 (56.5%) were positive according to RT-qPCR and the median Ct of the samples was 29.3 (IQR, 33.7–37.1). Of those specimens, 54 were collected from 30 patients during the early phase, which was defined as within 9 days of symptom onset, and the remaining 31 samples from 14 patients during the late phase, which was defined as 10 or more days after symptom onset (Table 1). Samples derived from patients of the early and late phases of COVID-19 tested positive by conventional RT-qPCR at 59.3% and 48.4%, respectively, but there was no difference in the detection rates of viral RNA at both phases ($p < 0.371$). Samples from the early and late phases tested positive by high-speed RT-qPCR, RT-LAMP, direct RT-LAMP, and LUMIPULSE at rates of 41.9–63.0%, 45.2–72.0%, 35.5–50.0%, and 25.8–46.3%, respectively. There was no difference in the detection rate of viral RNA in saliva from each phase with any of the detection methods (Table 1).

The results of the comparative evaluation of RT-qPCR and other standard diagnostic methods using saliva specimens are summarized in Table 2. In this study, the κ coefficient value showed diagnostic agreement between RT-qPCR and all tests performed using saliva.

The detection rate with all nucleic acid amplification tests and fully-automated antigen testing using saliva specimens evaluated in this study was significantly lower than those in previous reports, demonstrating positive rates of 90% or higher.[9] Above all, the oral cavity is kept clean and wet by salivary flow, and normal physiological activity is maintained by a saliva washout mechanism.[10] Our study suggested that a decrease in saliva production and changes in the oral environment that depended on the patient's conditions, including their age, symptoms such as fever, and disease severity, could affect the detection rate of viral RNA and antigens. The procedure for sampling saliva is advantageous compared with the use of NP swabs: it can be performed easily, does not require specialized personnel, and is comfortable compared with NP swab sampling. However, in a pandemic situation where there is a need to accommodate a large number of patients, it is difficult to standardize the conditions for saliva collection. Further validation is still needed to conclude if saliva-based screening tests for COVID-19 should always be recommended.

Declaration of Competing Interest

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Table 1
Summary of the results of molecular diagnostic tests and the antigen test for COVID-19 using saliva specimens.

Methods	Positive sample no. (%) at the indicated collection time since the onset of symptoms		
	Early phase (≤9 days, $n=54$)	Late phase (≥10 days, $n=31$)	p-value
RT-qPCR	32/54 (59.3)	15/31 (48.4)	0.371
High-speed RT-qPCR	34/54 (63.0)	13/31 (41.9)	0.073
RT-LAMP	39/54 (72.2)	14/31 (45.2)	0.020
Direct RT-LAMP	27/54 (50.0)	11/31 (35.5)	0.258
LUMIPULSE SARS-CoV-2 Ag test	25/54 (46.3)	8/31 (25.8)	0.070

Table 2
False positive and false negative results when compared with RT-qPCR using saliva specimens.

	RT-qPCR		Overall agreement	Kappa value (95% CI)
	Positive $n=48$	Negative $n=37$	(%) (95% CI)	
High-speed RT-qPCR				0.833
Positive	44	3	91.8	(66.3–
Negative	4	34	(83.7–96.6)	100)
RT-LAMP				0.83
Positive	47	6	91.8	(66.0–
Negative	1	31	(83.7–96.6)	100)
Direct RT-LAMP				0.721
Positive	37	1	85.9	(55.1–
Negative	11	36	(76.6–92.4)	89.1)
LUMIPULSE				0.633
Positive	33	1	81.2	(46.3–
Negative	15	36	(71.2–88.8)	80.3)

CI, confidence interval.

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First isolation of vancomycin-resistant *Enterococcus faecalis* from cattle and swine in Xinjiang, China

Dear Editor,

A recent article in this Journal pointed out that tigecycline- and vancomycin- resistant *Enterococcus faecalis*(*E. faecalis*) associated with worse clinical outcome and pose a big risk factor to public health[1]. Actually, *E. faecalis* are important nosocomial pathogens, causing diseases such as endocarditis, bacteremia, urinary tract infections and acute cholangitis in humans.[2,3] Renctenly, many evidence had indicated that *E. faecalis* may be a zoonotic pathogen with potentially highly significant influence on human health.[4_6] It had been isolated from different animal species, such as swine, poultry, cattle and horse.[7,8] Moreover, pathogenic isolates are often multidrug resistant, and display virulence factors such as hemolysis, extracellular gelatinaseand adhesion of collagen.[9,10] So it is important to carry out an epidemiological survey and etiological study on the zoonotic pathogen in different animal which would be a potential risk for public health.

To investigate the resistance phenotype and the prevalence of virulence genes of *E. faecalis* isolated from animals in Xinjiang, China, a total of 287 *E. faecalis* isolates, obtained from 650 faeces samples taken from swine, bovine, sheep, chicken and dung beetle between 2016 and 2019 in different areas of Xinjiang, China. All of the isolates were screened for the presence of nine virulence genes(*Ace, asa1, asa373, efaA, cylA, gelE, sprE, esp*, and *hyl*). Detection of these all above virulence genes by PCR showed that the positive rate of *gelE* gene was the highest(123/287 = 42.86%), the detection rates of other virulence genes from high to low were *sprE* (60/287 = 20.91%), *esp*(34/287 = 11.85%), *asa1*(20/287 = 6.97%), *hyl*(19/287 = 6.62%), *Ace*(18/287 = 6.27%), *asa373* (17/287 = 5.92%), *cylA* (17/287 = 5.92%), *efaA* (12/287 = 4.18%) (Table 1). Notably, some isolates (35/287 = 12.20%) contained three or more virulence genes, which may greatly contribute the infection of the *E. faecalis.*

Vancomycin-resistant Enterococci (VRE) are a major public health problem worldwide, since they are commonly implicated in nosocomial infections in various regions in the world.[11] In order to investigate the distribution of VRE from different resources, the DNA from all the isolates were amplified with primers specific for vancomycin resistance genes(VanA,VanB,VanC,VanD,VanE,VanG,VanL,VanM and VanN) as previous.[12] Results show that two isolates from swine and cattle were detected positive as VanC type, named JZ15 and D4, respectively. In order to further understand the antibiotic resistance patterns of the two VRE strains, the susceptibility to different antibiotics were determined using the disk diffusion method on Mueller–Hilton agar plates according to recommendations of the clinical and laboratory standards institute (CLSI). The results show that swine isolate JZ15 was resistant to penicillin, amoxicillin, streptomycin, erythromycin, tetracycline and vancomycin. The cattle strain D4 was resistant to penicillin, amoxicillin, streptomycin, erythromycin, tetracycline, ciprofloxacin and vancomycin. Consequently, the two VRE isolates all are the multidrug-resistant (MDR) bacteria. Moreover, both of them carried more than two virulence genes (Table 2).

In this study, we reported the first case of Vancomycin-resistant *E. faecalis* isolated from different animals in Xinjiang, China. Additionally, as a zoonotic pathogen, both of the VRE isolates are MDR bacteria and contain multiple virulence genes, which may contribute to easily infection and then may posed a high threat to animals and humans. These findings suggest that *E. faecalis* from swine and cattle should be cause concerned because they can be reservoirs for antimicrobial resistance and virulence genes. The measure should be put into effect to address this public health threat and more surveillance in animals and humans should be carried out.

Declaration of Competing Interest

All authors: No conflicts.

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Asymptomatic COVID-19 cases among older patients despite BNT162b2 vaccination: A case series in a geriatric rehabilitation ward during an outbreak

Dear Editor,

We read with great interest the work of Tré-Hardy et al.[1] in your pages, studying antibody response in healthcare workers after one and two doses of SARS-CoV-2 mRNA-1273 vaccine. As with other mRNA vaccines[2], they found high antibody titers following vaccination, which has been associated with effectiveness in preventing symptomatic disease in clinical trials.[3,4]

The SARS-CoV-19 pandemic is having a dramatic impact,[5] particularly on the elderly, and there is great hope that vaccination against SARS-CoV-2 will reduce mortality and ease the burden of the disease.[6] However, the impact of vaccination on the spreading of the disease is still not well known.[7] In particular, the effectiveness of vaccines for reducing asymptomatic SARS-CoV-2 infection is unknown. This aspect is important, because asymptomatic infection is a major contributor to viral transmission.

In this regard, we observed four patients who developed asymptomatic SARS-CoV-2 infection despite previous complete vaccination with BNT161b2, an mRNA vaccine. All were inpatients in a 40-bed geriatric rehabilitation ward, where a cluster of B.1.1.7 (VOC-202012/1) variant COVID-19 cases occurred. Over a period of

7 days, eight symptomatic cases happened among patients on this ward (Table 1), one of them in a vaccinated patient. In addition, seven cases occurred among ward staff. In an effort to limit the spread of the infection, all inpatients underwent nasal swabbing for reverse transcriptase polymerase chain reaction (RT-PCR) testing for SARS-CoV-2, immediately and 7 and 14 days afterwards. Of the 32 patients without clinical signs, eight had a positive SARS-CoV-2RT-PCT. Four of them had previously completed two doses of BNT161b2 vaccine (Table 1). The characteristics of previously vaccinated patient, four asymptomatic and one with symptoms, are shown in Table 2. None of the asymptomatic patients developed any symptom of COVID-19 during the follow-up or had negative outcomes. The patient with symptomatic COVID-19 infection despite previous BNT161b2 vaccination was immunocompromised due to a hematological condition (chronic lymphoid leukemia) and developed a moderate COVID-19 related pneumonia, from which he recovered.

This small case series shows that frail older patients vaccinated with BNT161b2 can develop asymptomatic SARS-CoV-2 infection and thus participate to viral transmission. In fact, it is striking to note that the attack rate was the same between vaccinated and unvaccinated patients (Table 1), the main difference being that the proportion of asymptomatic cases was much higher between vaccinated patients. This observation, however, was obtained in the context of a COVID-19 outbreak in a geriatric rehabilitation ward. The four patients described here had received a full vaccination with the BNT161b2 vaccine and the delay between their first dose and the outbreak was sufficient to allow a complete immunization. All four were fully asymptomatic at all times, despite being very old, having several comorbidities and being infected by the B.1.1.7 variant, which is associated with a higher risk of severe disease and mortality[8]. Three of them were tested again 12 days after and SARS-CoV-2 was detected in only one patient by RT-PCR in the nasal swabs.

Asymptomatic infection is a matter of concern from the point of view of epidemic control, as they can transmit the virus without being aware.[9,10] Recently, Tande et al. analyzed the results of pre-procedural or pre-surgical SARS-CoV-2 screening testing realized in 3 US hospitals as function of the patients' vaccination status for the SARS-CoV-2.[11] They observed asymptomatic SARS-CoV-2 in 1.4% of the individuals who had received SARS-CoV-2 vaccination, mainly the BNT161b2 vaccine. Even if this rate was significantly lower than the rate observed among unvaccinated persons (3.2%), their findings show that asymptomatic SARS-CoV-2 infection may occur in vaccinated persons, even out of the context of an outbreak, like in our case series. In contrast, Benenson et al. have found a dramatic decrease of new SARS-CoV-2 infections in healthcare workers after vaccination, to less than 1 case per 1000 workers tested.[12]

The number of patients we report is very small and they occurred in a specific setting, so it is not possible to draw any generalizable conclusion. However, these findings suggest that asymptomatic SARS-CoV-2 infection may be frequent in vaccinated frail older patients, and that the main effect of vaccination in this population might be a decrease of the severity of the disease rather than completely avoiding it. That has implications when designing measures for limiting the spread of SARS-CoV-2. Further studies are needed to determine the importance of asymptomatic SARS-CoV-2 infection among vaccinated persons in the transmission of the disease.

Declaration of Competing Interest

J.B. received personal fees from Pfizer and Novartis.
C.D., A.R., A.G. and C.L.L have no interest to declare.

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Effect of varying storage conditions on diagnostic test outcomes of SARS-CoV-2

Dear Editor,

Iwasaki et al. previously demonstrated the usefulness of saliva polymerase chain reaction (PCR) in diagnosing coronavirus disease (COVID-19).[1] Thereafter, several studies reported the simplicity and usefulness of saliva PCR and antigen tests.[2,3] However, the most appropriate method for storing saliva specimens remains elusive.[4]

Any samples for COVID-19-related investigations should ideally be processed immediately after procurement. However, limited availability of measuring equipment and manpower may warrant the need to preserve samples in certain conditions until they are tested. Additionally, preserved samples may contribute to retrospective research. However, the differences in the results of several COVID-19-related tests under variable storage conditions (temperature, freezing/thawing, and solute medium) are not evaluated. We aimed to examine how variations in sample storage conditions affected results of antigen testing, PCR, and virus infectious titers for SARS-CoV-2. Antigen testing was performed by LUMIPULSE® (Fujirebio, Inc.,Tokyo, Japan).[5,6] We analyzed specimens from eight patients who were examined at Hokkaido University Hospital from May 2020 to January 2021. The correspondence between patients and specimen numbers is as follows: Patient1 (swab1, saliva1), Patient2 (swab2), Ptatient3 (saliva2), Patient4 (swab3), Patient5 (saliva3), Patient6 (saliva4), Patient7 (saliva5), Patient8 (saliva6).

Nasopharyngeal samples were obtained using FLOQSwabs® (COPAN, Murrieta, CA, USA). The swab was inserted into the nostril up till the posterior nasopharynx and then gently removed while rotating. Thereafter, the swabs were placed in saline prior to evaluation. Saliva samples were self-collected by the patients. We instructed them to spit saliva o sterile PP Screw cup 50® (ASIAKIZAI Co., Tokyo, Japan) that naturally accumulates in the mouth into a cup twice and not to eat, drink, gargle, or brush their teeth for 10 min before specimen collection. Subsequently, 200 μL saliva was added to 600 μL phosphate-buffered saline, mixed vigorously, and centrifuged (20,000 x g; 5 min; 4 °C); the supernatant (140 μL) was used as a sample.

To investigate the PCR cycle threshold (Ct) values and antigen titers, swabs (swab1, 2) placed in saline were diluted using saline or CELLBANKER® (ZENOAQ RESOURCE Co., Fukushima, Japan), then

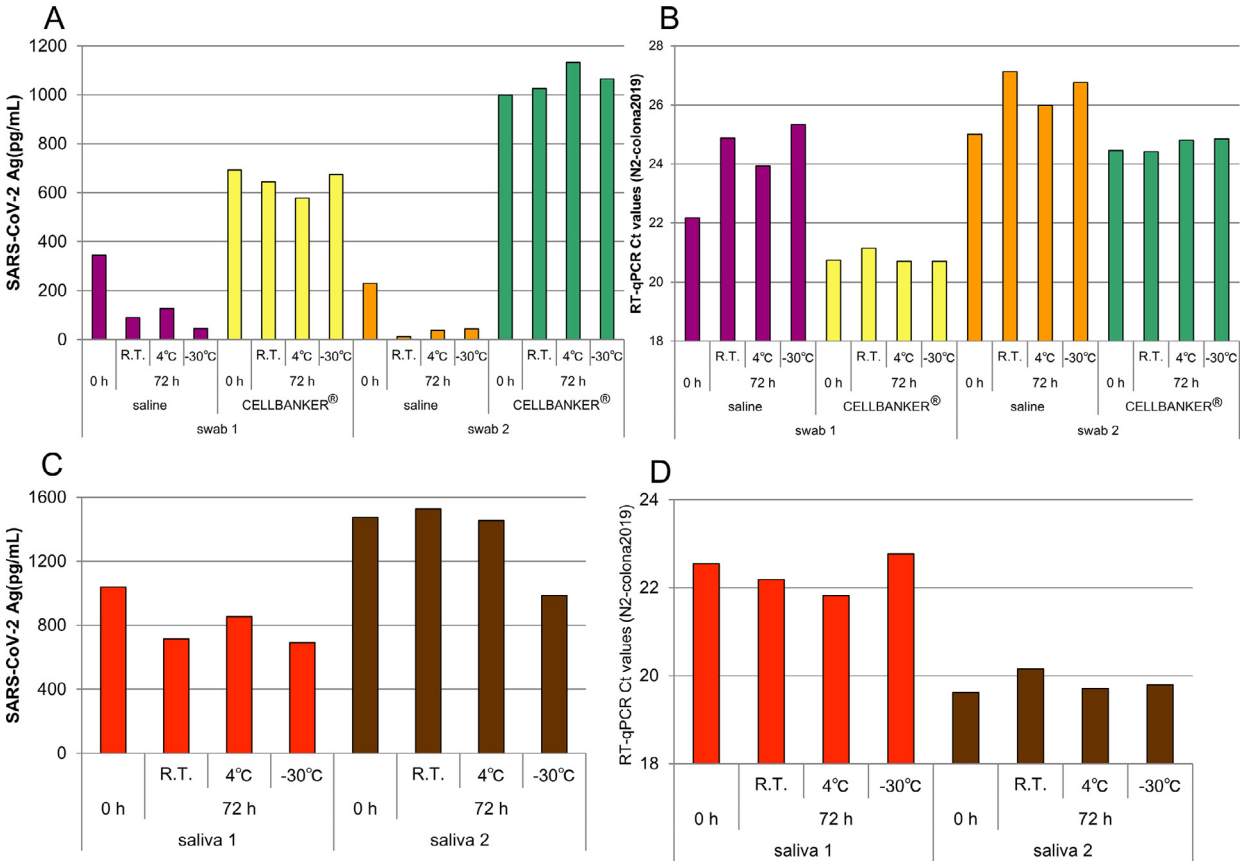

Fig. 1. Changes in virus antigen titer and PCR Ct values in Nasopharyngeal swabs and saliva samples. (A) virus antigen titer of nasopharyngeal swab, (B) RT-qPCR Ct value of nasopharyngeal swab, (C) virus antigen titer of saliva, (D) RT-qPCR Ct value of saliva.

preserved either at ambient temperature (AT), 4 °C, or −30 °C. Saliva samples (saliva1, 2) were preserved either at, 4 °C, or −30 °C, and diluted with PBS immediately before testing.

To investigate whether freezing and thawing affected virus cultures, we measured the virus infectious titer of one swab (swab 3) and four undiluted saliva (saliva 3,4,5,6) samples before freezing and after freezing and thawing. The detailed methodologies are reported previously[7] and in online supplementary material.

Comparing the stability of saline and CELLBANKER® as preservation media for nasopharyngeal swabs

Fig. 1A shows the changes in antigen titers of the samples preserved in saline and CELLBANKER®. The antigen titers for samples preserved in saline decreased until 72 h (AT and 4 °C). Contrastingly, no such reduction was seen for samples preserved in CELLBANKER® (AT and 4 °C). When samples were frozen at −30 °C and then thawed, the antigen titers decreased in samples preserved with saline but not in those preserved with CELLBANKER®. However, Fig. 1B shows that the PCR Ct values remained unchanged under all preservation conditions.

Our study revealed that antigen titers were significantly lower in nasopharyngeal swab samples that were preserved in saline than in CELLBANKER®. This may be due to the trapping of antigen on the wall of the tube for non-specific binding with the former method. The decrease over time in antigen titers of samples preserved in saline relative to those preserved in CELLBANKER® persisted after 72 h (supplemental data). Therefore, preserving nasopharyngeal swab specimens in saline may incur false negative results.

CELLBANKER® was developed to prevent the degradation of nucleic acids and protect the cell membrane, particularly during experiments. Although the exact mechanism influencing the virus' stability remains unclear, our findings indicate that CELLBANKER® is more effective than saline as a preservation medium for nasopharyngeal swab samples collected for antigen testing.

Stability of saliva samples in varying preservation conditions

For saliva samples preserved at AT and 4 °C, antigen titers and Ct values remained unchanged even after 72 h (Fig. 1C). Interestingly, for samples frozen at −30 °C and thawed, antigen titers decreased (Fig. 1C), whereas Ct values remained unchanged (Fig. 1D).

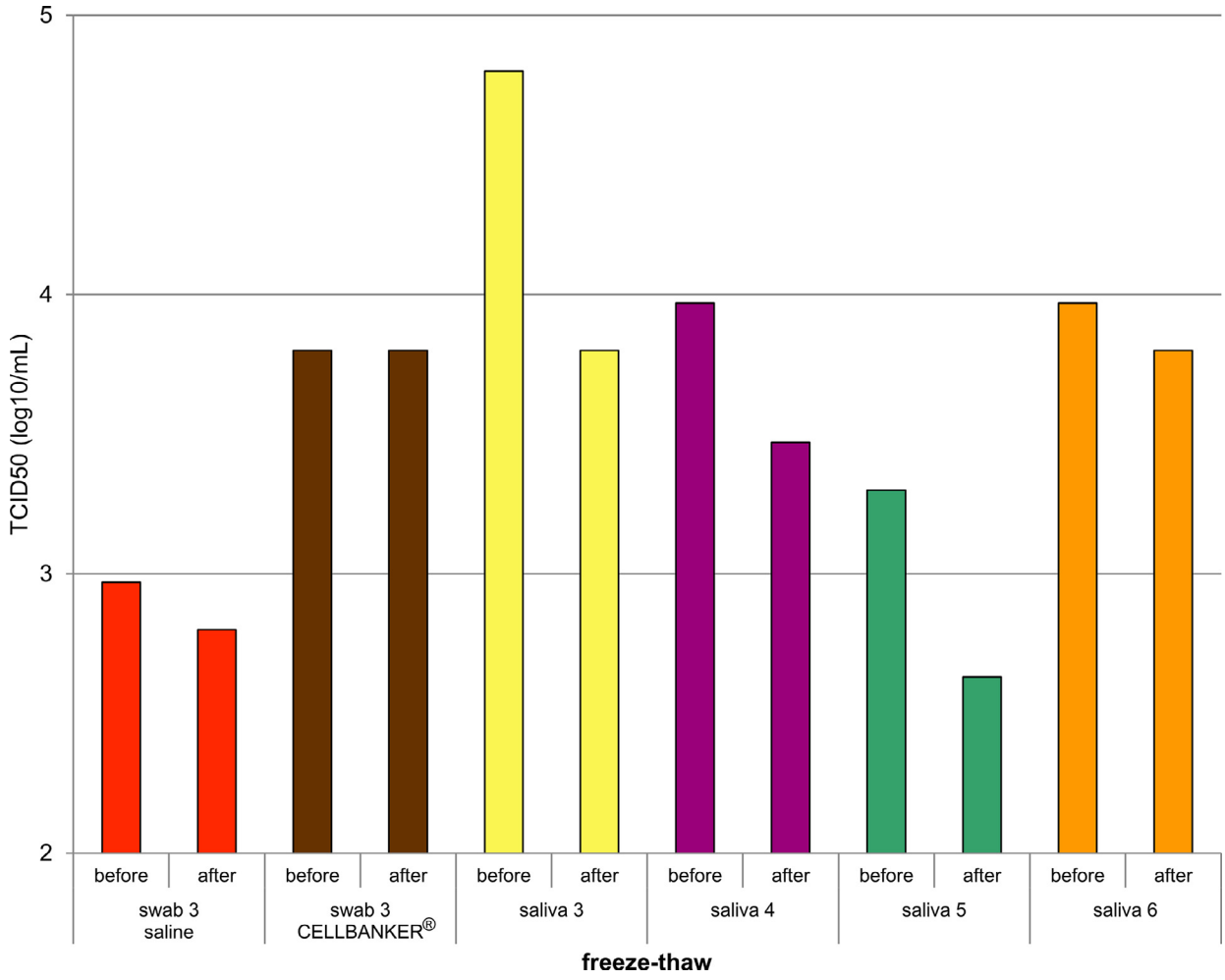

Fig. 2. Changes in culture virus titer before and after freezing and thawing.

Effects of specimen storage methods and freezing and thawing on culture tests

Fig. 2 shows the comparison of virus titers before freezing and after freezing and thawing in various storage solutions. When nasopharyngeal swab specimens were stored in CELLBANKER®, the infectious titers were unaffected; however, when stored in saline, the infectious titers decreased after freezing and thawing. Freezing and thawing of saliva samples also reduced infectious titers in culture tests.

Our results highlight the vulnerability of saliva samples to freezing and thawing. Although saliva was inferior to CELLBANKER® as preservation media, it maintained higher antigen titers than saline did. However, when the specimens were frozen and thawed, the titers decreased. Additionally, virus titers in the cultures may also decrease compared to that before freezing. Notably, freezing and thawing of saliva specimens may result in false negative results in antigen and culture tests.

The epitope recognized by LUMIPULSE® is speculated to be on the virus surface envelope. Therefore, freezing and thawing saliva degrades the epitope. We also found that saliva can be preserved for at least 72 h in AT or 4 °C. Contrastingly, the Ct value detected using PCR remained the same across all preservation conditions.

Our study has several limitations. First, the sample size is small due to the limited volume of cases at early phase of the disease, wherein the viral load and infectiousness quotient is high. This is because government policy demands suspected patients to either stay home or move into accommodation facilities after disease onset. It was difficult to collect nasopharyngeal swab specimens with positive viral cultures, because our institution recommended testing saliva specimens, and we were not able to match the number of both specimens. Second, there is the issue of heterogeneity of saliva as a medium. Salivary adulterants vary greatly among individuals, and the effect of freezing and thawing may also vary greatly among individuals. In fact, we found that the antigen titer decreased in saliva1 even before freezing.

Despite the limitations, the findings may greatly benefit clinical setting scenarios where antigen testing for COVID-19 cannot be performed immediately. Future studies must further investigate better storage solutions for saliva specimens.

Declaration of Competing Interest

The authors declare that they have no competing interests.

Ethics

This study was approved by the Institutional Ethics Board (Hokkaido University Hospital Division of Clinical Research Administration Number: 020–0116 and 020–0111), and informed consent was obtained from all patients. All patients enrolled in this study were from the Hokkaido University Hospital.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.jinf.2021.03.026.

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Retrospective serosurveillance for anti-SARS-CoV-2 immunoglobulin during a time of low prevalence: A cautionary tale

Dear Editor,

We read with interest the study in the Journal by Gurgel and colleagues from Sergipe, Brazil investigating asymptomatic circulation of SARS-CoV-2 in Northeastern Brazil prior to the first case reported on the 26th February 2020.[1] The authors obtained 987 anonymised serum samples collected between January and April 2020 and tested them for anti-SARS-CoV-2 IgG and IgM antibodies using two in vitro diagnostic tests: the Nantong lateral flow immunochromatography test and iChroma2 lateral flow sandwich detection immunofluorescence COVID-19 antibody test. The study found 16 (1.6%) participants who tested positive on both assays with seven (43.8%) being IgM positive, three (18.8) IgG positive, and six (37.5%) positive for both. The authors state that positive samples were not screened for the presence of SARS-CoV-2 antigen. The paper concludes that SARS-CoV-2 may have been circulating among the lower income population of the southern districts of Sergipe prior to the first confirmed cases.

A similar paper by Apolone and colleagues detailed the retrospective testing of 959 asymptomatic individual samples from Italy between September 2019 to March 2020 when the first confirmed case occurred in February 2020.[2] Using a receptor-binding domain specific enzyme-linked immunosorbent assay (ELISA), which detects Spike (S) protein immunoglobulin, the study found 111 (11.6%) positive samples, with the majority being in February (20%) followed by October (16.3%). The authors claim that SARS-CoV-2 could have been circulating in a high rate of asymptomatic carriers prior to the first confirmed case.

In a study similar to Gurgel and Apolone, we present the data from a small retrospective serosurveillance project of the Leicestershire patient population. 428 randomly selected serum samples (mean age: 39, s.d 23.3, range 0–95) from 1st October 2019 to 31st March 2020 were selected for serological testing. All samples were from patients undergoing viral serological screening and had previously been stored at −40 °C. All samples were thawed at 4 °C prior to testing on the DiaSorin SARS-CoV-2 S1/S2 Assay (Diasorin Ltd., Dartford, England) a chemiluminescent assay (CLIA) detecting IgG antibodies to the SARS-CoV-2 spike protein S1/S2 domain and all positive samples repeated on the Siemens Aptima SARS-CoV-2 Total Assay (Siemens, Erlangen, Germany) an ELISA detecting IgG and IgM antibodies to the SARS-CoV-2 spike protein S1 receptor-binding domain. Diasorin state a sensitivity and specificity of 97% (95% CI 86.8–99.5%) and 98.9% (97.5–99.2%), Siemens state theirs as 96.4% and 99.9% respectively. The Diasorin and Siemens assays were directly compared in a study analysing the performance of five SARS-CoV-2 serological assays with Diasorin being reported to have a sensitivity and specificity of 95% (92.8–96.7) and 98.6% (97.6–99.2) and Siemens as 98.1% (96.6–99.1) and 99.9 (99.4–100).[3]

Our screening revealed 10 (2.3%) anti-SARS-CoV-2 IgG positive samples using the Diasorin assay; of which 5 were from patients sampled in 2019 (Table 1). However, upon testing on the Siemens assay all 10 samples were anti-SARS-CoV-2 IgG/IgM negative, all samples were tested in triplicate with no inconsistency in results. Additional archived samples suitable for SARS-CoV-2 PCR testing from IgG positive patients were sought, but none were available.

A systematic review by Bastos and colleagues examined 40 studies on the diagnostic accuracy of serological testing for COVID-19[4]; examining the overall sensitivity and specificity of samples using ELISAs, lateral flow immunoassays (LFIAs) and CLIAs. They reported that LFIAs have demonstrated the lowest sensitivity (66%) compared to ELISA (84.3%) and CLIAs (97.8%), but that specificity varied minimally between methods (96.6–99.7%). Although Bastos and colleagues highlight a high level of agreement between the methods, they note a high risk of patient selection bias for 98% of the analyses in the 40 studies, because most studies selected cases and controls from different populations and did not use random or consecutive sampling.[4]

This has significant implications for the interpretation of assay performance characteristics, as the prevalence of infection in these studies will not be reflective of those in populations where these assays may be applied. Whilst there is a clear connection between prevalence and the positive and negative predictive values of a diagnostic test, prevalence can also impact sensitivity and specificity due to the spectrum effect.[5] This is particularly important where the comparator test used to define the gold standard is imperfect (such as using SARS-CoV-2 PCR results to define likely anti-SARS-CoV-2 antibody status) and when the test is used in a population where prevalence may differ significantly from those in the initial evaluations (such as a lookback exercise to identify potential early cases of SARS-CoV-2 infection).[5] Care must therefore be taken when interpreting results from SARS-CoV-2 serological assays in scenarios where prevalence differs significantly from those in the original evaluations, as seen in our data when samples were taken during a period of very low prevalence, resulting in 10 potential false positive results using the DiaSorin assay.

Our study and those mentioned highlight the difficulties of interpreting serological results during low levels of prevalence but also the need for additional molecular testing. A positive or negative PCR result gives strength to serological results; however, when working with a novel agent molecular testing can be difficult to perform given the required time to develop a specific assay.[3,6]

Gurgel used two LFIAs during their study but did not use a different serological technique for confirmation. Apolone used a single ELISA test and again did not use a different serological technique for confirmation. We used two separate assays which employ different serological detection techniques revealing that reliance on a single technique can lead to false results, a problem exacerbated during periods of low prevalence.

Our study along with Gurgel and Apolone were conducted retrospectively when SARS-CoV-2 would have had a low prevalence within the population and be at risk of higher rates of false positive results. Therefore, while high specificity of these antibody tests during periods of higher disease prevalence makes them ideal for seroprevalence work assessing how widely spread COVID-19 is within the population and determine the degree of asymptomatic transmission, caution must be urged in their use for identifying COVID-19 introduction to a population.

Authors' contributions

PWB and JWT conceived the original study idea. PWB and OF performed the testing of samples. PWB and VB performed the analysis of data. All authors discussed the result, reviewed and revised drafts of the manuscript and gave final approval to submit the manuscript.

Ethical approval and consent to participate

Ethical approval and patient consent was not required for this study.

Declaration of Competing Interest

No authors have any conflicting interests to declare.

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Table 1
Characteristics of SARS-CoV-2 IgG positive patients from retrospective serum sampling of Leicester population.

Patient	Sex	Age When Sample Taken (years)	Alive/ Deceased	Co-morbidities	Symptoms	Recent Travel History	Serum Collection Date	DiaSorin SARS-CoV-2 S1/S2 Assay Results	Siemens SARS-CoV-2 IgG Assay Results
1	F	3	Alive	Non-resolving tonsillitis	Fever, sore throat, inability to ingest solids	Unknown	29/10/2019	Positive	Negative
2	F	5	Alive	Unknown	Unknown	Unknown	07/11/2019	Positive	Negative
3	M	18	Alive	Unknown	Asymptomatic	Unknown	22/11/2019	Positive	Negative
4	F	52	Alive	None	Asymptomatic	Unknown	04/12/2019	Positive	Negative
5	M	35	Alive	Hepatitis B Positive	Asymptomatic	Unknown	04/12/2019	Positive	Negative
6	M	0	Alive	Unknown	Unknown	Unknown	14/02/2020	Positive	Negative
7	F	37	Alive	Medical Termination of Pregnancy	Vomiting and abdominal pain	Unknown	19/02/2020	Positive	Negative
8	M	60	Alive	Latent TB, hypertension, gout	Asymptomatic	Unknown	22/02/2020	Positive	Negative
9	F	38	Alive	Endometrial polyp	Unknown	Unknown	04/03/2020	Positive	Negative
10	M	56	Alive	ESRF, T2DM, chronic osteomyelitis of right foot	Unknown	Unknown	11/03/2020	Positive	Negative

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First report of Autochthonous Furuncular Myiasis caused by *Dermatobia Hominis* in Europe.

Dear Editor,

In this Journal, Chong and colleagues reported evidence that global warming is influencing influenza epidemiology.[1] We believe this climatological change could be determinant for the geographic distribution of parasitic diseases.

Myiasis is defined as the infestation of vertebrate animals by dipterous larvae of the genus *Dermatobia* ("human" botfly), *Cordylobia* and *Chrysomia* among others, which feed on the host's dead or living tissue.[2] Skin involvement is the most frequent reported location but, oral, auricular, ocular, nasal, gastrointestinal and genitourinary manifestations have also been described. Cutaneous myiasis presentations include furuncular, migratory and wound myiasis.[2,3]

Each fly species has specific geographical distribution but, in general, they all have a greater abundance in tropical countries.[3] Most reported cases of furuncular cutaneous myiasis in Europe involve infestation by *Dermatobia hominis* in travelers returning from Central and South America.[3,4] Cases of cutaneous myiasis in Europe acquired by patients without a history of recent travel are distinctly unusual.[5]

A 28-year-old previously healthy woman was admitted to the emergency department with a 4-day history of pruritus, foreign body and movement sensation on her leg. Physical examination revealed an erythematous infiltrated nodule of 5–7 mm in diameter, on the posterior aspect of her right leg, which presented a central *punctum* (Fig. 1A). She had no systemic symptoms associated but had noticed what she described as a "small mobile bug coming out from the lesion". She had not recently traveled outside Spain but she had been in the rural area of Valladolid, Castilla y León (Spain), in the vicinity of a horse stable, where she could have possibly noticed an insect bite. An occlusive ointment (petroleum jelly) was applied over the lesion for twenty-four hours without response. We then decided to perform surgical debridement after local anesthesia with the extraction of the larva (Fig. 1B-D). The organism was examined and identified morphologically as a second instar larva of *Dermatobia hominis*. Nor topical either systemic therapy was initiated as no signs of bacterial superinfection were present and her tetanus toxoid vaccination was updated; complete resolution was observed after six months of follow-up.

Table 1
Summary of the most frequent human cutaneous myiasis.

Larvae	Common name	Geographic location	Clinical presentation
Dermatobia hominis	Human bot fly, *torsalo*	Central and South America	Furuncular, solitary
Cordylobia anthropopahga	Tumbu fly, Mango fly	Africa	Furuncular, multiple
Cordylobia rodhaini		Tropical Africa	Furuncular, multiple
Gasterophilus intestinalis	Horse bot fly	Worldwide	Migratory
Hypoderma lineatum	Heel fly, cattle grubs	Northern hemisphere	Migratory, furuncular
Oestrus ovis	Sheep fly	Worldwide	Ophtalmomyiasis, ear-nose-throat myiasis
Curetebra species	Rabbit bot fly, rodent fly	North America	Furuncular
Chrysomya bezziana	Old World screwworm	Africa, India, Asia	Wound

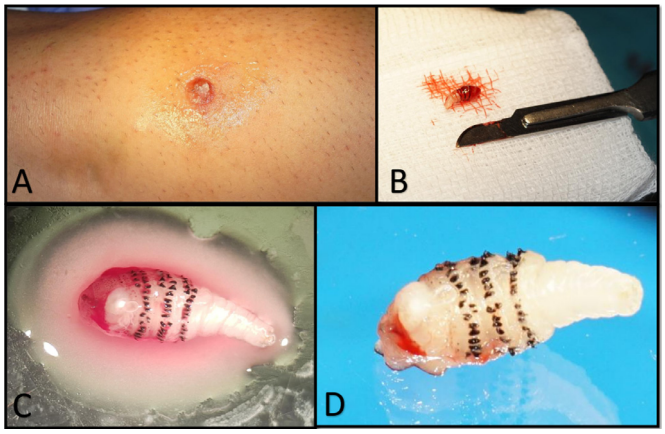

Fig. 1. (A). Clinical image: erythematous infiltrated nodule of 5–7 mm, on the posterior aspect of her right leg; residual petroleum jelly can be noted on the surface of the lesion as it was applied as occlusive ointment. (B-D). Images of *Dermatobia hominis* larva: size and morphological structures of the second instar larva (bottleneck shape, enlarged anteriorly; backward projecting spines encircling the thorax; respiratory spiracles on the posterior abdominal segment).

Dermatobia hominis causes an obligatory myiasis because the parasite depends on its host to complete its life cycle.[2] This cycle is complex as the female fly does need a phoretic vector, usually a mosquito, to carry the eggs to the host tissue. During the blood meal, these eggs penetrate the host's skin.[3] The larvae feed inside a subdermal cavity for 5–10 weeks where they develop as first, second and third instar larvae. Each instar has a distinctive shape.[4] When mature, they emerge from the skin, fall to the ground and pupate in the environment. After a month after abandoning the host, the adults emerge and complete their live cycle.[5]

When there is a high suspicion of cutaneous myiasis, careful observation is important. A preliminary diagnosis can be made by immersing the lesion in water, with bubbling indicating the presence of live insects. If this occurs, dermoscopy of the lesion is recommended.[6] Doppler ultrasound can be very useful in the detection of the larva; an oval-shaped structured with hypoechoic rim and hyperechoic center, spontaneous movement and peripheral blood flow are the features that have been described for the sonographic diagnosis of cutaneous *Dermatobia hominis* myiasis.[7]

Treatment of furuncular myiasis is directed to cause paralysis, asphyxiation or direct removal of the larva.[3,6] A number of original, but mostly unsuccessful, remedies have been described to facilitate the removal of the larva: heavy oil, occlusive ointment, meat… to interrupt the larva's respiration, forcing it to migrate to the surface where it can be removed easily. Local anesthesia has been demonstrated to paralyzed the larva and therefore, facilitating its extrusion. Isolated extrusion is usually ineffective.[7]

Dermatobia is also known as the human botfly, *berne, torsalo* and maggot, among others, depending on the geographical area.[1] This fly has been classified in the *Oestridae* family, which is an endemic species from southern Mexico to northern Argentina. Cutaneous myiasis is an increasing problem in travelers to these tropical countries.[8] However, autochthonous cases of cutaneous myiasis in Europe are very unusual.

Zammarchi et al.[9] reported three cases of human conjunctival myiasis caused by *Oestrus ovis* acquired in Florence, Italy; this fly species is highly widespread in Italy and it is responsible for most autochthonous cases of human myiasis. Rappelli et al.[10] described a case of hypodermal myiasis caused by the infestation of *Hypoderma lineatum* in a 63-year-old farmer, who referred closed contact with cattle and presented with pruritus, migratory edema and papular dermatitis. Each fly species has its characteristic clinical presentation (Table 1). To date, no cases of autochthonous human cutaneous myiasis by any *Dermatobia* species have been described in Europe.

To our knowledge, this is the first report of human furuncular myiasis caused by *Dermatobia hominis* contracted in Europe. Although unusual, this diagnosis should be considered not only in patients returning from tropical countries when classical clinical features are present.

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