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Letters to the Editor

Quantifying the impact of public health protection measures on the spread of SARS-CoV-2

Dear Editor,

Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) emerged in Wuhan, China in December 2019 and spread, mainly by sustained human-to-human transmission, so rapidly that it threatened to saturate health services[1]. This led the governments of several countries to employ strict lockdown to slow the progression of the pandemic at the start of 2020[2,3]. The SARS-CoV-2 epidemic has resumed in European countries, including France, since the end of the summer. As a result, several large cities have taken measures to limit the transmission of the virus[4]. A recent study published in this journal assessed the impact of different strategies including lockdown to contain the spread of the SARS-CoV-2 in the London population during the first phase of epidemic in March 2020[5]. In a complementary way, we evaluated here the effectiveness of each individual public health measure chosen as alternatives to a new strict lockdown, on the basis of the real SARS-CoV-2 dynamics. modeling the global evolution of the epidemic in France has become very complicated since the strategies used differ between regions and cities[4]. But their effectiveness could be assessed by quantifying the local impact of protective measures against SARS-CoV-2. These could then be adapted for use in other contexts.

Our statistical model is based on a diffusion and transmission coefficient that varies with an individual's age, the likelihood of contagion, and a reduction coefficient that accounts for the impact of public health measures on virus transmission in the French city of Toulouse. We have used models to measure how the dynamics of the SARS-CoV-2 infection is influenced by each individual public health measure.

Consider the variables $(S_n,\ P_n,\ I_n,\ \widehat{S_n},\ \widehat{P_n},\ \widehat{I_n})$. A is defined as the age class variable:

$$A \in \{< 18\ y.o,\ \ 18-64,\ \ 65-74, \geq 75\ y.o\}.$$

On day n, for a given age class A, $S_{n,A}$ is the real number of healthy people and $\widehat{S_{n,A}}$ is the estimated number. $P^i_{n,A}$ and $\widehat{P^i_{n,A}}$ are the real and the estimated numbers of contagious carriers infected for i days $(1 \leq i \leq N_T)$. $I_{n,A}$ and $\widehat{I_{n,A}}$ are the real and estimated numbers of people in a given age class A who are immunized or have died. We assume that the risk of reinfection by SARS-CoV-2 after a first infection is negligible.

N_T is defined as the number of days a person is contagious. R_0 is the number of healthy people who a contagious person contacts and infects. We assume that the contagion coefficient varies over time and peaks when the virus load is maximal: 7 days after the start of infection[6]. N is the total population at the start of the epidemic phase, c is the real multiplier for the rate at which the epidemic spreads according to the protective measures applied $(0 \leq c \leq 1)$, $\hat{c}$ is the estimated coefficient. c and $\hat{c}$ are equal to 1 when no protective measures are in use.

$$P_n = \sum_A \sum_{i=1}^{N_T} P^i_{n,A}$$

N is given by:

$$N = \sum_A N_A = \sum_A S_{n,A} + P_{n,A} + I_{n,A}$$

On the transition from day n to day $n+1$, we have:

$$\forall\ 1 \leq i \leq N_T - 1,\ \ P^{i+1}_{n+1,A} = P^i_{n,A} \tag{1}$$

$$P^1_{n+1,A} = \frac{S_{n,A}}{N_A} \cdot \left[\sum_i P^i_{n,A}.R^i_0.c \right] \tag{2}$$

$$I_{n+1,A} = I_{n,A} + P^{N_T}_{n,A} \tag{3}$$

The model is a discretized version of a Susceptible Infectious and Recovered (SIR)-type model[7]

We have set $R_0 = 1.17$ based on current data in our area.[8]

We estimated the initial model settings using data collected by the Toulouse Virology Laboratory. $\forall\ n \in [\![d+1,\ +\infty]\!]$ $\hat{c}$ is corrected by fitting the predicted data $\hat{P}_n$ to the real data P_n as:

$$\hat{c} = \underset{c}{\operatorname{argmin}} \left| \hat{P}_n - P_n(c) \right|_{n \in [\![1,\ d]\!]}$$

The data obtained by the Toulouse Virology Laboratory using serological tests showed that the real seroprevalence in the Toulouse urban area was around 1.5% at the end of lockdown (May 11, 2020). The population of the Toulouse urban area is around 1 million, which implies a number of immune individuals I_0 close to 15,000 in mid-May. The number of SARS-CoV-2 cases gradually increased from July 21, 2020. Date d corresponds to October 6, 2020.

We estimated $\hat{c}$ to be 82% at the end of July 2020, with no specific local protective measures in place. The epidemic is evolving as shown by the blue curve, with a peak of nearly 14,000 new cases on November 11 (Fig. 1). But the local authorities decided to make the wearing of masks compulsory in certain areas of the city on August 5. This reduced the virus coefficient of transmission ($\hat{c}$ =75%). The epidemic then evolved as shown by the red curve with a maximum of new cases approaching the 10,000 expected on December 2 (Fig. 1). Mask wearing was made compulsory throughout Toulouse on August 21, and the estimated value of $\hat{c}$ became 72%. The epidemic therefore evolved as shown by the green curve with a peak around 8500 new cases on December 13 (Fig. 1). Finally, some public spaces, such as bars, were placed under curfew and certain sports structures were added to

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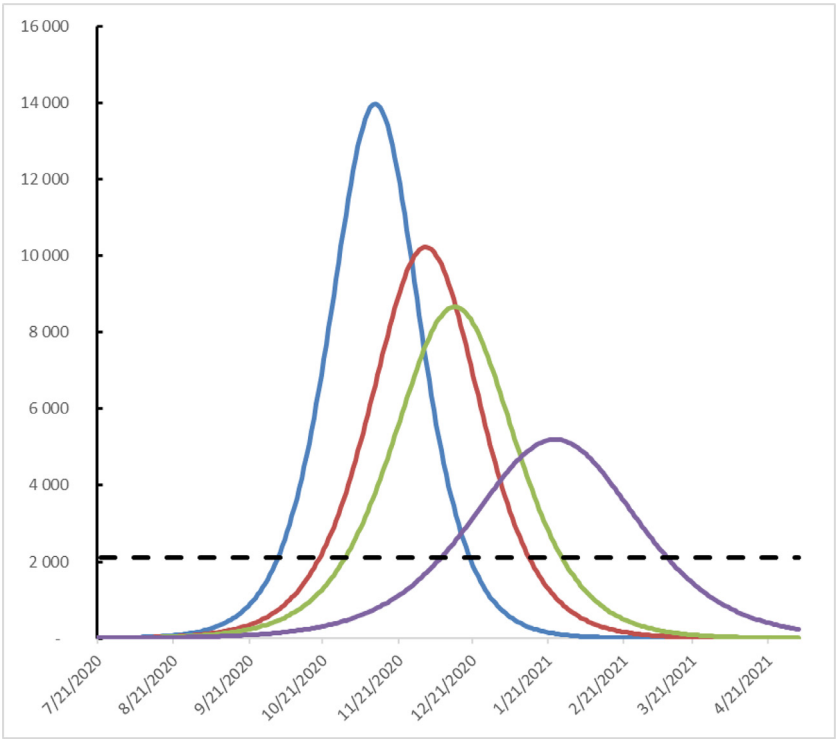

Fig. 1. Dynamics of SARS-CoV-2 infection per day from July 21, 2020 to May 5, 2021 according to the decisions on the protective measures .blue: no specific local measure red: masks are mandatory in some urban areas, green: masks are mandatory in the all Toulouse urban area, purple: closure of some public spaces and compulsory masks. The black dotted line corresponds to the new infections threshold at which the intensive care units could be saturated.

the measures already in force on September 26. This resulted in a $\hat{c}$ value of 65% and the epidemic evolved as indicated by the purple curve with a peak approaching 5000 new cases on January 22, 2021 (Fig. 1). Making compulsory the mask wearing and restricting access to some spaces open to the public reduced the maximum number of new infected cases per day by more than 75% and delayed the infected peak by approximately 2 months (from blue curve to purple curve). But these measures did not make it possible to avoid the saturation of the intensive care units in Toulouse (2100 new cases per day: black dotted line), which led the government authorities to add a global curfew on October 16. Its impact on the spread of the SARS-CoV-2 remains to be assessed.

We believe that this provides a basis for estimating the effect of specific restrictions on the dynamics of the SARS-CoV-2 epidemic. Its application could well indicate how local strategies can be tailored to avoid saturation of health services.

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SARS-CoV-2 positive virus culture 7 weeks after onset of COVID-19 in an immunocompromised patient suffering from X chromosome-linked agammaglobulinemia

Dear Editor,

Walsh et al. recently published a review in this journal focusing on the duration of infectiousness in SARS-CoV-2 infected individuals.[1] Based on available data from SARS-CoV-2 culture studies and contract tracing studies included in this review, the authors reported that patients with mild-to-moderate Coronavirus Disease 2019 (COVID-19) are considered to be without infectious potential beyond day 10 after the onset of symptoms. In contrast, immunocompromised and severe-to-critical patients may have prolonged viral shedding and, thus, may also provide prolonged infectiousness. Data addressing prolonged viral shedding and potential spread of SARS-CoV-2 are matter of public concern. Whereas isolation precautions as recommended by the United States Centers of Disease Control and Prevention[2] and the European health care authorities[3] are considered to fit for most SARS-CoV-2 infected patients, uncertainty remains in those patients with underlying immunodeficiency. Walsh et al. included a total of 15 relevant studies, but only two of them identified immunosuppressed patients from whom SARS-CoV-2 was isolated for up to 20 days beyond onset of disease.[4,5] Here, we report SARS-CoV-2 positive viral culture 7 weeks after onset of COVID-19 in a patient with an underlying immunosuppressive disorder, so-called X chromosome-linked agammaglobulinemia (XLA), demonstrating the potential of prolonged SARS-CoV-2 spreading beyond widely accepted isolation precautions.

Our patient had been tested positive for SARS-CoV-2 ribonucleic acid (RNA) by reverse transcription real-time polymerase chain reaction (RT-PCR) from upper respiratory specimen first on March 11 2020,. At this point of time, symptoms comprised fever and fatigue. In the patient's medical history, XLA, obstructive respiratory disorder, impaired alveolar diffusion capacity and non-cystic fibrosis bronchiectasis were recorded. Due to worsening of fever, cough and dyspnea, the patient required for hospitalization 16 days after the initial diagnosis of COVID-19. The patient received antibiotic treatment with amoxicillin/clavulanic acid and azithromycin, was switched to piperacillin/tazobactam, followed by moxifloxacin and meropenem. Based on local recommendations valid at this point of time, the patient was treated with hydroxychloroquine and then by an antiretroviral combination of lopinavir/ritonavir. Furthermore, posaconazole was administered for Aspergillus positive sputum culture and intravenous immunoglobulin substitution (IVIG) was performed regarding the absence of endogenous antibody production in underlying XLA. Due to persistent fever up to 40.4 °C, progressive respiratory insufficiency and deterioration of laboratory parameters expressing an increasing inflammatory activity, the patient was transmitted to the Intensive Care Unit (ICU) on April 9. Interleukin-6 (IL-6) receptor blockade by tocilizumab and convalescent plasma were administered on April 10. No adverse effects related to this treatment regimen were recorded. The rationale behind this approach was to restrain the inflammatory response by IL-6 blockade and to provide neutralizing COVID-19 immunoglobulins by convalescent plasma. Afterwards, we observed a rapid recovery regarding clinical and laboratory parameters. Ferritin and C-reactive protein (CRP) significantly declined as compared to pre-transfusion whereas the lymphocyte count returned to normal. We observed an increase in IL-6 after treatment followed by a fast and almost complete decline within the next days. Body temperature did not exceed a limit of 38 °C as compared to measurements of up to 40.4 °C pre-transfusion. Oxygen demand decreased resulting in an increase of PaO2/FiO2 ratio (143 before versus 223 after treatment). A chest radiograph showed a significant decline in infiltrative opacities. On April 15, five days after tocilizumab and convalescent plasma administration and five weeks after the initial diagnosis of COVID-19, SARS-CoV-2 RNA was not detectable for the first time. The patient showed progressive clinical recovery, but an alternating course of three negative followed by three positive SARS-CoV-2 RT-PCR results was subsequently observed. Convalescent plasma transfusion was repeated on April 21. Despite full clinical recovery, SARS-CoV-2 RT-PCR showed six positive and also six negative SARS-CoV-2 RT-PCR results in an alternating order in the time span between April 23 and May 4. SARS-CoV-2 PCR from oropharyngeal swabs and sputum obtained on April 24 showed 10^3 (sputum) to 10^5 (oropharyngeal swabs) SARS-CoV-2 copies/mL. A SARS-CoV-2 PCR-positive oropharyngeal swab with a low cycle threshold (Ct) value of 25 was inoculated onto Vero E6 cells for viral culture. A cytopathic effect was observed four days after inoculation, and the presence of SARS-CoV-2 in the cell culture supernatant was confirmed by RT-PCR (Ct value of 16 at a 10-fold higher dilution than the original swab). In contrast to this find-

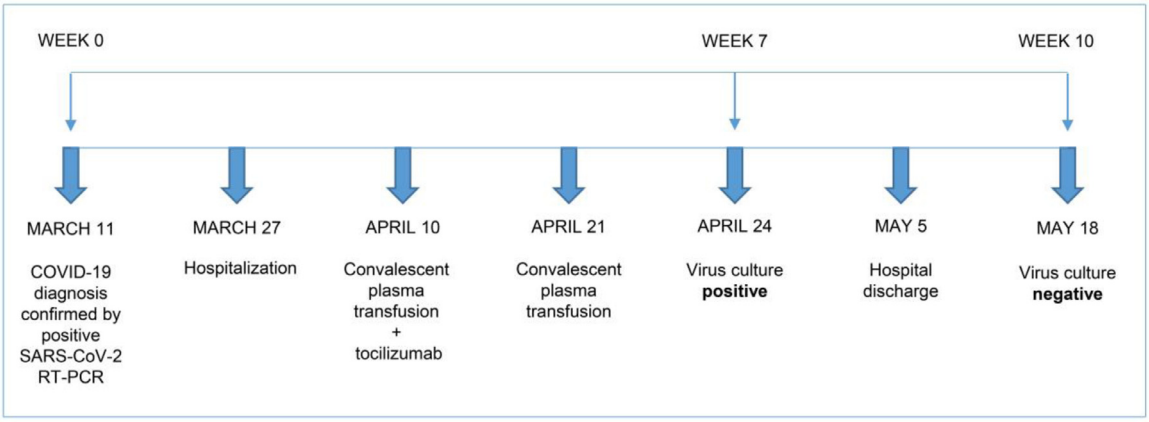

Fig. 1. Timeline presenting milestones of disease from onset of COVID 19 up until negative virus culture. Our patient was diagnosed with COVID-19 based on a positive result from SARS-CoV-2 RT-PCR first on March 11. Admission to the hospital was required on March 27 due to progressive clinical deterioration. Administration of an experimental treatment approach comprising convalescent plasma transfusion and interleukin-6 receptor blockade by tocilizumab was performed on April 10 and convalescent plasma was administered for a second time on April 21. A virus culture within week 7 of disease still showed a positive result and, thus, confirmed prolonged viral shedding in this patient. Viral culture control in week 10 presented a negative result. COVID-19 = coronavirus disease 2019; SARS-CoV-2 = severe acute respiratory syndrome coronavirus 2; RT-PCR = real-time polymerase chain reaction.

ing, previously published reports revealed that the viral burden measured in respiratory specimens obtained from mild coronavirus disease 2019 (COVID-19) cases declined after onset of symptoms and was considered without infectious potential beyond day 9 or 10 of symptoms with less than 10^5 viral ribonucleic acid (RNA) copies/mL of sputum.[6,7] Based on the clinical improvement and three negative follow up SARS-CoV-2 PCR results the patient was discharged on May 5 and isolated at home. Almost 2 weeks later a SARS-CoV-2 PCR showed 500 copies/mL in transport medium (containing the oropharyngeal swab) and the viral culture was negative. Fig. 1 presents an overview by timeline from the initial diagnosis of COVID-19 up until negative viral culture.

In summary, we have to assume that in our patient shedding of infectious SARS-CoV-2 stopped between week 7 and 10 of disease. Our patient suffers from XLA, also known as Bruton's agammaglobulinemia, which is caused by a mutation in Bruton's tyrosine kinase resulting in an inability of endogenous antibody production du to a developmental arrest of pre B cells.[8] In contrast with a previously described 3-day mild course of COVID-19 in a patient with underlying XLA,[9] our patient experienced a prolonged and severe course COVID-19 requiring ICU, oxygen support, treatment of cytokine storm with tocilizumab and administration of convalescent plasma to overcome a lacking antibody response. To conclude, our case demonstrates that in immunocompromised patients caution is warranted in applying generally accepted clinical management and public health strategies to prevent the spread of COVID-19. Additionally, our observations in a patient with severe COVID-19 in underlying immunodeficiency encourages the conclusion drawn by Walsh et al. requiring for further reserch in certain subgroups, particularly.

Declaration of Competing Interest

None to declare.

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Impact of immunosuppressive therapy on the severity of COVID-19 in solid organ transplant recipients

Dear Editor,

We read with interest the article by Minotti et al. on immunosuppression on severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) infection.[1] Variable clinical courses of Coronavirus-19 disease (COVID-19) were reported [2–4] in solid organ transplant (SOT) recipients, but few data are available on the impact of immunosuppression on clinical severity. Since immune system plays an essential role in the pathophysiology of COVID-19 by developing a hyperinflammatory state,[5] immunomodulators were found to improve clinical course.[6] In addition, CNIs inhibits T cells activation and may reduce monocyte-macrophages activation [7] thus preventing cytokine-release syndrome, but a suboptimal T cells response may hamper the clearance of SARS-CoV-2.[8]

SOT recipients with confirmed SARS-CoV-2 infection admitted to two transplant centers in Northern Italy, ASST Grande Ospedale Metropolitano Niguarda (Milano) and ASST Ospedale Papa Giovanni XXIII (Bergamo), between February 21 and May 31, 2020 were retrospectively analyzed to assess the role of immunosuppressive therapy in clinical presentation and severity of COVID-19 and to describe its management during the course of the disease. Disease severity was classified as (1) mild (WHO Clinical Progression Scale grades 1–3), (2) moderate (WHO Clinical Progression Scale grades 4–5), (3) severe or critical (WHO Clinical Progression Scale grades 6–9).[9] The immunosuppressive regimen before and after diagnosis of SARS-CoV-2 infection was recorded and its major changes after admission were categorized as (1) CNIs withdrawal/dose reduction, (2) anti-metabolite withdrawal, (3) steroid dose increase. Continuous variables were reported as median with interquartile range (IQR), categorical variables as absolute (%) values. Kruskall–Wallis and Mann–Whitney *U* test to compare continuous variables and Chi-square/Fisher exact test to compare categorical variables among groups were used. Survival curves were calculated by Kaplan-Meier method and compared by Log-rank test. Data were analyzed using GraphPad Prism v8.

Thirty-nine patients were analyzed. Major baseline demographic, clinical and biochemical characteristics are described in Table 1. Twelve patients (31%) were classified as mild, 15 (38%) as moderate and 12 (31%) as severe or critical. Five patients (13%) required admission to intensive care unit. SARS-CoV-2 infection was detected 5 (1–10) days after symptoms onset, while the peak of the disease was observed 10 (7–14) days after clinical presentation. The overall survival was 82% (n=32): 92% (n=11/12) in mild, 100% (n=15/15) in moderate and 50% (n=6/12) in severe patients. Five patients died due to COVID-19-related respiratory failure and 2 to concomitant diseases (diffuse large B cells lymphoma and pancreatic cancer progression).

All patients were on immunosuppressants: 69% was on a combined regimen, mainly based on CNIs (n=37; 95%) and a large proportion was receiving mycophenolate (44%) and steroid (42%). No significant association of type, dose or level of immunosuppressive agents at the time of COVID-19 diagnosis with clinical severity has been observed (Table 2), even when the analysis was limited to liver transplant recipients (data not shown). Patients on tacrolimus showed lower lymphocyte count compared with those on cyclosporin (0.58 (0.61–0.96] vs 1.12 [0.98–1.69], p=0.033), while no significant difference was observed among those on mycophenolate compared to the others (0.81 [0.52–1.16] vs 0.75 [0.48–1.18], p=0.064).

The management of immunosuppressive treatment was heterogeneous after confirmed SARS-CoV-2 infection: 18/39 (46%) underwent modifications, especially among patients with moderate and severe disease (53% and 59%) compared to those with mild disease (25%). Mycophenolate withdrawal (mild 75% vs moderate 63% vs severe 70%, p=0.882) was the most frequent change, together with increased steroid dose (mild 50% vs moderate 43% vs severe 40%, p=0.954). CNIs reduction or withdrawal were more common in patients with moderate to severe respiratory failure (mild 17% vs moderate 39% vs severe 59%, p=0.213). Overall survival was not significantly different between patients who changed immunosuppressive regimen and the others (30-day survival 81% vs 65%; p=0.368).

Overall, we did not find any association between chronic immunosuppressive regimen at the time of COVID-19 presentation and its severity. Even though we could not analyze SARS-CoV-2 viral load, which might be influenced by CNIs [8] and it is associated with disease severity,[10] similar plasma trough levels of CNIs were observed among severity groups.

High mycophenolate dose (>1000 mg/day) was found an independent risk of severe COVID-19 in liver transplant recipients,[3] even though not confirmed in another study.[4] In our sample mycophenolic use and its dose tended to be lower in mild compared to moderate and severe patients, though no significant difference was observed, and more frequent withdrawals were recorded in mild compared to severe patients, possibly supporting the previous observation.[3]

Current assumption and doses of steroid were similar among severity groups at admission, although its use improved the outcome in SARS-CoV-2 related respiratory failure.[6] Nonetheless, the dose of prednisone (or equivalent) at the time of infection was lower than those recommended in COVID-19.[6]

Reduction of CNIs dose, mycophenolate withdrawal and increase in steroid dose were more frequent in patients with moderate and severe disease, as expected.[2,3] Nonetheless, the consequences of these adjustments on disease progression are difficult to assess, given the heterogeneity of patients in terms of disease severity, comorbidities, length of symptoms and the limited and variable data on the efficacy of antiviral and anti-inflammatory treatments. Moreover, the timing of immune modulation might be a relevant issue: the reduced immune defense may favor viral replication and expose patients to a severe course,[10] while the potential effect of immunosuppression [6] might be beneficial only after cytokine release syndrome have been elicited by SARS-CoV-2.

As already shown in the general population arterial hypertension was strikingly associated with disease severity (Table 1), suggesting that risk factors other than immunosuppression may have a major role also in SOT recipients.

Even though the retrospective nature of the analysis and the small and heterogenous study population limit the strength of the conclusions, CNIs levels do not appear to influence the course of the infection. Since inadequate immunosuppression may expose patients to an increased risk of graft rejection, their withdrawal is not encouraged, even though transient reduction could be considered if concomitant anti-inflammatory treatment for COVID-19 is administered. Together with other currently available data,[3] transient dose reduction or withdrawal of mycophenolate appears advisable.

Table 1
Major baseline characteristics of the three severity groups.

	Overall *n*=39	Mild *n*=12	Moderate *n*=15	Severe *n*=12	*p*
Age *years*	62 (54–68)	63 (53–67)	60 (58–69)	62 (56–69)	0.813
Male gender *n*	28 (73%)	8 (67%)	10 (67%)	10 (83%)	0.566
Time-from-transplant > *1 year*	7 (18%)	2 (17%)	3 (20%)	2 (17%)	0.680
Charlson comorbidity index	3 (2–4)	2 (2–4)	4 (3–4)	3 (2–4)	0.755
Arterial hypertension *n*	16 (41%)	2 (9%)	6 (40%)	8 (67%)	0.045
Diabetes mellitus *n*	8 (21%)	3 (25%)	3 (20%)	2 (17%)	0.878
Chronic kidney disease *n*	6 (15%)	2 (9%)	3 (20%)	1 (8%)	0.719
Organ transplant *n*					
Liver	27 (69%)	10 (83%)	9 (60%)	8 (67%)	0.566
Kidney	9 (23%)	2 (17%)	5 (33%)	2 (17%)	
Kidney-heart	2 (5%)	–	1 (7%)	1 (8%)	
Kidney-pancreas	1 (3%)	–	–	1 (8%)	
Leukocyte count 10^3 cells/mcL	5.5 (4.5–7.9)	5.5 (4.5–6.4)	3.2 (1.1–7.3)	4.5 (3.1–8.6)	0.205
Lymphocyte count 10^3 cells/mcL	0.76 (0.54–1.15)	0.81 (0.56–1.03)	1.14 (0.72–1.54)	0.62 (0.51–0.76)	0.289
C-reactive protein mg/dL	4.9 (2.2–10.7)	3.5 (1.9–6.4)	4.3 (2.2–7.9)	13.6 (8.2–23.8)	0.110
Creatinine mg/dL	1.4 (1.0–2.24)	1.4 (1.2–1.6)	2.1 (0.7–2.8)	1.37 (1.2–1.86)	0.918
Lactate dehydrogenase U/L	96 (92–99)	225 (198–250)	311 (287–457)	381 (340–432)	0.023
Alanine aminotransferase U/L	25 (15–44)	41 (21–51)	20 (13–29)	23 (15–75)	0.882
Bilirubin mg/dL	0.7 (0.5–1.1)	0.7 (0.7–1.1)	0.7 (0.5-1.0)	0.8 (0.4–1.3)	0.788
COVID-19 treatment*					
Lopinavir/ritonavir	10 (26%)	1 (8%)	4 (27%)	5 (42%)	–
(Hydroxy-)Chloroquine	18 (46%)	2 (17%)	8 (53%)	8 (67%)	
High-dose steroid	10 (6%)	1 (8%)	5 (33%)	4 (33%)	
Tocilizumab	3 (8%)	–	–	3 (25%)	
Convalescent plasma	1 (3%)	–	–	1 (8%)	

Different treatment combinations were administered.

Table 2
Immunosuppressive regimens at admission.

	Overall *n*=39	Mild *n*=12	Moderate *n*=15	Severe *n*=12	*p*
Single-agent *vs* combined regimen	12/27	5/7	4/11	3/9	0.614
CNIs					
Cyclosporine *n*	11 (28%)	1 (8%)	7 (47%)	3 (25%)	0.041
Tacrolimus *n*	26 (66%)	11 (92%)	6 (40%)	9 (75%)	
Mycophenolate mofetil *n*	17 (44%)	4 (33%)	8 (53%)	5 (42%)	0.574
Steroid *n*	18 (46%)	5 (42%)	8 (53%)	5 (42%)	0.777
Belatacept *n*	1 (3%)	–	1 (7%)	–	–
Everolimus *n*	1 (3%)	–	1 (7%)	–	–
Cyclosporine level mg/L	75 (46–89)	46 (46–46)	75 (43–94)	80 (24–113)	0.853
Tacrolimus level mg/L	5.5 (3.7–8.2)	6.7 (2.4–8.4)	4 (3.5–7.4)	5.1 (3.8–8.9)	0.821
Mycophenolate dose g/day	1.40 (1.00–1.75)	1.25 (0.63–1.50)	1.47 (1.02–1.94)	1.44 (1.00–2.00)	0.574
Prednisone dose mg/day	7.5 (5–10)	10 (5–17.5)	6.5 (2.5–9.4)	10 (3.75–15)	0.530

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Zero measles after COVID-19 pandemic in Taiwan

Dear Editor,

In recent issue, Rana et al.[1] reported that a sudden reduction of measles cases during COVID-19 pandemic was observed in Pakistan. Although the decline of respiratory infectious disease, such as invasive pneumococcal disease, tuberculosis and influenza was also reported during early 2020 in Taiwan,[2–4] we did not know whether the similar scenario could be replicated for measles like Rana et al's study.[1] In Taiwan, measles had been listed as a reportable disease since 1985, and has been under control after implementation of effective vaccination and the strengthened quality of the surveillance system. Although imported- and local-transmission cases were still sporadically reported in recent years, most of the imported cases contracted the disease in China and South East Asia and most of non-imported cases were linked to the imported cases in Taiwan.[5] To assess the impact of COVID-19 on the epidemiology of measles, we conducted this study to compare the case number of measles in Taiwan before and after COVID-19 outbreak.

Because measles is a notifiable disease in Taiwan, it is mandatory to report measles cases once it has been identified to the Centers for Disease Control (CDC). Therefore, we can use the open data website provided by Taiwan's CDC to extract the reported case numbers for measles between January and August from 2015 to 2020 for comparsion.[6]

First of all, zero measles case was reported in 2020, which was lower in the same period in 2019 ($n=125$), 2018 ($n=33$), 2017 ($n=5$), 2016 ($n=13$) and 2015 ($n=27$). Second, neither imported nor locally transmitted case was identified in 2020, which was in contrast to the previous years – 2015 to 2019 (Fig. 1).

In this study, we found the measles case number was dramatically reduced to zero since the start of 2020 – COVID-19 outbreak. This "zero" phenomenon was not achieved in the previous five years. Zero measles in Taiwan during COVID-19 pandemic could be attributed to the implementation of aggressive infection control measures within this period. First, the implementation of "border control", particularly for China since the early COVID-19 outbreak in Wuhan might directly lead to "zero imported measles cases" and indirectly reduce the occurrence of "imported cases associated locally-transmission measles. Second, the infection control measures, such as mask wearing, avoid visiting crowd area and social distancing might help reduce the risk of the measles spreads through the air by breathing, coughing or sneezing. These interventions could prevent the circulation of locally-transmission measles cases, like other respiratory infectious diseases in Taiwan.[2–4]

However, this study had one limitation. Under report or under diagnosis due to the fear of visiting hospital is possible during COVID-19 pandemic, which may cause under-estimate of measles cases. Further study comparing the number of visiting clinics for respiratory symptoms during COVID-19 pandemic with those in previous years may help estimate the effect of this issue.

In conclusion, this study demonstrated an additional benefit of COVID-19 preventive measures - "zero measles" in Taiwan during this pandemic. Therefore, it is worth aggressively implementing the infection control and prevention measures during COVID-19 pandemic, not only for SARS-CoV-2 outbreak but also for other infectious diseases.

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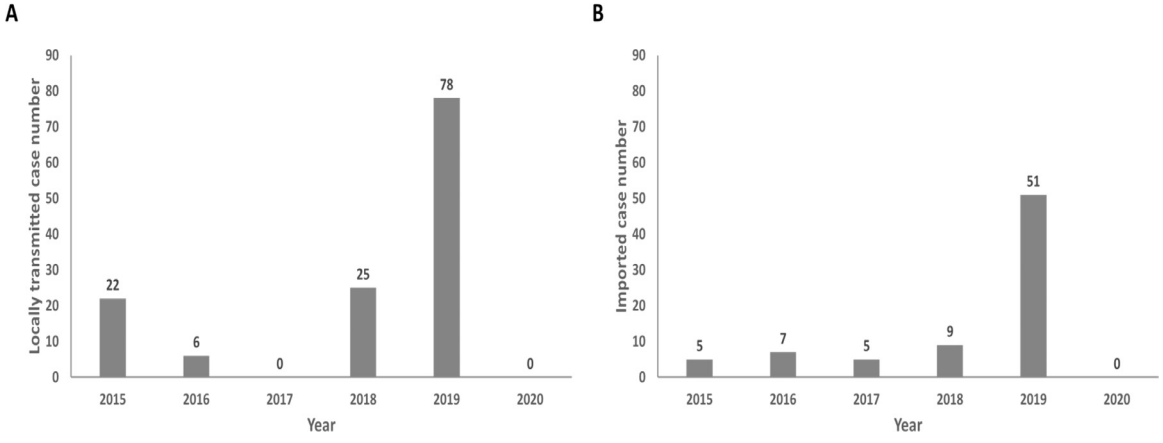

Fig. 1. The locally-transmitted (A) and imported (B) measles case number between January and August from 2015 to 2020.

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COVID-19: Spatial resolution of excess mortality in Germany and Italy

Dear Editor,

Stang et al.[1] explored age-specific numbers of weekly deaths in Germany from 2016 to June 2020. We wish to complement their results and conclusion that excess mortality existed for two months with analyses of higher *spatial resolution*.

Importantly, analysing weekly deaths can offer virus test-independent information on mortality effects during the SARS-CoV-2/COVID-19 pandemic. Thus, the authors contribute to a fuller epidemiological picture. However, looking at countries alone can miss relevant information on the pandemic's course and toll in smaller spatial units.

To exemplify, let us look at effects of SARS-CoV-2/COVID-19 on mortality in Germany and Italy[2] in the first six months of 2020. Assuming otherwise constant determinants of death, we chose Standardized Mortality Ratio (SMR) methodology[3] to analyse excess mortality by state in Germany and by region in Italy. Monthly and weekly all-cause mortality data from January 2016 to June 2020, published by the Federal Statistical Office in Germany and the National Institute of Statistics in Italy, respectively, for all age groups, <65 years and ≥ 65 years and individual states or regions, were used for our explorations. SMRs were evaluated by comparing the index year 2020 with our reference years 2016–2019 with a focus on trend detection.[4–6]

In analyses for Germany, higher mortality in April was followed by a decline: For January-June, we calculated the SMR as 1.00 (95% CI: 0.97–1.04), including an SMR of 1.10 in April (95% CI: 1.06–1.13). As one noticeable – as yet unappreciated – result in individual states, in Mecklenburg-Western Pomerania, reduced SMRs in January turned monotonically into significantly increased SMRs until June with an SMR of 1.07 (95% CI: 0.99–1.16). Poisson trend models estimated an SMR increase of 2.5% per month, observed in both age groups (always $p<0.05$). SMRs were most increased in April in Hamburg (1.24; 95%CI: 1.12–1.37), Bavaria (1.21; 95%CI: 1.17–1.26) and Bremen (1.20; 95%CI: 1.06–1.37). In Italy, SMRs of 1.43 (95%CI: 1.39–1.47) were observed in March and 1.31 (95%CI: 1.3–1.33) in April with highest SMRs in Lombardy in March (2.89; 95%CI: 2.79–3.00) and April (2.12; 95%CI: 2.07–2.17), Valle d'Aoste (April: 1.71; 95%CI: 1.47–1.99) and Emilia-Romagna (March: 1.69; 95%CI 1.61–1.77).

Importantly, in the same time windows with significant excess mortality there were also federal states and regions with no elevated SMRs: In Germany, in April for instance in Schleswig-Holstein (0.96; 95%CI: 0.91–1.02) or Saxony-Anhalt (0.98; 95%CI: 0.93–1.04). In Italy, for instance in Basilicata (March: 0.83; 95%CI: 0.72–0.95), Friuli Venezia (March: 0.86; 95%CI: 0.78–0.94; April: 0.88; 95%CI: 0.83–0.94) or Latium (March: 0.97; 95%CI: 0.93–1.0; April: 0.91; 95%CI: 0.88–0.94). Clearly, the variation of SMRs may hold important clues regarding effects of the pandemic, and of counter-measures, which can vary over space and time. This heterogeneity should be explored rather than remain masked by exclusively looking at the countries as a whole.

Overall, when Stang et al.[1] point out that the course of the pandemic across Europe is different and that pooling of mortality data[7] may mask relevant differences at national levels we do agree. But exclusively focusing on Germany, or Italy, as a whole can also mask relevant effects in individual states or regions. In this vein, extending the authors' aim to provide estimates of excess mortality during the "first wave" of the pandemic would offer added value: SMR analyses with appropriate *spatial resolution* are needed to directly compare the burden of disease not only between but also within countries. They should be used to assess desired, and undesired, effects of measures against SARS-CoV-2/COVID-19.

In conclusion, to "fly with high visibility"[8] when working to cope with the pandemic, SMR analyses – with appropriate spatial resolution – are needed in Germany, and in other countries, on a regular basis. For independent analyses, national authorities should expedite the publication of raw data on mortality and populations – expanded by detailed information on age, sex and causes of death.[9]

Declaration of Competing Interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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SARS-CoV-2 re-infection: a case report from Qatar

Dear Editor,

We read with interest the study of Gousseff et al.[1] The study reported a second acute COVID-19 episode in 11 patients. A novel coronavirus (SARS-CoV-2) caused a pandemic end of 2019. Common signs of SARS-CoV-2 include fever, cough and shortness of breath with no definitive treatment to date.[2] Reverse transcriptase polymerase chain reaction (RT-PCR)–based assays are the current reference diagnostic test.[3] Positive result does not necessitate the presence of infection and viral RNA shedding declines following the resolution of symptoms. Viral nucleic acid could be detectable in throat swabs up to 6 weeks after symptom onset.[2] Different reports have proposed the reactivation of SARS-CoV-2 infection, with 2 RT-PCR positive results following resolving symptoms and interim RT-PCR negative results.[1,4–10] Early studies reported patients with negative results having positive results within one week of discharge. Later, reports used 3 weeks (21 days) as the cut-off point, following which another positive RT-PCR result with symptoms would support the possibility of reinfection.[1] A recent report confirmed the possibility of reinfection with genetically distinct SARS-CoV-2 infection in 2 different cases.[11] Here we describe a case with 2 positive RT-PCR results in a symptomatic female with 84 days apart, patient had a complete resolution of symptoms and interim negative swab results on day 13.

A 46-year-old female attended primary healthcare settings in Qatar with a history of SARS-CoV-2 contact and sore throat on the 23rd of May 2020. The patient SARS-CoV-2 RT-PCR results swab result was positive. Her vital signs, blood investigations and chest X-ray were normal. The patient had a past medical history of mild asthma, which was controlled with only occasional use of salbutamol inhaler. The patient was admitted to a quarantine facility for observation. The patient PCR swab result was negative on the 5th of June and she was discharged on the 6th June.

On the 11th of August she presented with fever, sore throat and body pain following a second contact with SARS-CoV-2 positive case. SARS-CoV-2 RT-PCR results swab result was inconclusive on 13th August. The patient also reported chest pain, cough, and mild dyspnea. Her vital signs were normal, but blood investigations showed leucocytes of 1.7×10^9 cells per L and lymphocytes 0.9×10^9 cells per L. A repeat COVID-19 PCR swab was positive on the 15th of August with CT value of 25.49. Chest X-ray was normal and patient progression to recovery was unremarkable. She was discharged home on the 29th of August 2020. During both events, the patient received only symptomatic treatment.

Early studies reported that re-detectable positive virus nucleic acid among patients with SARS-CoV-2 with an average duration of 15 days from discharge to a re-positive results.[12] Patients in those early reports did not show signs of infection with the second positive results and had negative swab results within one week later. Researchers have suggested the persistence of viral RNA with no recurrence of infection.

The COCOREC (Collaborative study COvid RECurrences) study suggested that recurrence of infection is likely if the patient has two confirmed SARS-CoV-2 RT-PCR positive results over 15 days apart with one major clinical sign and no other cause to explain the symptoms. The study identified 11 patients similar to our case. The longest time to second positive test results in this cohort of patients was 49 days.[1]

Our case presented with symptoms, positive contact history and positive swab results with a timeline significantly longer than any reported case. The persistence of positive RT-PCR for SARS-CoV-2 is reported only up to 6 weeks.[1] A re-infection or to the least a re-activation following long-lasting carriage seems more likely in this patient report. Reports of such occurrences are rare to date in view of the number of worldwide reporting of the infection rates which is reassuring. This case report adds to current evidence of possibility of reinfection and provides a basis for future cohort studies. Detection of viral RNA in symptomatic[1] patients following complete remission of symptoms and full recovery should be considered as reinfection or recurrence at the least.

Informed consent

The patient gave consent for the material to appear in scientific publication.

Ethics statement

The case report received ethical approval from Primary Health Care corporation (PHCC) research committee. The patient consented to publication and reporting is fully anonymized.

Authorship

All Authors have contributed to the drafting and the critical revision of the article. The final version approved by all authors.

Declaration of Competing Interest

None.

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Lack of detrimental effect of corticosteroids on antibody responses to SARS-CoV-2 and viral clearance in patients hospitalized with COVID-19

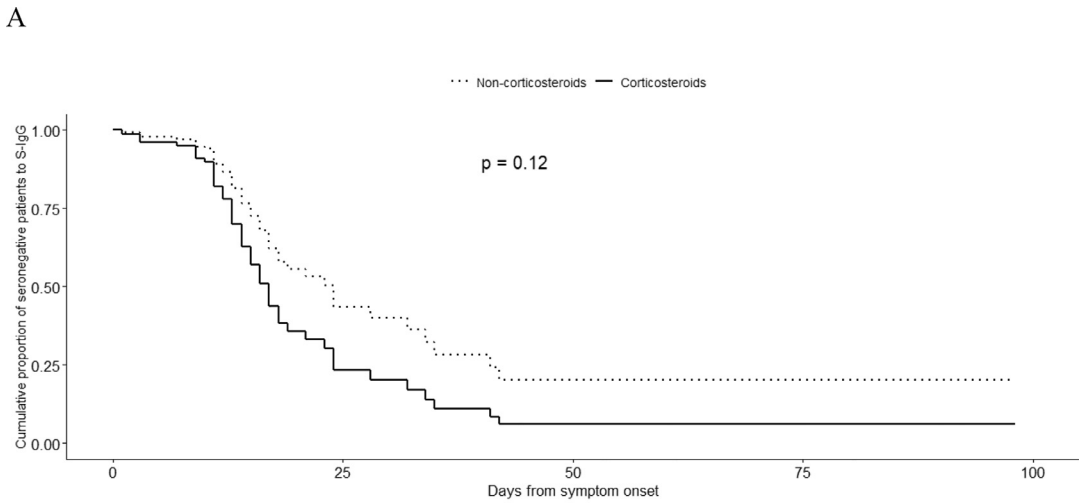
Dear Editor,

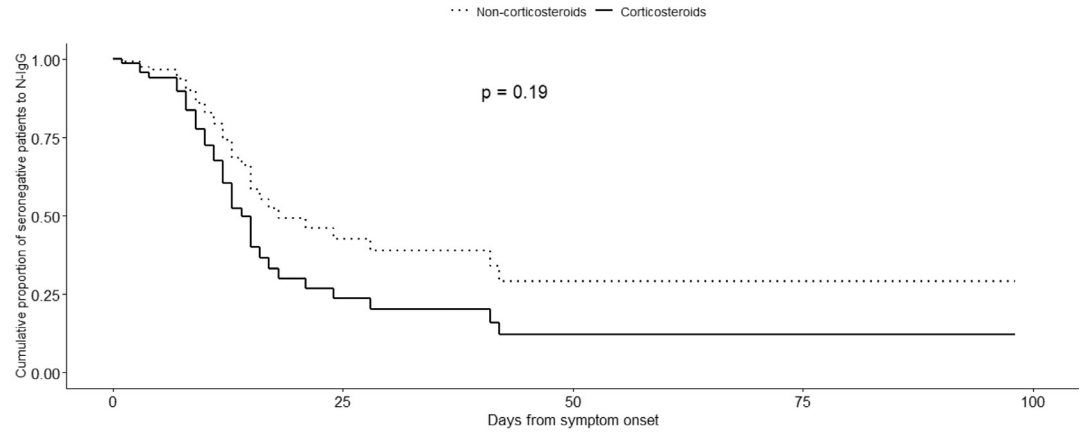
Recent articles in this Journal have described the beneficial effects of corticosteroids on outcome of COVID-19, and have also suggested that corticosteroids may not impair the natural immune response.[1–3] Corticosteroids are the only immunomodulatory agents that have shown so far a reduction in mortality in a randomized multicenter trial,[4] and have accordingly been recommended for moderate-to-severe COVID-19. Besides the benefits derived from their potent anti-inflammatory properties, the potential negative consequences of corticosteroids' immunosuppressive effects on SARS-CoV-2 dynamics remain to be characterized. Particularly, information about their impact on the humoral immune response against the virus is limited. Short courses of corticosteroids have been associated with a decrease in serum IgG and IgA concentrations.[5] Short-term and long-term reduction in antibody production might have negative effects on viral clearance and protection against reinfection. In addition, data regarding the effect of corticosteroids on SARS-CoV-2 clearance remain controversial.[6,7]

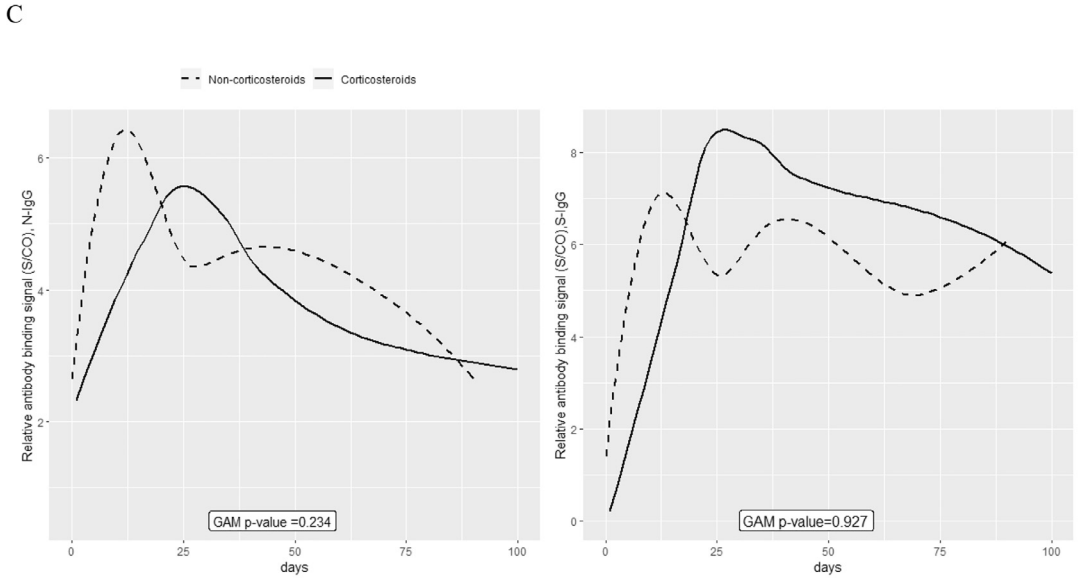
We analyzed the longitudinal impact of therapy with corticosteroids on the antibody response to SARS-CoV-2 and viral clearance in patients admitted with COVID-19.

A prospective study was carried out in hospitalized patients with COVID-19 confirmed through real-time polymerase chain reaction. Serial nasopharyngeal and plasma samples were obtained at different time points for SARS-CoV-2 RNA and antibody measurement during hospital stay and after discharge. IgG antibody plasma levels against the SARS-CoV-2 internal nucleo-capsid protein (N-IgG) and surface S1 domain of the spike protein (S-IgG) (Anti-SARS-CoV-2 IgG ELISA, Euroimmun, Lubeck, Germany) were determined. Of 210 adults admitted with COVID-19, 77 participants with positive SARS-CoV-2 RNA in more than one nasopharyngeal sample, more than two plasma samples at least 14 days apart, and who did not receive other sole immunomodulatory agents were selected; of them, 27 received corticosteroids given in daily pulses of 250–500 mg during 3 days. Participants receiving corticosteroids had higher severity of disease and tended to be older (see clinical characteristics in Table 1). Median follow-up for antibody detection was 71 (62–83) days. Detectable titers of both S-IgG and N-IgG were observed in 23 (92%) patients receiving corticosteroids and 25 (62.5%) not on corticosteroids ($p = 0.009$) after a median (Q1-Q3) of 13 (10.5–14.5) days vs 16 (13–24) days from symptom onset, respectively, for S-IgG ($p = 0.008$); and of 10 (9–13) days vs 14 (8–21) days, respectively, for N-IgG ($p = 0.043$). Kaplan-Meier curves showed a higher cumulative proportion of patients with detectable S-IgG ($p < 0.001$) and N-IgG ($p = 0.012$) levels among those receiving corticosteroids. After Cox regression adjustment for the significant variables associated with S-IgG and N-IgG response in the univariate analysis (specifically, SARS-CoV-2 viral load, Charlson comorbidity index and C-reactive protein levels), no significant differences in antibody response were observed between the two groups (Fig. 1A and B). Median (Q1-Q3) peak S-IgG titers were 6.5 (5.4–7.4) vs 4.5 (0.1–6.5) absorbance/cut-off (S/CO) in patients with and without corticosteroids, respectively, ($p = 0.005$), and 4.9 (4.0–5.4) vs 3.8 (0.1–5.5) S/CO for N-IgG, respectively ($p = 0.037$). Temporal changes in S-IgG and N-IgG titers analyzed with local polynomial regression did not differ according to corticosteroid therapy group (Fig. 1C). SARS-CoV-2 viral clearance occurred in 21 (77.8%) patients receiving corticosteroids and 44 (88%) not on corticosteroids after a median (Q1-Q3) of 30 (22–46) days from the first positive sample ($p = 0.325$). Kaplan–Meier curves exhibiting the probability of SARS-CoV-2 clearance by treatment group are shown in Fig. 1D.

In contrast to other studies, we analyzed the effects of corticosteroids on both viral kinetics and the humoral immune response to SARS-CoV-2. We did not find a detrimental effect of corticosteroid pulses on the intensity and duration of antibody responses, and the same was observed with time to viral clearance in this cohort of patients who were thoroughly investigated with multiple sequential samples. Although differences in antibody response even favored patients receiving corticosteroids, probably due to their







S/CO, absorbance/cut-off; GAM, generalized additive model

Fig. 1. Effects of corticosteroids on antibody responses and viral clearance. A, Adjusted Kaplan Meier curve to estimate the cumulative proportion of patients with negative titers of S-IgG according to therapy with corticosteroids. B, Adjusted Kaplan Meier curve to estimate the cumulative proportion of patients with negative titers of N-IgG according to therapy with corticosteroids. C, Temporal changes in N-IgG titers (left) and S-IgG titers (right) analyzed with local polynomial regression. D, Kaplan Meier curve to estimate the cumulative proportion of patients with detectable viral RNA according to therapy with corticosteroids.

Table 1
Clinical data of patients admitted with COVID-19 confirmed with real-time polymerase chain reaction.

Variable	Corticosteroids $N=27$	Non-corticosteroids $N=50$	P
Sex, male	18 (66.7)	25 (50.0)	0.229
Age, years	71 (58–81.5)	63.5 (46.8–74.0)	0.059
Active smoking	17 (70.8)	27 (57.4)	0.311
Charlson comorbidity index	3.0 (1.0–5.5)	3 (1–5)	0.490
Days from symptom onset to admission	7 (3–10)	6.5 (3–11)	0.797
SOFA score on admission	3 (2–3)	2 (2–3)	0.035
SpO2/FIO2 on admission	344.6 (321.4–350)	353.6 (343.8–380.8)	0.035
SARS-CoV-2 RNA, copies/sample	3.9 (3.4–4.4)	2.2 (2.0–3.7)	<0.001
Peak S-IgG, S/CO	6.5 (5.4–7.4)	4.5 (0.1–6.5)	0.005
Peak N-IgG, S/CO	4.9 (4.0–5.4)	3.8 (0.1–5.5)	0.037
Interleukin-6, pg/mL	35.3 (17.4–97)	13.4 (8–29.8)	0.021
Ferritin, ng/mL	299.5 (190–640)	180.5 (115.5–333)	0.056
C-reactive protein, mg/L	80.1 (35.1–141.7)	34.5 (4.9–53.5)	0.001
Fibrinogen, mg/dL	614 (429.7–851.3)	443 (323.1–552.5)	0.028
Lymphocytes, x103/μL	1.0 (0.8–1.2)	1.4 (1.2–2.1)	<0.001
Hospital stay, days	19 (13.5–24.5)	9 (6–12)	<0.001
Death	1 (3.7)	1 (2.0)	1
ICU admission	2 (7.4)	4 (8.0)	1
HCQ-based combinations	27 (100.0)	49 (98.0)	1
Azithromycin	27 (100.0)	44 (88.0)	0.085
Lopinavir/ritonavir	26 (96.3)	39 (78.0)	0.047
Remdesivir	0	1 (2)	1
Interferon-$\beta-1$b	2 (7.4)	7 (14.0)	0.481
Concomitant tocilizumab use	24 (88.9)	0	<0.001

Categorical variables are expressed as no. and (%), and continuous variables as median (Q1-Q3). Mann-Whitney-Wilcoxon test was used to compare continuous variables, and Fisher's exact test to compare categorical variables. SOFA, Sequential Organ Failure Assessment; TCZ, tociluzumab; SpO2/FIO2, peripheral blood oxygen saturation/fraction of inspired oxygen rate; S/CO, absorbance/cut-off; ICU, Intensive Care Unit; HCQ, hydroxychloroquine.

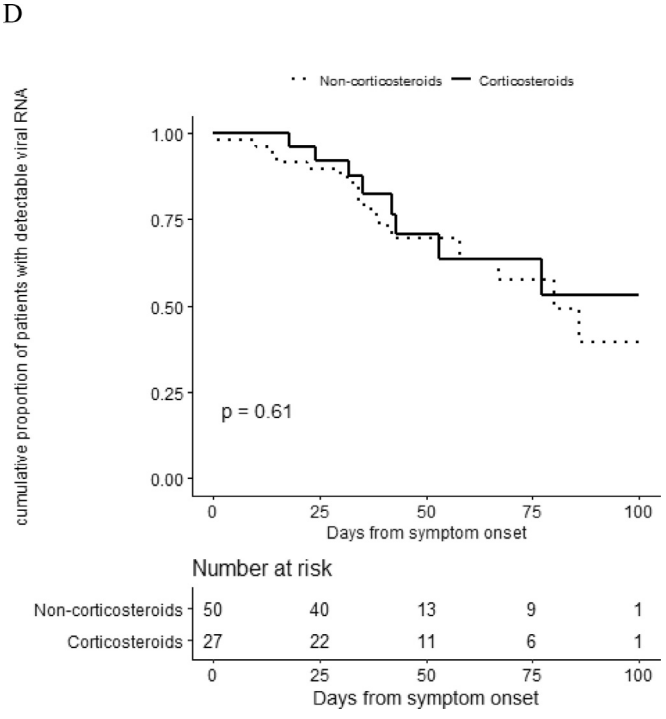


Fig. 1. Continued

higher severity of disease and initial SARS-CoV-2 viral load, such differences vanished after adjustment. Remarkably, most patients on corticosteroids also received anti-interleukin-6 (IL-6) therapy with tocilizumab. Our results show that, even in combination with IL-6 blocking agents, corticosteroids do not negatively impact viral clearance or the humoral immune response against SARS-CoV-2. Further carefully designed studies are warranted to confirm these findings.

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C_t values from SARS-CoV-2 diagnostic PCR assays should not be used as direct estimates of viral load

Dear Editor,

We read with interest the review by Walsh et al.[1] summarizing data on detection patterns and viral loads of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) during the course of infection. We agree with them that determination of SARS-CoV-2 viral load in clinical samples will aid the interpretation of laboratory assays and in the management of isolation and contact tracing protocols, but it should be noted that currently there is no standard measure of viral load in clinical samples. It is becoming common to assume that the C_t values from real-time (quantitative) reverse transcription polymerase chain reaction (qPCR) diagnostic tests are direct measures of viral load, and the use of C_t values has been proposed as a tool to identify those patients who might not be infectious in spite of being positive[2] or to correlate the PCR results with infectivity in cell cultures in order to predict which samples are actually infectious.[3,4] While it is true that C_ts are related to the starting amount of template in the reaction this is not a linear relation and the use of raw C_t values understates the dispersion of the measurements.[5] Another problem is that most diagnostic SARS-CoV-2 qPCR tests are done on suspensions from nasopharyngeal swabs, and these are samples from a surface and have an intrinsic variability that depends on the operator and on the tolerance of the patients.[6] Moreover, the concept of viral load itself is dubious in the absence of a reference mass or volume unit. Finally, the different nucleic acid extraction and amplification systems used by are additional variability sources. For these reasons the assumption that there is a direct relation between the qPCR signal, the amount of virus collected and the amount of virus in the patient's nasopharynx may be misleading and should be taken with care.[7]

To illustrate these points, we take advantage of the design a commercial SARS-CoV-2 RT-qPCR that targets two SARS-CoV-2 genes (E and N) in two different reactions (SARS-CoV-2 Real Time PCR kit, Vircell, Granada, Spain). The tube targeting gene N includes an unrelated (and undisclosed) internal amplification control (primers, probe and a target RNA) in the reaction mix, while the tube targeting gene E includes primers and probes for human RNAse P. The housekeeping RNAse P is a ribozyme expressed in many tissues. Specific primers and probes detect both the gene (DNA) and its RNA product and are included for sample quality control in many SARS-CoV-2 and influenza virus commercial assays.[8] To explore the use of human RNAseP to normalize the data we collected the results of a series of 145 randomly selected positive assays from our registers (March and April 2020). In this set the internal control in the N tube had an average C_t of 30 (range 25.3 to 35.5, IQR=2.1 cycles) (Fig. 1A). The human RNAse P control in the E tube had an average C_t of 28.8 and a broader distribution: range 20.9 to 36.3 and IQR=3.5 cycles (Fig. 1B). The C_t values of the target genes were independent of those of the controls in both cases (Figs. 1A,B, r^2 values not significantly different from zero), meaning that there were no interferences between the target and the control reactions. The variability of the internal controls in the N tubes must be due to experimental errors during the setting up of the PCR reactions, while the higher variability of the human RNAse P controls in the E tubes reflects, in addition, the variations in the amount of material collected with the swabs and in the nucleic acid extraction process.

To correct for sampling variability we used the human RNAse P as a reference to normalize the viral load by the comparative C_t method (ΔC_t)[9] that transforms the C_ts into relative loads (ratios of viral target to human target). Fig. 1C shows a plot of SARS-CoV-2 gene E C_ts normalized with the human RNAse P C_t values against the gene E C_ts. The plot shows an inverse linear correlation, which is expected because C_t values reflect, indeed, viral loads, but the dispersion of the data may reach up to four log units (ten thousand-fold) for any given C_t (black arrow). This is not a problem of this particular brand or PCR design, it could be observed in other commercial (TaqMan 2019-nCoV Assay Kit v1, Thermo Fisher Scientific, Waltham, MA, USA) and in-house[10] assays.

Normalization is not as straightforward as suggested by this example. A full characterization of the linear ranges and a calibration using standards[11] should be done for every different target and primer/probe design. Other reference genes might be explored as

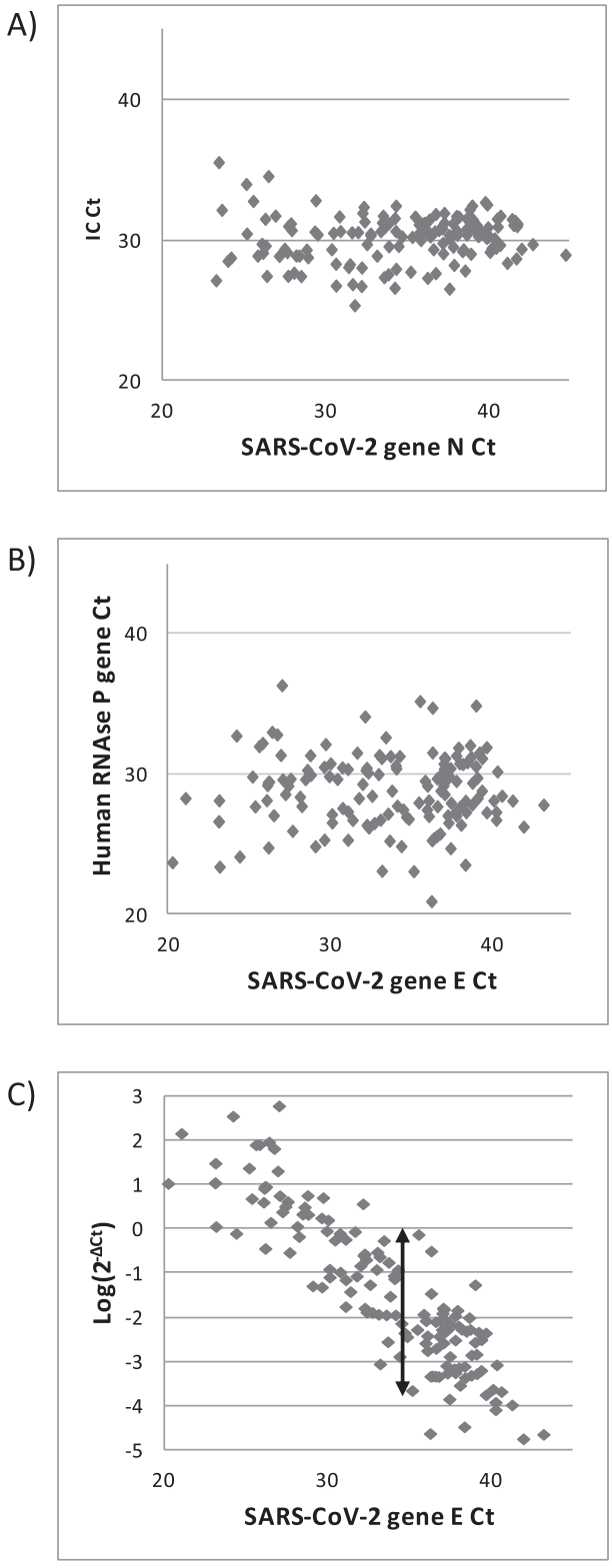

Fig. 1. Analysis of the SARS-CoV-2 C_t values obtained using a commercial RT-qPCR assay (Vircell) in a set of clinical samples. A) C_ts of the Internal Control RNA plotted against the SARS-CoV-2 N gene C_ts ($r^2 = 0.004$). B). C_ts of the human RNAse P plotted against the SARS-CoV-2 E gene C_ts ($r^2 = 0.007$). C) Normalized SARS-CoV-2 gene E C_t values ($\log(2^{-\Delta C_t}) = \log(2^{-C_t\text{target}-C_t\text{reference}})$) plotted against the SARS-CoV-2 E gene. The normalized C_t values are relative loads (ratios of viral target to human target) and are transformed to logarithmic scale for graphical representation. The black arrow illustrates the broad range spanned by any particular C_t value.

well, although human RNAseP has been widely used and might enable to exploit the huge amount of data already collected in many laboratories around the world.

Using C_t values obtained in diagnostic PCR reactions as direct measures of SARS-CoV-2 viral loads is simple, but at the cost of introducing errors that cannot be neglected. Normalization using some marker of the cell mass or the mucosal surface sampled should be integrated into commercial diagnostic kits to make the different assays comparable and to evaluate the potential of quantitative PCR for the clinical management of COVID-19 patients.

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Comparing the 4C mortality score for COVID-19 to established scores (CURB65, CRB65, qSOFA, NEWS) for respiratory infection patients

Dear Editor,

We have taken great interest in Su et al.'s comparison of CRB-65 and qSOFA for predicting intensive respiratory support in COVID-19 patients.[1] The initial assessment of severity is a key part of clinical decision making, guiding management and treatment escalation. This is particularly pertinent with the recent publication of Knight et al.'s 4C mortality score for patients hospitalised with COVID-19 and the upcoming winter respiratory infection season.[2]

Respiratory illnesses often present with symptoms similar to that of COVID-19; fever, cough, shortness of breath and fatigue. This presents a challenge in clinically differentiating patients with COVID-19 from other viral or bacterial infections. Therefore, for clinical assessment and prognostication, a scoring system that can be applied to a wide range of respiratory infections would be beneficial.

We compared the newly validated 4C mortality score to the established CURB65, CRB65 and qSOFA scores in the prediction of 30-day mortality in a variety of existing respiratory infection cohorts in an exploratory analysis. Data from various previous studies performed in Dundee,[3] Hull[4] and South Yorkshire[5] of community-acquired pneumonia (CAP), invasive pneumococcal disease (IPD), and influenza (flu), respectively, plus a COVID-19 cohort (local ISARIC study patients[2]) were analysed.

A total of 606 patients with required data for 4C calculation were analysed from the existing databases described above. Baseline characteristics are presented in Table 1. Overall, the mean age was 60 years old, 30-day mortality was 12% and the median time to death was 5 days. The area under the receiver operating curve (AUROC) with associated 95% confidence intervals was calculated for each scoring system in the respective cohorts (see Table 2).

Table 1
Clinical characteristics of patients included.

	Flu	COVID-19	CAP	IPD
Total	68	53	381	104
Average Age (Years)	43	60	70	65
Male	31 (46%)	28 (53%)	183 (48%)	50 (48%)
Death at 30 days	5 (7%)	6 (11%)	44 (12%)	17 (16%)
Median time to death (Days)	3	10	4	3

The 4C mortality score had the greatest AUROC in COVID 19, CAP and IPD patients (0.83, 0.78 and 0.74, respectively) and had a similar AUROC, compared to the other scores (except NEWS, which was not calculable), in the influenza cohort (0.88). The 4C score was the only score that performed statistically significantly better than chance across all four cohorts.

This supports the findings of Knight et al.,[2] which showed that the 4C mortality score outperformed existing scores in COVID-19 patients. The findings of our analyses also suggest the potential for application of the 4C score in other common, but potentially fatal respiratory infections. A larger prospective validation study of the 4C mortality score versus established scoring systems is needed this winter to confirm its utility in undifferentiated respiratory infection, focusing on the potential for ongoing use of the 4C mortality score, even after the pandemic has ended and the incidence of COVID-19 is much lower.

In conclusion, the 4C mortality score performed well (AUROC of 0.74 to 0.88 across all the cohorts) in predicting 30-day mortality in COVID-19 and other common respiratory infection populations. The 4C score has the potential to be applied broadly this winter, guiding initial escalation and management plans in patient's presenting with symptoms of respiratory infection, prior to a formal diagnosis and regardless of whether they are subsequently confirmed to have COVID-19.

Declaration of Competing Interest

All authors have no conflict of interest to declare.

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Table 2
Area under the receiver operating curve for CURB65, CRB65, qSOFA, NEWS and 4C scores for COVID-19, Flu, CAP and IPD.

Area under the receiver operating curve – 30 day mortality												
	COVID-19 ($N=53$)			Flu ($N=68$)			CAP ($N=381$)			IPD ($N=104$)		
	Value	95% CI	*P*	Value	95% CI	*P*	Value	95% CI	*P*	Value	95% CI	*P*
CURB65	0.62	(0.36–0.88)	0.38	0.92	(0.86–0.99)	<0.01	0.73	(0.67–0.79)	<0.01	0.65	(0.5–0.8)	0.05
CRB65	0.63	(0.41–0.85)	0.25	0.90	(0.83–0.98)	<0.01	0.78	(0.73–0.83)	<0.01	0.63	(0.48–0.8)	0.08
qSOFA	0.61	(0.37–0.84)	0.38	0.89	(0.79–1.0)	<0.01	0.70	(0.62–0.76)	<0.01	0.59	(0.43–0.76)	0.22
NEWS	0.48	(0.23–0.73)	0.89	–	–	–	0.67	(0.60–0.74)	<0.01	0.61	(0.43–0. 79)	0.16
4C	0.83	(0.71–0.95)	<0.01	0.88	(0.79–0.96)	<0.01	0.78	(0.72–0.83)	<0.01	0.74	(0.60–0.88)	<0.01

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Genetic characterization of an H5N6 avian influenza virus from chickens in Guangdong, China

Dear Editor,

Recently, A highly pathogenic H5N6 avian influenza virus (AIV), isolated from Greylag goose, has been documented in this journal[1]. Migratory waterfowl are the major reservoir hosts of AIV.[2] The migration flyways serve as an important source of AIV across distant sites by facilitating fecal-oral transmission.[3] Ten genetically unique clades have been reported for H5 HA, while clade 2.3.4.4 of H5N6 highly pathogenic avian influenza virus(HPAIV) that first identified from China in late 2013 has replaced H5N1 as the dominating HPAIV subtype in southern China.[2,4,5] Since the first human infection with H5N6 was reported in May 2014, a total of 19 cases of infection and 9 deaths were reported in China (www.who.int/influenza/human_animal_interface). The increasing genetic diversity and geographical distribution of H5N6 pose a serious threat to the poultry industry and human health.[6] In this study, we reported the genetic characterization of a HPAI H5N6 strain, isolated from a chicken farm in Jiangmen, Guangdong province in China.

A highly pathogenic H5N6 strain, designated A/chicken/Jiangmen/1001/2019 (H5N6)(JM/19), was identified from the liver, and spleen of dead chickens from a chicken farm in Jiangmen city of Guangdong province. To clarify the genetic characteristics of JM/19, we performed an in-depth sequence analysis of all eight segments of JM/19 by comparing with AIV sequences from GenBank and GISAID (Global initiative on sharing all influenza data). Viral RNA was purified by the AxyPrep Body Fluid Viral DNA/RNA Miniprep kit (Axygen, China) according to the manufacturer's protocol. The Maxima H Minus First Strand cDNA Synthesis Kit (Thermo Fisher Scientific, USA) with a Uni-12 primer (5'-AGCGAAAGCAGG-3') was used for reverse transcription of full-length cDNA. All eight viral segments were successfully amplified by Phusion Hot Start II High-Fidelity PCR Master Mix (Thermo Fisher Scientific, USA) with a set of universal primers as previously designed by Hoffman et al.[7] PCR products were subcloned into the pMD-18T vector and then sequenced. The size of each gene segment of JM/19 is shown in Table 1. The G+C contents were calculated using BioEdit and were 43.95% (PB2), 42.52% (PB1), 44.26% (PA), 41.39% (HA),47.43% (NP),44.42% (NA),48.07% (M), and 44.84% (NS) (Table 1). JM/19 was found to be a HPAIV based on the presence of multiple basic amino acid sequences (KGRRRKR/GLF) at the HA proteolytic cleavage site. Interestingly, our blast results for the cleavage site suggested 327KG328 was first shown in JM/19, while most of the recent H5N6 AIVs had a 327RE328 near the proteolytic cleavage site.

A blast search of viral sequences in the GeneBank database showed a high nucleotide identity of the HA of JM/19 with A/chicken/Vietnam/HU9-842/201 and A/Muscovy duck/Japan/AQ-HE30-77C2/2018, with an identity of 97.71% and 97.65%, respectively. Phylogenetic trees were constructed by MEGA5.1, and the results suggested that the HA gene of JM/19 belonged to clade 2.3.4.4 (Fig. 1A). There are up to 9 recognized wild bird migratory flyways, and our results suggest that the East Asian–Australasian flyway passing through Vietnam, Japan, and China is involved in the transmission of HA gene of H5N6 AIV. Interestingly, NA and the internal genes shared the closest relationship with viruses isolated from geese in China since 2016 (Fig. 1B). NA gene of JM/19 contains a 11-aa deletion at the residue 59–69, which is known as a biomarker for adaptation to terrestrial poultry.

The avian host-specific residues Q226 and G228 (H3 numbering) were found at the receptor binding site (RBS) of HA protein. However, JM/19 possessed 137A and 160A at the RBS, which was found in the human H5 viruses from clade 2.3.4.4 that had dual-receptor specificity. Moreover, JM/19 possesses known mammalian adaptation markers, such as D101N, I121T, S137A, K193D, Q196K, and S227R mutations in HA RBS.[8] Those observations suggested that JM/19 may exhibit binding affinity to human receptors while maintaining a high affinity to avian receptors.[9]

We further checked the N-linked glycosylation (abbreviated G) of two surface glycoprotein HA and NA based on the sequence N-X-S/T (Fig. 1C). The HA protein of JM/19 contained eight pre-

Table 1
Nucleotide Sequence information of the H5N6 viruses and the closest homologs in the GeneBank database.

Gene	Length of coding region	G+C content (%)	Homologous strains	GenBank accession no.	Identity (%)
PB2	2280	43.95	A/goose/China/GS42-1.seq/2016(H5N6)	MN173479	98.95
PB1	2274	42.52	A/goose/China/GS45-2.seq/2016(H5N6)	MN173495	97.99
PA	2151	44.26	A/goose/China/GS42-3.seq/2016(H5N6)	MN173481	98.79
HA	1701	41.39	A/chicken/Vietnam/HU9-842/2018(H5N6)	LC497177	97.71
NP	1497	47.43	A/mink/Eastern China/032/2018(H5N6)	MK812974	99.06
NA	1413	44.42	A/goose/China/GS42-6.seq/2016(H5N6)	MN173484	97.97
M	982	48.07	A/goose/China/GS24-M.seq/2016(H5N)	MN173398	98.37
NS	823	44.84	A/goose/Guangdong/A-Goose-Guangdong-GS014-2015-NS/2015(H5N6)	MN128315	99.15

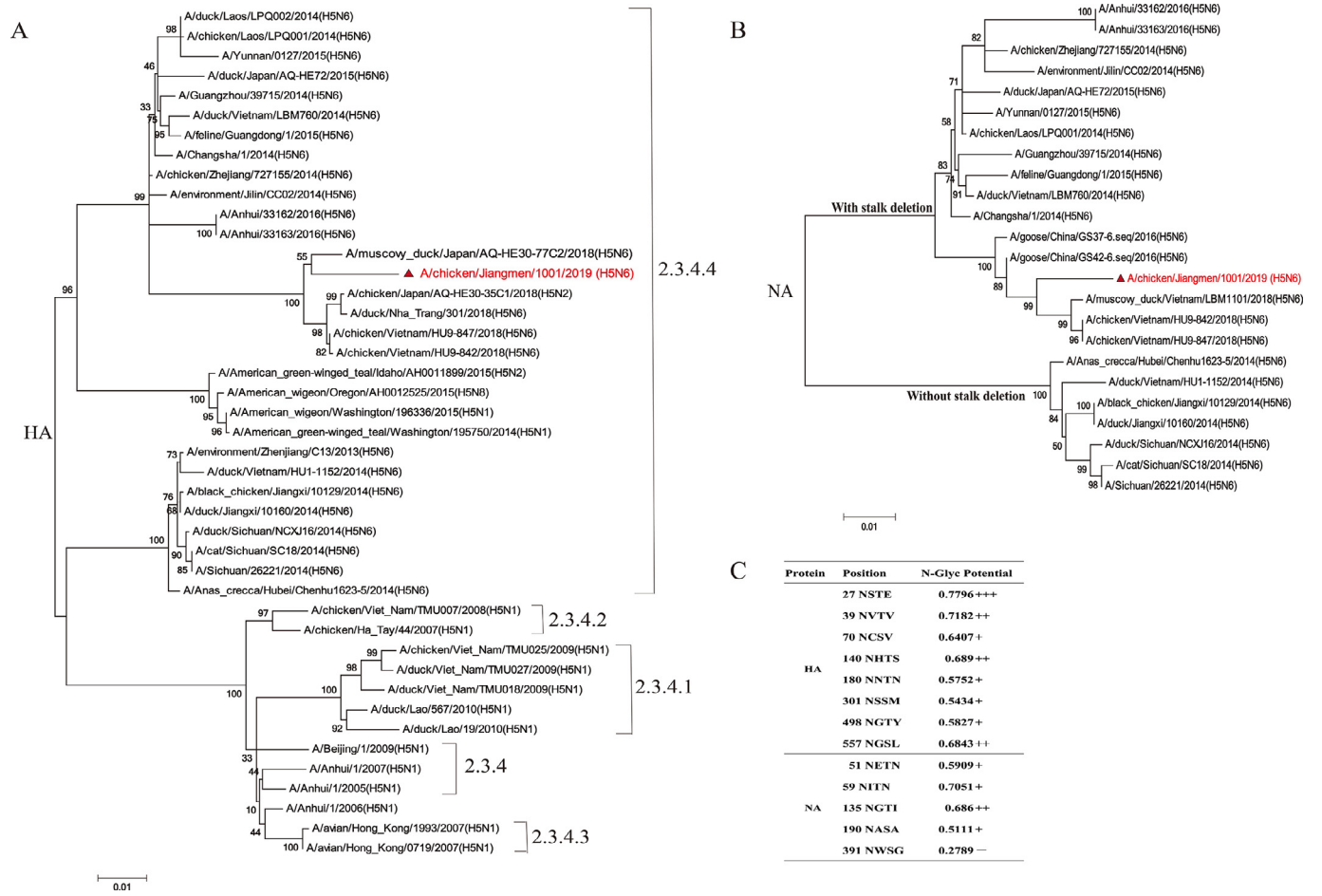

Fig. 1. Phylogenetic trees for the haemagglutinin (HA) and neuraminidase (NA) genes of the A/chicken/Jiangmen/1001/2019 (H5N6)(JM/19). Phylogeny analysis was performed using the maximum likelihood method with 1000 bootstrap replicates in MEGA5.1, The location of JM/19 reported here is indicated with red triangles. (A) Phylogenetic tree of HA gene for JM/19 suggested it belongs to clade 2.3.4.4. (B) Phylogenetic tree of NA gene for JM/19. (C) N-Glycosylation analyses of the HA and NA protein for JM/19 by the NetNGlyc 1.0 Server(http://www.cbs.dtu.dk/services/NetNGlyc/).

dicted glycosylation sites (16G$^+$, 28G$^+$, 59G$^+$, 129G$^+$,169G$^+$,290G$^+$, 487G$^+$, and 446G$^+$). Like most of the 2.3.4.4 (H5) viruses, JM/19 lacked of a glycosylation site at residues 158–160 due to a 160A substitution, which facilitates airborne transmission of AIV in the mammalian hosts. Mutations in M1 (N30D, T215A), and NS1 (P42S, D92E) that associated with the pathogenicity of IAV in mammals were found in JM/19.[8] Nevertheless, PB2 of JM/19 lacked of a mammalian pathogenicity marker 627K. The oseltamivir resistance residues, such as 274H and 294S were found in NA protein.[10] In addition, HA gene contained K166M, S167I, N276Y, and E339G mutations compared to homologous sequences. The significance of these mutations remains to be further explored.

In summary, we reported the gene characterization of a highly pathogenic H5N6 AIV during routine surveillance in chicken farms. Our results suggested that the H5N6 AIV circulating in chickens is more likely a reassortment of H6N6 virus and H5N8 virus with the clade 2.3.4.4 HA. The importance of East Asian-Australasian flyway in the evolution and transmission of H5N6 AIV highlights the need for enhanced AIV surveillance in wild birds.

Declaration of Competing Interest

The authors have no conflicts of interest to declare.

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Low impact of SARS-CoV-2 infection among paediatric acute respiratory disease hospitalizations

Dear Editor,

We read with interest the article by Pagani et al.[1], in which the authors report that 0 to 19-year-old children exhibit the lowest SARS-CoV-2 IgG seroprevalence among all the age-groups. In our centre, which is a 250-bed reference paediatric hospital located in Barcelona, 960 patients with fever and/or respiratory symptoms were tested for SARS-CoV-2 in the emergency department during the regional pandemic peak (week 11–20/2020) and only 56 of them were positive (6%). Thirty-one of them were admitted to hospital (Table S1, Supplementary data) and only 7 with an acute-lower respiratory disease (ALRD). Our centre captured most of regional paediatric hospitalizations due to the closure of paediatric services of general hospitals during the pandemics.

Lower respiratory tract infections are one of the leading causes of paediatric mortality and morbidity worldwide, and they also cause a high number of hospitalizations in well-developed countries.[2] Several reports have described that SARS-CoV-2 infection causes a much milder respiratory disease in children in comparison to adults,[3–6] but the real burden of SARS-CoV-2 infection in children requiring for hospital admission due to ALRD during the pandemics has not been addressed specifically. This study describes the clinical, epidemiological, and microbiological characteristics of children requiring admission with an ALRD during the first pandemic wave. Comparison of variables was made between patients with SARS-CoV-2 confirmed infection (SARS-CoV-2(+)) and those in whom SARS-CoV-2 was not detected (SARS-CoV-2(-)).

Data of patients < 18 year-old with ALRD (pneumonia, bronchiolitis, bronchospasm, or bronchopneumonia) requiring hospital admission were prospectively collected. The study was performed during the first pandemic peak in Spain (week 11–20/2020) after imposition of strict social contact measures (a state of emergency was declared on week 10/2020).[7] Nasopharyngeal samples were collected from all children < 18 year-old with respiratory symptoms and/or fever in our emergency department. They were tested at admission using a real-time polymerase chain reaction assay for RNA detection of SARS-CoV-2. Influenza and respiratory syncytial viruses were also routinely tested using automated molecular assays until the end of these viral epidemics (week 16/2020). A real-time PCR for multiple pathogens was performed in those in whom respiratory specimens were available for re-test after routine microbiological diagnosis. Specific information about microbiological methods and definitions can be found at Supplementary data. This study was approved by the institutional ethical research committee.

411 patients were admitted during the study period, 125 (30%) with a diagnosis of ALRD. Informed consent was obtained from 110 (88%) and they were included in the study. Of them, 7 (6%) were SARS-CoV-2(+).

Median age of SARS-CoV-2(+) children was 16.9 year-old (interquartile range (IQR):11.7–17.7), being significantly higher in comparison to SARS-CoV-2(-) (3.5, IQR:0.9–7.5; $p = 0.004$). Only 2 patients had comorbidities (1 obesity, 1 leukaemia) in the SARS-CoV-2(+) group, whereas pre-existing respiratory conditions (recurrent viral-induced wheezing chiefly) and neurologic chronic conditions were not found despite being quite common among those SARS-CoV-2(-) (44% and 13%, respectively)). Only 3/7 SARS-CoV-2(+) patients had a household confirmed contact Table 1.

There were not significant differences in symptoms between SARS-CoV-2(+) and SARS-CoV-2(-) patients Table 1. Nevertheless,

Table 1
Main epidemiologic, clinical, analytical, and microbiological characteristics of patients with an ALRD admitted during the pandemics in a tertiary care hospital in Catalonia.

	Total ($n=110$)	SARS-CoV-2 (+)($n=7$)	SARS-CoV-2 (-)($n=103$)	p-value
Age (year-old)*	3.7 (0.9–8.4)	16.8 (11.7–17.6)	3.5 (0.9–7.5)	**0.004**
Sex (males)	55 (50%)	4 (51%)	51 (49%)	1
Previously healthy (n)	47 (43%)	5 (71%)	42 (41%)	0.236
- Pulmonary conditions	45 (41%)	0 (0%)	45 (44%)	**0.040**
- Neurologic condition	13 (12%)	0 (0%)	13 (13%)	1
- Cardiovascular conditions	4 (3.6%)	1 (14%)	3 (3%)	0.234
- Haematologic malignancy	2 (2%)	1 (14%)	1 (1%)	0.124
Ethnicity (Caucasian) (n)	64 (58%)	5 (71%)	59 (57%)	0.657
Household confirmed contacts (n with > 1 confirmed contact)	6 (5%)	3 (43%)	3 (2%)	**0.003**
Symptoms at hospital admission (n):				
- Cough	105 (95%)	6 (86%)	99 (96%)	0.285
- Wet cough	61 (55%)	2 (29%)	59 (57%)	0.238
- Fever	80 (73%)	7 (100%)	73 (71%)	0.186
- Gastrointestinal	21 (19%)	2 (29%)	19 (18%)	0.618
- Exanthem	4 (4%)	1 (14%)	3 (3%)	0.236
Time-lag from the onset of symptoms to hospital admission (days)*	4 (2–7)	7 (4–9)	4 (2–7)	0.052
HbSat at admission (%)*	94 (92–96)	94 (91–96)	94 (92–96)	0.909
Chest-X-ray at admission (n/total in whom the test was performed)				
- Normal	12 / 80	0 / 7	12 / 72	0.587
- Lobar pneumonia	22 / 80	4 / 7	18 / 72	0.089
- Interstitial pneumonia	39 / 80	2 / 7	37 / 72	0.431
- Pleural effusion	6 / 80	1 / 7	5 / 72	0.442
Required respiratory support during admission (n)**:**				
- NNCC	78 (71%)	4 (57%)	74 (72%)	0.413
- HFNO	14 (13%)	0 (0%)	14 (14%)	0.592
- NIV	7 (6%)	1 (14%)	6 (6%)	0.380
- MV	2 (2%)	1 (14%)	1 (1%)	0.125
Length of fever (days)*	4 (2–6)	5 (3–11)	4 (2–6)	0.231
Length of oxygen requirements (days)*	2 (1–4)	4 (2–11)	2 (1–4)	0.097
PICU admission (n)	11 (10%)	2 (29%)	9 (95)	0.145
Need for inotropes (n)	2 (2%)	2 (29%)	0 (0%)	**0.004**
Other viral pathogens (n/total tested)				
- RSV	4 / 69	0 / 4	4 / 65	1
- Parainfluenza 1	1 / 32	1 / 4	0 / 28	0.125
- Parainfluenza 4	1 / 32	0 / 4	1 / 28	1
- Influenza A	3 / 70	0 / 4	3 / 66	1
- Influenza B	6 / 70	1 / 4	5 / 66	0.307
- Pre-pandemic coronaviruses	1 / 32	0 / 4	1 / 28	1
- HRV/EV	11 / 32	1 / 4	10 / 28	1
- Adenovirus	2 / 32	0 / 4	2 / 28	1
- Metapneumovirus	2 / 32	0 / 4	2 / 28	1
Clinical classification:				
- Bronchiolitis	29 (26%)	1 (14%)	28 (27%)	0.520
- Bronchospasm/viral-induced wheezing	33 (30%)	0 (0%)	33 (32%)	0.134
- Viral pneumonia	23 (21%)	4 (57%)	19 (18%)	0.030
- Bacterial suspected pneumonia	25 (23%)	2 (29%)	23 (22%)	0.737
Analytical features at admission*:				
- Haemoglobin (g/L)	11.7 (10.9–12.7)	12,4 (11.6–12.7)	11,7 (10.8–12.9)	0.417
- Leukocytes (cells x 10^9 /L)	11.6 (6.9–18.2)	5.5 (3.1–7.9)	13.8 (7.2–19.4)	**0.001**
- Lymphocytes (cells x 10^9 /L)	2.4 (10.5–3.7)	0.7 (0.4- 1.7)	2.8 (1.1–4.1)	**0.007**
- Neutrophiles (cells x 10^9 /L)	7.0 (3.9–12.5)	3.4 (1.5–5.8)	7.5 (4.3–12.6)	**0.010**
- Platelets (cells x 10^9 /L)	338 (243–448)	164 (127–262)	355 (264–468)	**0.012**
- C-reactive protein (mg/L)	37 (13–81)	48 (15–138)	36 (12–82)	0.662
- Procalcitonin (ng/mL)	0.24 (0.08–0.95)	0.06 (0.04–2.42)	0.25 (0.09–1.01)	0.098
- D-dimer (mg/L)	0.95 (0.67–1.91)	0.80 (0.49–4.22)	1.09 (0.76–2.58)	0.602
- Ferritin (µg/L)	183 (88–449)	350 (144–2580)	99 (85–269)	0.072
- Alanine transaminase (UI/L)	13 (8–20)	38 (17–89)	13 (8–17)	0.064
- Aspartate Aminotransferase (UI/L)	24 (18–38)	50 (21–57)	24 (18–33)	0.229
- Creatinine (mg/dL)	0.51 (0.42–0.64)	0.77 (0.67–0.93)	0.48 (0.40–0.59)	**0.007**
Hospital stay (days)*	3 (2–5)	11 (3–16)	3 (2–5)	**0.024**

* median (interquartile-range)Proportions between the groups (SARS-CoV-2(+) Vs SARS-CoV-2(-)) were compared using Pearson Chi-square o Fisher exact test. For continuous variables, the Mann-Whitney U test was performed.NNCC: nasal cannula; HFNO: high-flow nasal cannula; NIV: non-invasive ventilation; IMV: invasive mechanical ventilation; RSV: respiratory syncytial virus; HRV: human rhinovirus; EV: enterovirus.

SARS-CoV-2(+) children required admission after 7 days (IRQ:4–9) since symptoms onset and this time-lag tended to be higher than in those SARS-CoV-2(-) (4, IQR:2–7; $p=0.052$). Regarding physical examination, bronchospasm was not observed in SARS-CoV-2(+) (0/7 vs 77/103, $p<0.001$). Pneumonia was the main clinical diagnosis in SARS-CoV-2(+) children (6/7). Differences in chest-X-ray radiologic patterns were not observed between those SARS-CoV-2(+) and (-). Lower values of leucocytes, lymphocytes, neutrophils, and platelets and higher values of creatinine were found in SARS-CoV-2(+) Table 1. The only patient with bronchiolitis and SARS-CoV-2(+) was a new-born male who required oxygen for less than 24 h.

Seventy patients were tested for influenza infection, 69 for RSV and 32 for multiple viral pathogens. Viral codetection was observed in 1 of 4 SARS-COV-2(+) patients (1 with influenza B, human-rhinovirus/enterovirus (HRV/EV) and parainfluenza), while viral codetection was observed in 25 of 28 SARS-CoV-2(-) patients tested for multiple viral pathogens. Among SARS-CoV-2(-) patients,

HRV/EV was the main viral detection (10/28). There were not significant differences in weekly rates of respiratory virus detections across the study period. Eleven patients, all in the SARS-CoV-2(-) group, had a confirmed bacterial pneumonia (6 *Mycoplasma pneumoniae*, 2 *Streptococcus pneumoniae*, 2 Gram-negative bacteria and 1 *Staphylococcus aureus*).

Despite there were not differences in PICU admission rates between SARS-CoV-2(+) and (-) children, two SARS-CoV-2(+) patients required inotropic support whereas none of SARS-CoV-2(-) required this treatment ($p = 0.004$). Hospital stay was longer (11 days (IQR:3–16) vs 3 (IQR:2–5); $p = 0.024$) and 1 patient died in the SARS-CoV-2(+) group. This was an 11 y-old boy with an influenza B coinfection and a graft-versus-host disease after an allogenic transplant due to an acute lymphocytic leukaemia. Table S2, Supplementary data.

Our results suggest that, despite conducting the study during the pandemic peak, those children who required hospital admission were infected more often by other respiratory microorganisms. Pneumonia was the main clinical diagnosis among SARS-CoV-2(+) children, and they showed indistinguishable clinical and radiological characteristics at hospital admission from those SARS-CoV-2(-), as observed by others.[4] Nonetheless, the two groups showed different analytical features and SARS-CoV-2(+) patients were significantly older.

There are some cases reports of infants with bronchiolitis in whom SARS-CoV-2 was the only infection detected.[8] Literature is scarce addressing this topic, but SARS-CoV-2 does not seem to be a main trigger of bronchiolitis.[9] It caused only 1/29 episodes in our series. In the pre-COVID-19 era, viral coinfection in infants with bronchiolitis caused by coronaviruses was very common (85%).[10] Since the pandemic did not coincide with the bronchiolitis season, the behaviour of the emergent virus in coinfection with other well-known triggers of bronchiolitis is uncertain.

The main limitation of this study was its observational design, as heterogeneous tests were performed in each patient depending on their clinical condition and the epidemiological context. Most patients with ALRD had fever (73/103), therefore a presumable viral infection could be assumed, however a low rate of patients was tested for multiple pathogens. On the other hand, a low number of SARS-CoV-2(+) respiratory patients was found.

To conclude, most of the ALRD episodes identified during the pandemics and under strict lockdown measures were not related to SARS-CoV-2 infection in this study. SARS-CoV-2 was mainly found causing pneumonia in older children, whereas the impact of SARS-CoV-2 infection among ALRD hospitalizations was low in young children.

Authorship

All authors have made substantial contributions to all the following: the conception and design of the study, the acquisition, analysis, and interpretation of data, drafting the article and revising it. The Kids-Corona Paediatric Hospitalist group carried out a very important task collecting data and taking care of patients.

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The authors declare no potential conflicts of interest.

Ethics statement

The institutional ethics board approved the study and informed consent was obtained from parents or carers.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.jinf.2020.10.013.

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Sex-disaggregated data confirm serum ferritin as an independent predictor of disease severity both in male and female COVID-19 patients

Dear Editor,

The current COVID-19 pandemic is revealing profound differences between men and women in disease outcomes. Available sex-disaggregated data for COVID-19 show equal numbers of cases between sexes with fatality rates higher in men than in women. The Italian data confirm this trend with male patients undergoing a worse outcome and a significantly higher lethality at all age groups (13, 9%) compared to females (9, 4%).[1]

We recently reported that increased levels of ferritin were directly related with COVID-19 severity.[2] Particularly, patients who needed admission to the ICU showed 5.8 times higher serum ferritin compared to patients with mild disease. In light of the reported sex differences in COVID-19 severity and lethality, in this letter we present sex disaggregated data of routine serum laboratory testing performed on admission, including serum ferritin, according to disease severity.

141 patients confirmed as COVID-19 were admitted to the isolation ward of Emergency Department at Policlinico Umberto I Hospital in Rome, Italy, between March 2020 and June 2020, were studied. Serum samples were collected from patients upon admission before starting any treatment and tested by Laboratory Department. The patients were 60 females and 81 males, aging 64,48 (16,58) years. The 60 female patients included 44 patients (73.3%) with mild disease and 16 (26.7%) with acute respiratory distress syndrome (ARDS) and systemic inflammation (severe group).The 81 male patients included, 37 patients (45.7%) with mild disease and 44 (54.3%) in the severe group with a significantly higher number of severe cases in males (Chi-squared test $p<0.001$).Disaggregating data by sex, the only parameter that showed a significant difference between male and female patients was ferritin (Table 1, Fig. 1A). Serum ferritin levels were positively correlated with severity of COVID-19 both in male and female patients (Fig. 1B-C). Moreover, ROC curve analysis confirmed the excellent prognostic accuracies of serum ferritin in discriminating patients with severe clinical conditions in both sexes (male patients: AUC 0.961, CI: 0,921 to 1000 $p < 0.001$; female patients: AUC 0.930, CI: 0,865 to 0,996 $p<0,001$) (Fig. 1D-E) with different associated criterion (males: ferritin > 717 µg/L – sensitivity 88.64% specificity 97.30%; females: ferritin > 596 µg/L – sensitivity 81.25%, specificity 93.18%). Based on the severity of pulmonary impairment in CT scan and respiratory failure, the patients were divided in 4 groups according to the WHO guidelines.[3] As shown in Table 2A regarding 60 female patients, 18 did not present CT alterations and did not need mechanical ventilation (Group 0-mild); 17 had changes in CT scan but did not need mechanical ventilation (Group 1-moderate); 15 presented CT scan alterations and needed mechanical ventilation (Group 2-severe); 10 showed CT alterations and needed ICU admission (Group 3-critical). Out of 81 male patients (Table 2B) 11 patients belonged to Group 0-mild; 15 to Group 1-moderate; 23 to Group 2-severe; 32 to Group 3-critical. Sex-disaggregated data obtained by re-analyzing the four groups of patients confirm that male patients have a worse disease severity than women (Chi-squared test for trend $p<0.001$). Table 2A and B also report age and serum levels of C Reactive Protein (CRP), D-Dimer (D-D), Lactate Dehydrogenase (LDH), Neutrophil to Lymphocyte ratio (NLR) and ferritin in the different groups of female and male patients and Fig. 2A-B-C-D shows categorized box and whisker plots of ferritin and age according to COVID-19 severity in female and male patients. Multivariate logistic regression model including age, CRP, D-D, LDH, NLR and ferritin, demonstrated that serum ferritin resulted as an independent predictor of disease severity in male (OR = 1,0058, 95% CI, 1,0013 to 1,0103 $p < 0,001$) and female patients. (OR = 1,0048, 95% CI, 1,0018 to 1,0078 $p < 0,001$).

The outcome of COVID-19 appears to be influenced by the interaction among several genetic, environmental, gender and sex-dependent factors. COVID-19 can trigger a cytokine response storm (CRS) that is associated with high mortality but for which the underlying pathophysiology and diagnostics are not yet well characterized.[4] Loss of balance between adaptive and innate immune systems may result in a hyper-inflammatory state and CRS.[5] It has been reported that male patients have a greater inflammatory reaction, with higher levels of LDH, ferritin, and CRP but a lower lymphocyte count than females, adjusted by age and comorbidity.[6] It is well known that the innate and adaptive immune responses to viral infections are more intense in females and that sex hormones act as important modulators of the immune response.[7] Taken together our sex-disaggregated data show that serum ferritin levels progressively increase with the severity of the disease but males require intensive care more frequently than women. To note, ferritin is a key mediator of immune dysregulation, especially in conditions of extreme hyperferritinemia, via direct immune-suppressive and pro-inflammatory actions, contributing to the CRS involved in COVID-19 complications.[2,8] Although it should be noted that the normal reference ranges for

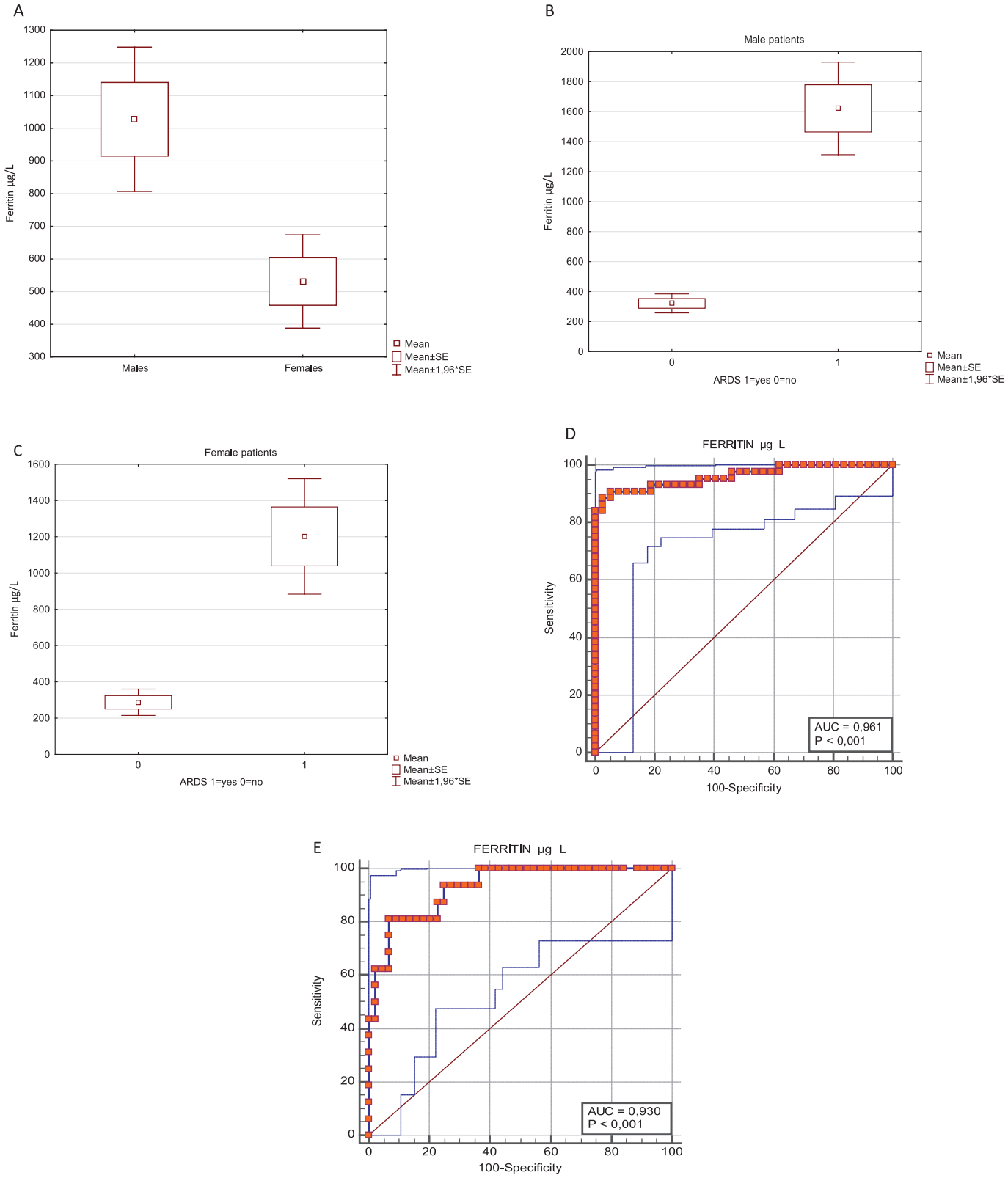

Fig. 1. A. Analysis of Variance of serum ferritin between male (81) and female (60) COVID-19 patients $p<0.001$. B Analysis of Variance of serum ferritin between severe (44) and non severe (37) male COVID-19 patients $p<0.001$. C Analysis of Variance of serum ferritin between severe (16) and non severe (44) female COVID-19 patients $p<0.001$. D ROC curve analysis of serum ferritin levels for the severity of COVID-19 in male patients. E ROC curve analysis of serum ferritin levels for the severity of COVID-19 in female patients.

Table 2A
Analysis of variance of serum parameters in female patients.

	number	Age years	CRP μg/L	D-D μg/dL	LDH IU/L	NLR	Ferritin μg/L
Group*	Mean (SD)	Mean (SD)	Mean (SD)	Mean (SD)	Mean (SD)	Mean (SD)	Mean (SD)
0	18	53.31(18.26)	5.84(11.85)	1624.36(1774.70)	306.57(175.15)	4.88(3.97)	315.85(396.98)
1	17	74.6513.54)	10.31(9.79)	2429.27(1609.08)	356.11(99.15)	6.71(5.65)	279.88(183.85)
2	15	69.73(12.34)	14.87(10.22)	2066.82(1565.46)	265.50(51.44)	6.93(7.43)	592.19(499.38)
3	10	68.00(14.43)	6.49(5.48)	2722.25(1919.16)	590.50(400.93)	11.11(7.26)	1254.19(733.54)
all	60	66.32(16.81)	9.20(10.466)	2160.27(1696.12)	335.68(170.22)	7.02(6.27)	531.13(563.67)
P		0.001	n.s.	n.s.	n.s.	n.s.	0.000

* for group description refer to the text.

Table 2B
Analysis of variance of serum parameters in male patients.

	number	Age years	CRP μg/L	D-D μg/dL	LDH IU/L	NLR	Ferritin μg/L
Group*	Mean (SD)	Mean (SD)	Mean (SD)	Mean (SD)	Mean (SD)	Mean (SD)	Mean (SD)
0	11	51.00(22.91)	2.52(3.31)	930.10(1244.80)	253.00(69.61)	6.37(9.54)	224.00(137.88)
1	15	55.93(16.60)	6.56(7.22)	1468.92(1479.56)	271.40(159.97)	8.13(6.66)	339.93(199.26)
2	23	62.30(13.89)	5.52(5.70)	1583.95(1263.37)	298.20(145.01)	9.90(9.44)	839.56(561.40)
3	32	71.90(10.74)	13.99(10.93)	2548.14(1644.12)	378.46(187.17)	11.59(10.85)	1761.19(1168.41)
all	81	63.16(16.39)	8.70(9.26)	1783.62(1525.03)	306.98(154.98)	9.80(9.69)	1027.54(1013.85)
p		0.000	0.000	0.019	n.s	n.s	0.000

* for group description refer to the text.

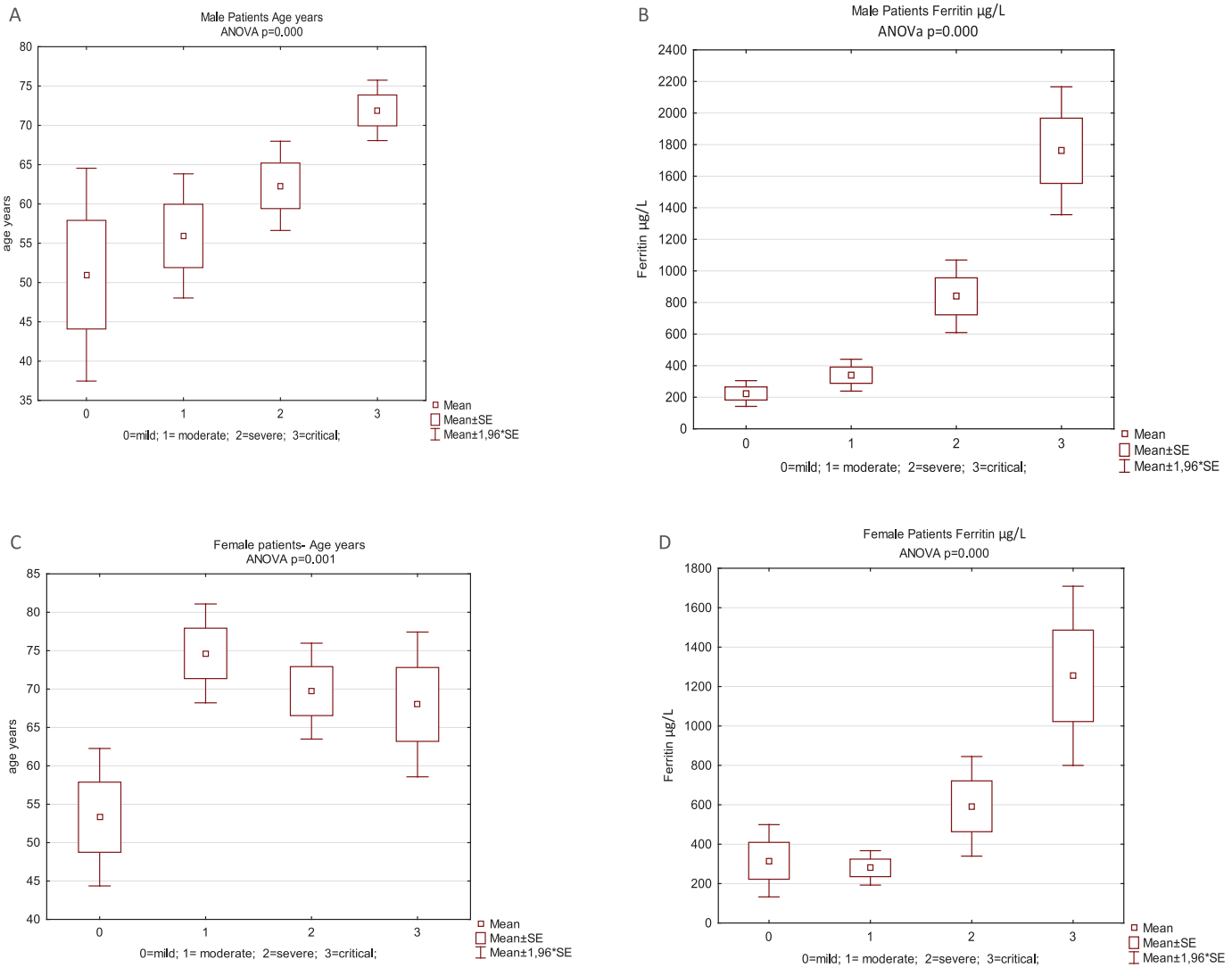

Fig. 2. A-B. Analysis of Variance – Categorized box and whisker plot of age and ferritin according to COVID-19 severity in male patients. C-D Analysis of Variance – Categorized box and whisker plot of age and ferritin according to COVID-19 severity in female patients.

serum ferritin in women show lower values than in men, it could be tempting to speculate that the higher serum ferritin status of males could contribute to the worse outcome of COVID-19 in male patients.

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Impact of COVID-19 pandemic on Measles surveillance in Pakistan

Dear Editor,

We read with great interest the article entitled “Decline in invasive pneumococcal disease during COVID-19 pandemic in Taiwan” by Hung-Jen Tang et at.[1] Authors discussed the decline in invasive pneumococcal disease and here we would like to present the decline in measles cases in Pakistan during the COVID-19 pandemic.

Measles is a highly contagious viral disease which affects susceptible individuals of all ages and remains one of the leading causes of morbidity and mortality among young children causing an estimated 2.6 million deaths each year across the globe. Because of vaccination, more than 21 million lives have been saved and measles deaths have been reduced by 80% since 2000. Despite the availability of a safe and effective vaccine for the last fifty years, many countries around the world still experience measles outbreaks. As of 5 November 2019, there have been 413,308 confirmed measles cases reported to the World Health Organization including more than 110,000 and 140,000 deaths due to measles in 2017 and 2018 respectively.[2]

The present study was conducted to compare the total number of measles cases reported during COVID-19 epidemic between January to August 2020 comapred to the same time period in 2019. The data source utilized for this study is based on the surveillance records available at the Sub-regional Reference Measles Surveillance Laboratory, National Institute of Health, Islamabad, which is the national public health institute supporting disease surveillance and epidemiology programs across Pakistan.

In Pakistan, Measles is a notifiable disease for which the case investigation and reporting is mandatory for all public as well as private clinicians.

A total of 3,253 Measles cases were reported during the first 8 months in 2020 compared to 6,536 measles cases reported during the same period in 2019.

Comparatively in 2020, there was almost 50% reduction with significant difference ($P<0.001$) in total number of reported measles cases as compared to 2019. During the first quarter of 2020 (January, $n=459$), (February, $n=652$) and (March, $n=846$), the reported number of measles cases were higher than in 2019 (January $n=245$), (February $n=326$) and (March $n=663$).

The sudden downward trend in reporting of measles cases in 2020 coincide with the peak of COVID-19 epidemic observed from April to August (April $n=230$, May, $n=124$, June, $n=236$, July, $n=347$ and August $n=359$) compared to a higher number of reported measles cases in 2019 (April, $n=1448$, May, $n=1618$, June, $n=1052$, July $n=810$ and August, $n=374$). According to the results of the study the gradual reduction in Measles cases was noted from April to August 2020, when the highest number of COVID-19 cases were reported such as in April, $n=14{,}778$, May, $n=55{,}643$, June, $n=141{,}010$, July, $n=65{,}676$ and August, $n=17003$ (Fig. 1).

During the current pandemic situation, there may be three important considerations related to the decline in the number of reported measles cases. First, as the measles is also respiratory disease, the strict implementation of preventive measures such as use of mask, frequent hand washing, use of sanitizers, social distancing and ban on public gatherings helped to prevent the measles virus transmission along with the SARS-CoV-2 transmission. The same strategy adopted to combat COVID-19 spreadmay also helped to prevent the transmission of other respiratory infections including tuberculosis, influenza and pneumococcal disease which is already reported by the previous studies.[1,3,4]

Secondly, the government of Pakistan, as many other countries, diverted majority of the available economic resources, towards containment of COVID-19 epidemic that might resulted in decline of major public health services including decline in disease surveillance activities. Thirdly, owing to the panic associated with COVID-19 pandemic and interventions like lockdown restricted public visits at the health care facilities for medical check-up.

The long term impact of COVID-19 epidemic on measles surveillance is unclear but it would affect the under-resource countries like Pakistan struggling on many other fronts such as measles, polio, dengue, tuberculosis, malaria, typhoid and influenza. Globally, nearly 120 million children are at risk of missing their measles vaccine shots this year.[5] Suspension of immunization activities might

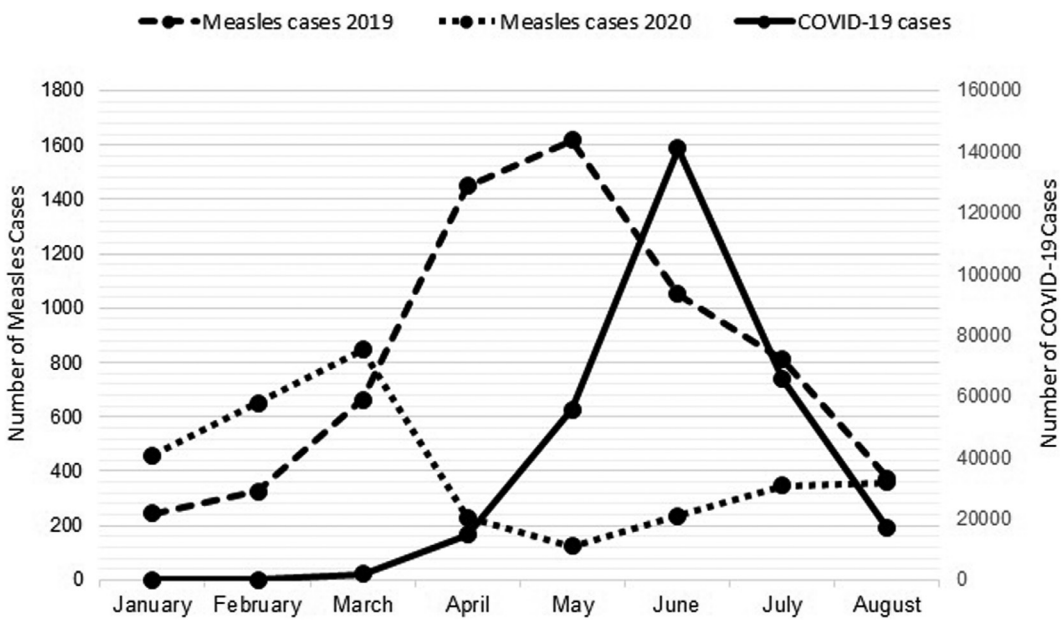

Fig. 1. Month wise Measles cases reported during 2019 and 2020 and COVID-19 cases in 2020.

be helpful in the control of COVID-19 pandemic but on the other hand it may also sow the seeds of other major public health disasters. The risks of measles epidemic is magnified in countries with already low routine immunization coverage. Measles vaccination coverage in Pakistan stood at 66 percent in 2018, instead of required 95 percent coverage which is required to prevent outbreaks.[6]

In conclusion, the health authorities must ensure that the surveillance systems built over years should remain sustained, operational and should not collapse. In-time planning for continuous surveillance and monitoring in the coming months will help to better understand the epidemiology of measles, particularly any resurgence of cases, once the containment measures are lifted. Importantly, under-diagnosis and under-reporting during the COVID-19 pandemic should be ruled out before concluding the unusual data figures generated during this period related to measles, other infectious disease and any other health condition.

Declaration of Competing Interest

All authors have declared that there is no conflict of interest.

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Authors contributions

MSR, AI, SSZZ and MS conceived and designed the study. MSR, MU, MMA, MT and AD were responsible for data collection, lab testing, data analysis. MSR, MMA and OM wrote and revised the manuscript draft.

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False negative RT-PCR and false positive antibody tests–Concern and solutions in the diagnosis of COVID-19

Dear Editor,

We read with interest that antibody testing using a rapid immunochromatographic assay is reliable in the diagnosis of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) infection.[1] However, the accuracy of antibody testing and RT-PCR does not meet the need for a large number of screening tests. False negative RT-PCR and false positive antibody tests are a concern.

Coronavirus disease 2019 (COVID-19), which is caused by SARS-Cov-2, was first detected at the end of 2019, and was named by the World Health Organization on January 12, 2020. COVID-19 is now a pandemic. It took only 25 days for newly confirmed cases to decrease to zero in Beijing in June, which revealed that timely discovery, accurate diagnosis, early isolation and treatment of COVID-19 are the most effective measures.

Suspected cases were judged by epidemiological history and clinical manifestations. Confirmed cases were diagnosed by real-time fluorescent reverse transcription-polymerase chain reaction (RT-PCR) which identified the new coronavirus nucleic acid and serum IgM/IgG antibody tests. Infected persons with false negative RT-PCR results may not be isolated and can infect others.[2] False positive results can cause panic among patients and doctors. Furthermore, unnecessary crowd isolation wastes human and material resources.

Different types of clinical specimens and thermal inactivation may cause false negative RT-PCR results. Nucleic acid detection has the limitation of a low positive rate in different types of clinical specimens. Wenling Wang's research revealed that bronchoalveolar lavage fluid specimens showed the highest positive rates (14 of 15; 93%), followed by sputum (72 of 104; 72%), nasal swabs (5 of 8; 63%), fibrobronchoscope brush biopsy (6 of 13; 46%), pharyngeal swabs (126 of 398; 32%), feces (44 of 153; 29%), and blood (3 of 307; 1%).[3] We recommend that upper respiratory tract specimens be collected in the acute phase and lower respiratory tract specimens or feces samples be collected in the non-acute phase.

Based on the knowledge of SARS-CoV and Middle East respiratory syndrome (MERS)-CoV, thermal inactivation at 56 °C was recommended to inactivate SARS-CoV-2 before nucleic acid testing. However, Pan Y's research demonstrated that thermal inactivation affected the efficiency of RT-PCR for SARS-CoV-2 detection and chemical inactivators, such as guanidinium-based lysis, are suggested. His study showed that approximately half of the weakly positive samples (7 of 15 samples, 46.7%) were RT-PCR negative after heat inactivation in at least one parallel test.[4]

At the initial stage of the SARS-CoV-2 epidemic in February 2020, we observed 57 suspected COVID-19 infected patients. Pharyngeal swabs were tested for nucleic acid and serum samples were obtained for 2019-nCOV IgM and IgG tests. The positive rate of COVID-19 nucleic acid was 42.10%. The positive detection rate of combined 2019-nCOV IgM and IgG for patients with COVID-19 negative and positive nucleic acid tests was 72.73% and 87.50%, respectively.[5] These data demonstrated that the detection of novel coronavirus antibodies is an important supplementary method for the diagnosis of COVID-19. In China's trial version 7 of the diagnosis and treatment guideline for the novel coronavirus disease (COVID-19), serum novel coronavirus specific IgM antibody and IgG anti-

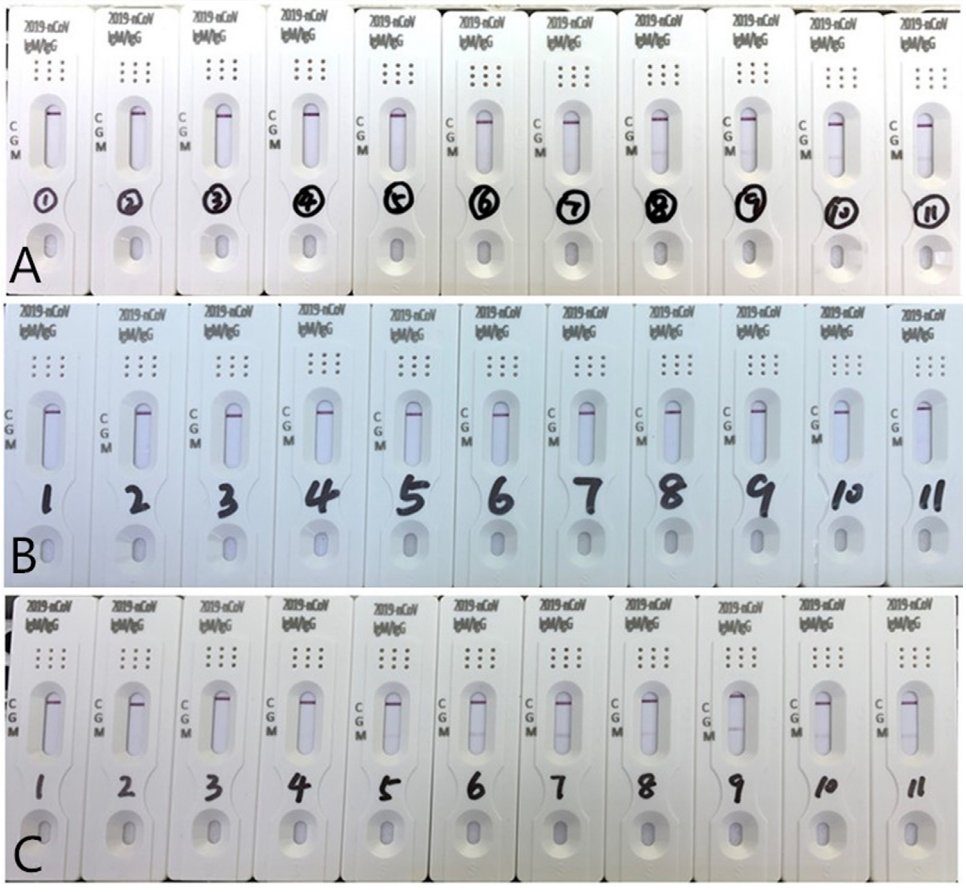

Fig. 1. Influence and elimination experiment resulting in a false positive test result, which was affected by serum rheumatoid factor levels.
Eleven different serum concentrations of rheumatoid factor were tested for 2019-nCoV IgM and IgG. From left to right, RF was 16.1, 85.0, 133.0, 187.5, 231.7, 284.3, 331.0, 390.5, 450.0, 490.6, and 539.7 (IU/mL). A is the original result, B and C are the results diluted five times with normal human serum and physiological saline, respectively.

body were regarded as criteria for confirming COVID-19 in patients on March 3, 2020.

A study involving 1779 patients in Iceland, showed that 91.1% of those who recovered from SARS-COV-2 infection tested seropositive.[6] It was found that the antibody test may be affected by other factors such as rheumatoid factor (RF) in the serum.[7] Rheumatoid patients often have different concentrations of RF, and RF has five types of immunoglobulins including IgM, IgG, IgD, IgA and IgE. A high concentration of RF-IgM may interfere with novel coronavirus IgM/IgG antibody detection. The effects of RF concentration from 16.1 IU/mL to 539.7 IU/mL on the detection of 2019-nCoV IgM and IgG tests were observed. It was found that RF may lead to false results when the serum RF level was higher than 231.7 IU/mL. The false positive antibody results could be eliminated after five times dilution with normal human serum, when the RF level was lower than 10 IU/mL. It was not eliminated after five times dilution with physiological saline[Fig. 1]. We also identified five patients with false antibody results, who had nasopharyngeal carcinoma, colon cancer, duodenal carcinoma, diabetes, and diffuse bronchitis, respectively. Serum RF level in these patients was lower than 100 IU/mL. The false positive antibody results could also be eliminated after 5 times dilution with normal human serum. Thus, further studies are needed to investigate the false results of this test.

We believe that no diagnostic technique has 100% sensitivity and specificity. Although the RT-PCR test has become the standard method for the diagnosis of SARS-CoV-2 infection, false-negative rates have been reported. For the serological antibody test, the detection time needs to consider the window period. Moreover, several factors should be considered when diagnosing COVID-19, including epidemiology, history of exposure and clinical symptoms, such as fever or respiratory disease.

Therefore, the combination of serum IgM/IgG antibody detection, the nucleic acid test, CT scan and clinical features improves the accuracy of COVID-19 diagnosis.

Declaration of Competing Interest

The authors declare that they have no conflict of interest.

Ethical approval

This study was approved by the Ethics Committee of Shenzhen Hospital, Southern Medical University (NYSZYYEC20200009).

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Co-infection in COVID-19, a cohort study

Dear Editor,

Co-infection in COVID-19 patients may inflence the outcome of the disease and needs more attention and investigations. In this journal, Lansbury and colleges reported a meta-analysis of co-infections in COVID-19 patients.[1] In this study, we investigated a COVID-19 cohort in Shanghai, China. We screened viruses include *Human parainfluenza virus 1, Human parainfluenza virus 2, Human parainfluenza virus 3, Human parainfluenza virus4, Influenza A virus, Influenza B virus, Human rhinovirus, Human metapneumovirus, Human respiratory syncytial virus, Human Bocavirus, Human adenovirus, Human Coronavirus 229E, Human Coronavirus NL63, Human Coronavirus HKU1, Human Coronavirus OC43;* bacteria include *Pseudomonas aeruginosa, Moraxella catarrhalis, Mycobacterium tuberculosis, Legionella pneumophila, Group A Streptococcus, Haemophilus influenza, Staphylococcus aureus, Acinetobacter baumannii, Streptococcus pneumonia, Klebsiella.peneumoniae, Escherichia coli and Mycoplasma pneumonia, Chlamydia pneumonia by a taqman-based real time PCR methods.*

Eighty-nine patients were enrolled with disease outcomes include mild, moderate, severe and critical (Chinese clinical guidance for COVID-19 pneumonia diagnosis and treatment (7th edition) published by China National Health Commission on March 4, 2020. http://www.gov.cn/zhengce/zhengceku/2020–03/04/content_5486705.htm). Nine patients showed severe or critical symptoms. As reported, age is a risk factor for severe symptoms (Table 1).[2,3] Nucleic acids from the throat swab samples of the COVID-19 patients were used as template for Real-time PCR. Real-time PCR was performed by using One Step PrimeScript RT-PCR kit (Takara) in a 25μl reaction mixture as following: 2.5μl of nucleic acid was added in the mixture of 12.5μl of 2 × one step RT-PCR buffer, 0.5μl of EX Taq HS, 0.5μl of RT Enzyme, 1.6μl

Table 1
Patients in this study.

Age	Patients (Total number)	Patients (Severe or critical symptom)
15–60	66	1
61–75	20	5
>75	3	3

of primer mix (10μM each), 0.4μl of probe (10μM) and H_2O up to 25μl. The R-PCR program was run with incubation at 42 °C for 10 min, followed by 40 cycles of a program (95 °C, 10 s; 95 °C 10 s, 60 °C, 1 min). The primers and probes for Influenza A virus were as following: sense (5′-CTT CTA ACC GAG GTC GAA ACG TA-3′), antisense: (5′-GGT GAC AGG ATT GGT CTT GTC TTT A-3′) and probe: (5′-FAM-TCA GGC CCC CTC AAA GCC GAG −3′-BHQ1); For *E. coli* were as flowing: sense (5′-GGA TAT CGT CTG GGA CTT CCG-3′), antisense (5′-GCG GAG CCA GAC CGA ATT T-3′) and probe (5′-FAM-GTG AAA TCG ATC AGT GCT TCA GGC CA −3′-BHQ1). Screening for other pathogens were performed by using real-time PCR detection kits from BioGerm (Shanghai, China) as following: human parainfluenza virus 1/human parainfluenza virus 3(PIV1/PIV3) (BioGerm, SJ-HX-215–2), human parainfluenza virus 2/human parainfluenza virus 4 (PIV2/PIV4)(BioGerm, SJ-HX-216–2), Influenza B virus (FLUB)(BioGerm, SJ-LG-006–2), human rhinovirus/human metapneumovirus/Human respiratory syncytial virus (HRV/HMPV/RSV)(BioGerm, SJ-HX-303–2), human Bocavirus/human adenovirus (HBOV/RADV)(BioGerm, SJ-HX-207–2), human Coronavirus 229E/human Coronavirus NL63 (BioGerm, SJ-HX-204–2), human Coronavirus HKU1/ human Coronavirus OC43 (BioGerm, SJ-HX-205–2), Pseudomonas aeruginosa (PA)(BioGerm, SJ-HX-039–2), Moraxella catarrhalis (MC)(BioGerm, SJ-HX-027–2), Mycobacterium tuberculosis (MTB)(BioGerm, SJ-HX-040–2), Mycoplasma pneumonia/Chlamydia pneumonia/Legionella pneumophila (MP/CP/LP)(BioGerm, SJ-HX-302–2), Group A Streptococcus/Haemophilus influenzae/Staphylococcus aureus (GA/HI/SA)(BioGerm, SJ-HX-319–2), Acinetobacter baumannii/Streptococcus pneumoniae/Klebsiella peneumoniae (AB/SP/KP)(BioGerm, SJ-HX-309–2).

As shown in the Table 2, we detected co-infections in 18 patients. We detected co-infection with *Kp (Klebsiella.peneumoniae)* in 6 patients, co-infection with *EC (E. coli)* in 5 patients, co-infection with *Mcat (Moraxella catarrhalis)* in 4 patients, co-infection with *Hi (Haemophilus influenzae)* in 4 patients, co-infection with *Ab (Acinetobacter baumannii)* in 2 patients, co-infection with *Sa (Staphylococcus aureus)* in 2 patients, co-infection with *PA (Pseudomonas aeruginosa)* in 1 patient, and co-infection with *GAS (Group A Streptococcus)* in 1 patients. Notably, 6 patients got coinfection with more than two bacteria. One patient with a moderate to severe disease and one patient with severe disease got co-infection with *Mcat (Moraxella catarrhalis).* One patient with a critical disease got co-infection with *Ab (Acinetobacter baumannii).* We didn't detect co-infection with any virus in these patients.

M. catarrhalis typically infect adults with a weakened immune system.[4] Elderly COVID-19 patients have impaired Cytotoxic CD8+ *T* Cell Responses, which may make them highly risk for infection.[5] *A. baumannii* is a common pathogen in Intensive Care Units (ICU) and evolves rapidly to be resistant to many antibiotics and should be seriously considered for critical COVID-19 patients.[6]

In summary, we carried out an extensive pathogen screening in a COVID-19 cohort. We didn't detect co-infection of SARS-CoV-2 with other viruses. Co-infection with bacteria was detected in 18 of the 89 patients. *M. catarrhalis* was detected in two patients with severe symptoms and *A. baumannii* was detected in one patient with critical symptoms. These bacterial co-infections should be taken care in managing the COVID-19 patients.

Ethics statements

This study was approved by the ethics committee of Shanghai public health clinical center under the study number YJ-2020-S077-02, and the procedures were carried out in accordance with approved guidelines. Informed consent was obtained from the subjects.

Authors' contributions

YZ conceived the manuscript; SW and JX performed experiments; ZX, LY collected samples; YZ all authors performed litera-

Table 2
Co-infection in the COVID-19 patients.

Patients	Pathogen	Age	Ct Value	Disease Severity
277#	*PA, Pseudomonas aeruginosa*	15	35.47	*Mild*
726#	*Mcat, Moraxella catarrhalis*	76	35.14	*Moderate to Severe*
144#	*Kp, Klebsiella.peneumoniae*	32	32.87	*Mild*
973#	*Hi, Haemophilus influenzae*	57	30.52	*Moderate*
994#	*Mcat, Moraxella catarrhalis*	35	24.16	*Mild*
	GAS, Group A Streptococcus		32.46	
903#	*Hi, Haemophilus influenza*	21	26.55	*Mild*
	Kp, Klebsiella.peneumoniae		35.82	
830#	*Ab, Acinetobacter baumannii*	65	30.17	*Critical*
14#	*Mcat, Moraxella catarrhalis*	37	34.49	*Mild*
	Hi, Haemophilus influenza		34.01	
	Kp, Klebsiella.peneumoniae		26.19	
743#	*Hi, Haemophilus influenza*	36	35.13	*Mild*
	Ab, Acinetobacter baumannii		32.11	
952#	*Sa, Staphylococcus aureus*	33	31.94	*Moderate*
	EC, E. coli		35.77	
976#	*Kp, Klebsiella.peneumoniae*	32	35.42	*Mild*
75#	*EC, E. coli*	45	26.40	*Mild*
1002#	*Sa, Staphylococcus aureus*	19	34.68	*Moderate*
225#	*Mcat, Moraxella catarrhalis*	67	28.62	*Severe*
63#	*Kp, Klebsiella.peneumoniae*	34	32.02	*Mild*
	EC, E. coli		27.38	
67#	*EC, E. coli*	27	23.01	*Mild*
74#	*EC, E. coli*	66	28.43	*Mild*
327#	*Kp, Klebsiella.peneumoniae*	64	33.97	*Mild*

ture search; YZ wrote the first draft of the manuscript; all authors reviewed it. SW and JX contributed equally.

Declaration of Competing Interest

The authors declare no conflicts of interest.

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Effect of administering subsequent immune checkpoint inhibition in cancer patients with prior COVID-19 infection

Dear Editor,

We read with interest the recent article published by Skevaki C et al who described the laboratory characteristics of patients infected with the novel SARS-CoV-2 virus,[1] which indicated that patients with COVID-19 have varying degrees of multiple organ dysfunction. Coronavirus disease 2019 (COVID-19) is characterized by symptomatology from immune hyperactivation against normal tissues such as the lungs. Because immune checkpoint inhibitors (ICIs) are intended to galvanize the immune system, it is imperative to evaluate the effect of ICI therapy in patients who have recovered from prior COVID-19.

In this study, we collected and analyzed data from 8 cancer patients with SARS-CoV-2 infection history (negative for viral RNA and positive for serum antibodies) who received subsequent immunotherapy from four hospitals, including Hubei Cancer Hospital, Union Hospital, People's Hospital of Dongxihu District and The Fifth Hospital of Wuhan. All patients had been diagnosed with COVID-19 via positive for nucleic acid testing before. We reviewed the medical records, including SARS-CoV-2 nucleic acid and antibody testing, chest computed tomography (CT), biochemical markers, and routine bloodwork and treatment data of all cancer patients with prior COVID-19 infection from March 9 to July 29, 2020. Confirmation of prior COVID-19 infection was defined as positive SARS-CoV-2 antibody testing by the colloidal gold immunoassay (Innovita, Tangshan, Hebei, China) and negative for viral nucleic acid by real-time reverse transcriptase PCR (rRT-PCR). The median age was 58 years (range: 41–72), and 63% patients were men. Four (50%) patients had a history of smoking and three (38%) patients had chronic diseases. All cases harbored positive serum antibodies: 1 was negative for immunoglobulin G (IgG^-) and positive for immunoglobulin M (IgM^+), 6 were IgG^+ IgM^- and 1 was IgG^+ IgM^+. Nasopharyngeal cancer was the most frequent neoplasm (3/8 [38%] patients), followed by lung cancer (2/8 [25%] patients) (Table 1). Two (25%) patients had received ICI therapy prior to initially developing COVID-19, and 6 (75%) patients were ICI therapy-naïve.

Three (38%) patients received a combination of anti-PD-1 agents with chemotherapy. Two (25%) patients were treated with immunotherapy alone. Two (25%) patients received combinatorial anti-PD-1 ICI with radiotherapy ± chemotherapy. One (13%) patient received anti-PD-1 with anlotinib.

The median follow-up from initial administration of ICI therapy was 83 days (IQR: 64–98). At the time of last follow-up, all patients remained negative for SARS-CoV-2 RNA nucleic acid, without suspicious changes on chest CT. Five (63%) patients experienced altered immunoglobulin test results. Specifically, 3 (38%) patients who were initially IgG^+ IgM^-became IgG^-IgM^- after 25, 42, and 62 days of ICI therapy. Another was initially IgG^+ IgM^+ but became IgG^+ IgM^-after 23 days, and the final patient (initially IgG^- IgM^+) became IgG^- IgM^- following 28 days of ICI therapy.

Systemic therapies were tolerated well in this cohort. Three patients developed grade 2 myelosuppression and one patient had hypothyroidism. Only one patient had grade 3 myelosuppression.

Cancer patients are a vulnerable population to COVID-19, and delaying therapies such as ICIs risks disease progression. It have reported that SARS-CoV-2 can reemerge in recovered (with negative viral RNA) patients,[2] and some studies have reported hepatitis B virus (HBV) or tuberculosis reactivation in cancer patients undergoing ICI therapy.[3, 4] Thus, it became essential to evaluate the safety of ICIs in patients recovered from prior COVID-19 infection.

Table 1
Clinical characteristics of cancer patients receiving systemic therapy with prior SARS-CoV-2 infection.

Patient No.	Sex	Age	PS	Cancer diagnosis	Staging	Chronic diseases	Systemic therapy	Time of nucleic acid testing
1	Female	59	1	NPC	rT0N1M0	None	2 cycles of GP and 2 cycles of GP +PD-1 inhibitor	April 21; May 15; June 3; June 6; June 30; July 20
2	Male	41	1	NPC	T3N2M0	None	2 cycles of GP + PD-1 inhibitor; radiotherapy + 2 cycles of DDP + PD-1 inhibitor	March 25; April 17; May 29
3	Female	48	1	NPC	T3N1M0	None	Radiotherapy + 2 cycles of PD-1 inhibitor; 1 cycle of TP	April 26, May 8; June 10; June 15
4	Male	72	1	NSCLC	T3N1M0	Hypertension; Cardiovascular disease; COPD	2 cycles of abraxane and 2 cycles of abraxane + nedaplatin + PD-1 inhibitor	March 23; April 1; April 3; May 5; June 3; June 29
5	Male	64	1	NSCLC	T2N2M0	Cardiovascular disease	2 cycles of PD-1 inhibitor	April 18; May 12; May 26; June 9; July 17
6	Male	71	1	Soft tissue sarcoma	T3N0M0 G3	None	2 cycles of gemcitabine + anlotinib + PD-1 inhibitor	May 20; June 3; June 4; June 29
7	Female	51	1	Rectal cancer	rT0N0M1	None	4 cycles of XELOX + PD-1 inhibitor	April 16; May 5; May 29; July 1
8	Male	57	1	Esophagus cancer	T4aN2M0	Hypertension; Diabetes	3 cycles of capecitabine + nedaplatin + PD-1 inhibitor	March 20; June 1; July 2

Abbreviations: NPC, nasopharyngeal cancer; NSCLC, non-small cell lung cancer; COPD, chronic obstructive pulmonary disease; GP, gemcitabine and cisplatin; PD-1, programmed cell death protein 1; XELOX, oxaliplatin + capecitabine.

Importantly, this study did not observe a single case of disease reactivation or immune hyperactivation from ICI therapy.

Patients with COVID-19 have varying degrees of multiple organ dysfunction.[5,6] The rates of liver dysfunction, acute kidney injury, and cardiac injury can be as high as 29%, 29% and 23%, respectively, in critically ill patients.[6] From our analysis, our data demonstrate that cancer patients with prior COVID-19 infection who undergo ICI therapy do not show an overtly increased susceptibility to organ dysfunction in the short term. Although the quality of evidence is overall low, our study may help add important data to this emerging issue.

Our study has several limitations, in addition to its retrospective nature. According to the COVID-19 Diagnostic Criteria,[7] viral serum antibodies are indeed valid for diagnosis; however, false positives/negatives can still occur. Additionally, the number of such cases herein remains relatively small, and a larger sample size in patients with cancer is needed to validate these findings, including a deeper understanding regarding the diverse types of ICIs as well as the impact of combinatorial therapies.

Declaration of Competing Interest

All other authors declare no competing interests.

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On-site Multiplex PCR for CSF diagnostics in an Acute Hospital versus Referral to Reference Laboratories: Assessing Economic Factors, Length of Stay and Antimicrobial Stewardship.

Dear Editor,

The Journal of Infection recently published a systematic review and validation of diagnostic prediction models in cases of suspected meningitis, performed by Ingeborg van Zeggeren and his colleagues[1]. The authors advise against the use of diagnostic prediction models in clinical practice, due to a variable sensitivity and specificity, inconsistent validation and poor performance.

Distinct clinical features of central nervous system (CNS) infection may not always be present, particularly in paediatric patients. We propose that increased access to on-site multiplex polymerase chain reaction (PCR) diagnostics for all cerebrospinal fluid (CSF) samples is both cost-beneficial and may alleviate diagnostic uncertainty in these settings. Our study, conducted in Cork University Hospital (CUH) from April 2019 to April 2020, evaluated the usefulness of on-site CSF PCR, versus sample referral to reference laboratories, and assessed differences in antibiotic prescribing, length of stay (LOS), and hospital expenditure.

The BioFire® FilmArray® Meningitis/Encephalitis panel (FA-M/E), bioMérieux (Marcy-l'Étoile, France) is a multiplex PCR assay, which allows for rapid (one-hour), simultaneous detection of fourteen common pathogens: *Escherichia coli* K1, *Haemophilus influenzae, Listeria monocytogenes, Neisseria meningitidis, Streptococcus agalactiae, Streptococcus pneumoniae*, Cytomegalovirus (CMV), Enterovirus (EV), Herpes simplex virus 1 and 2 (HSV1,2), Human herpesvirus 6 (HHV6), Human parechovirus (HPeV), Varicella zoster virus (VZV), and *Cryptococcus neoformans/gattii*.

All CSF specimens with an age-adjusted raised white cell count (>5 WCC/cm^2 adults, >15 WCC/cm^2 less than 1 year old, >30 WCC/cm^2 less than 28 days old) were tested on the FA-M/E. Samples were simultaneously sent to reference laboratories to correlate results and allow comparison of turn-around time (TAT).

Information on clinical presentation, antimicrobials and length of stay (LOS) was gathered by retrospective chart review. LOS data was retrospectively collected on a case-matched cohort admitted during the year prior to introducing the FA-M/E, to gather historical-control data for statistical analysis. Potential cost savings were calculated against standard of care (SOC), assuming the patient would remain in hospital until a PCR result was returned, and that antimicrobials would be continued until a negative viral PCR result was available, or bacterial culture was negative at 48hrs.

135 tests were performed on the FA-M/E, of which 72 CSFs meeting the study criteria were included in the analysis (*Fig. 1*). 46 (64%) were adults and 26 (36%) were children. Median age was 23.5 years (range 0-87). The most common documented symptom was pyrexia (55%), followed by headache (54%), gastrointestinal upset (35%), behavioural change (29%), neck stiffness (28%), photophobia (25%), rash (14%) and seizures (12.5%).

There were 33 positive detections (*Fig. 1*), all of which were considered clinically significant, apart from two detections of HHV6 attributed to chromosomal integration.

Two bacterial PCR detections had no growth on culture, however, antimicrobials had been administered prior to CSF sampling in both cases. Overall concordance with the reference laboratories' results was 94%. There were four discrepant results (*Table 1*).

Six CSFs, which were positive for viral targets at the reference laboratory, were not tested on the FA-M/E because of a normal WCC. These included four EV (WCC 0-2/cm^2), one VZV (WCC 1/cm^2) and one HSV-1 (WCC 4/cm^2).

Median TAT was 2.1 hours (CUH) versus 5.6 days (reference laboratories) ($p<0.0001$). Mean improvement in TAT was 5.4 days (95% CI [4.8, 5.9]).

LOS was compared between 'Cohort 1', a group prior to introduction of FA-M/E (n=50, 17 children, 33 adults), and 'Cohort 2', post-FA-M/E (n=72, 26 children, 46 adults). A Mann-Whitney U test revealed no statistically significant difference in total LOS ($p=0.38$), however, a subcategory analysis of Enterovirus positive cases, which comprised the majority of positive detections in both groups, indicated that LOS was greater in 'Cohort 1' (n=17, median 4.2 days) versus 'Cohort 2' (n=17, median 2.2 days) ($p=0.02$).

60 (83%) patients received broad-spectrum empirical antimicrobial therapy, of which only 23% was, in retrospect, appropriately directed at a subsequently diagnosed infection.

In 44 (61%) cases, antimicrobials were changed based on the FA-M/E result. Of those, 17 (39%) were immediate, 8 (18%) in <24hrs, 10 (23%) in 24-48hrs, 7 (16%) in 48-72hrs and 2 (4%) in >72hrs. A total of 283 doses of aciclovir, 91 doses of third-generation cephalosporin, 42 doses of amoxicillin and 7 doses of gentamicin were potentially avoided. In four cases, an essential antimicrobial agent was commenced based on the rapid acquisition of a FA-M/E result. A Chi-squared test of independence showed a significant correlation between a positive FA-M/E result and more rapid antimicrobial change ($p=0.04$).

Cost of antimicrobials was €245.59 (FA-M/E) versus €311.77 (SOC) per course of treatment ($p<0.0001$). Total cost of LOS per patient was €12,136.40 (FA-M/E) versus €13,622.57 (SOC) ($p<0.0001$). Subtracting cost of consumables (€150/FA-M/E), €1,402 was saved per case, resulting in a predicted total of €100,969 saved over one year.

Within positive FA-M/E results, significant reductions in antimicrobial consumption (€75 per case, $p<0.05$) and LOS (€1735 per case, $p<0.0001$) were seen in Enterovirus positive results compared with all other positive targets.

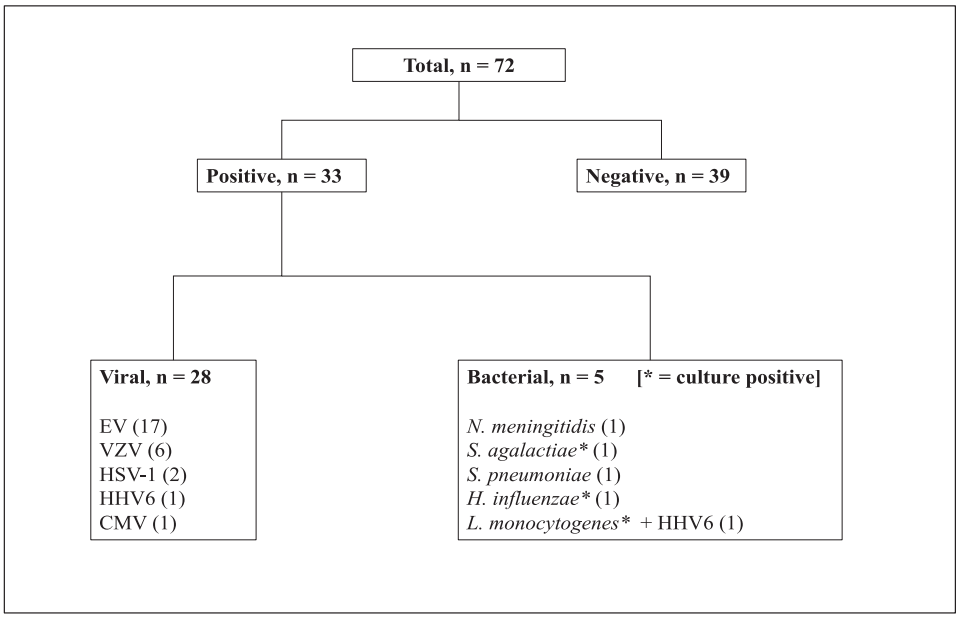

Fig. 1. Key: CNS = Central Nervous System, WCC = White Cell Count, EV = Enterovirus, VZV = Varicella zoster virus, HSV-1 = Herpes simplex virus 1, HHV6 = Human herpesvirus 6, CMV = Cytomegalovirus, * = culture positive (confirmed on standard bacterial culture).

Table 1
Discrepant results. Key: F = female, M = male, EV = Enterovirus, NTD = nil target detected, CMV = Cytomegalovirus, VZV = Varicella zoster virus, LM = *Listeria monocytogenes*

Case Presentation	FA-M/E	Reference laboratory	Clinical implications
21 F: headache, photophobia, neck stiffness	EV	NTD	Nil, managed conservatively
35 M: headache, confusion, behavioural change, left arm weakness, dysarthria	CMV	NTD	Switch of agent from aciclovir to ganciclovir
41 M: HIV complicated by AIDS, vesicular rash, diffuse infiltrative CNS process	VZV	VZV + HSV-2 (low level)	Nil, treated with aciclovir
1 M: pyrexia, diarrhoea, irritability, rash	LM	NTD	Commenced on amoxicillin and gentamicin

Our study illustrates that on-site PCR, as part of the diagnostic pathway for CNS infections, has considerable advantages. Financial savings were consistent with studies performed in other centres, which similarly depend on tertiary reference laboratories for PCR diagnostics[2]

In addition, rapid pathogen identification provides crucial diagnostic value, particularly in a paediatric population, where clinical signs and symptoms are often less discriminatory[3]. More rapid access to a final diagnosis, or exclusion thereof, affords reassurance to patients and families and has wider Public Health implications. In addition, FA-M/E has been shown to increase diagnostic yield in cases of paediatric bacterial meningitis where empiric antibiotics have been administered[4].

Only 23% of antimicrobials prescribed in this study were directed at a subsequently diagnosed infection, which further establishes the role of PCR diagnostics in Antimicrobial Stewardship.

CSF pleocytosis, a common parameter in meningitis prediction models, was a poor predictor of a positive FA-M/E result. Relying solely on WCC as an indicator for FA-M/E testing may result in missed diagnoses[5], as demonstrated by the six viral detections which were missed due to the WCC cut-off for this study. For these reasons, our institution now performs FA-M/E testing universally on all CSF samples.

The authors disclose no conflict of interest. This study received no specific funding.

This research has not been published elsewhere.

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Serial semiquantitative detection of SARS-CoV-2 in saliva samples

Dear Editor,

We read with interest the paper by Azzi and colleagues who report on the reliability of saliva testing for SARS-CoV2 infection.[1] We have carried out a study to analyze the efficiency of saliva testing in monitoring the viral load of confirmed patients and get a similar conclusion. Saliva testing has been widely used in diagnosing and screening suspected COVID-19 patients due to it being easy to collect and noninvasiveness and having a high positive rate.[2,3] For inpatients, the current standard for discharge is a negative RT-qPCR result from two sets of nasopharyngeal and throat swab specimens. Multiple throat swab specimens from each patient are needed to monitor the viral load, which will not only inevitably increase the risk of cross-infection but also increase the discomfort of the patient and cause possible complications such as bleeding.[4] There is no doubt that saliva testing can greatly improve patient comfort and reduce the risk of medical staff contracting the virus.

In this study, inpatients with a diagnosis of COVID-19 provided by real-time reverse-transcriptase polymerase chain reaction (rRT-PCR) on oropharyngeal swabs in Beijing Ditan Hospital, Capital Medical University from July 10, 2020, to July 20, 2020, were included. Saliva was collected and one-step rRT-PCR was performed using the Da'an Gene 2019-nCoV Detection Kit (fluorescent PCR method, batch number: 2020032). Ct values of the ORF1a gene and N gene were also tested simultaneously. The results were considered 'positive' when the cycle threshold (Ct) values of FAM and VIC channels were less than 40, and there were obvious amplification curves. SPSS 24.0 and Prism 8.0 were used for statistical analyses, the difference between groups was analyzed by ANOVA and Student's *t*-test, $P < 0.05$ was considered to be statistically significant.

A total of 34 patients were included (Table 1), and 709 nucleic acid tests, consisting of 150 saliva tests (average of 4.41±1.89 times per patient), 326 oropharyngeal swab tests (average of 9.59±2.63 times per patient), and 232 sputum tests (average of 6.82±2.61 times per patient) were performed. The Ct value of 91 saliva tests was recorded; the median Ct value of the ORF1a gene was 36.64 (range 24.10–39.90), and the median Ct value of the N gene was 33.99 (range 23.03–39.67). According to the number of weeks after hospitalization, the median Ct value of the two genes gradually increased, and the amplitude gradually decreased (Fig. 1A, B) (see Appendix Table A1). The Ct value of most patients increased with time. However, in some patients, the Ct value first decreased with increasing time and finally increased and became negative (Fig. 1C, D). Univariate analysis found that the reduction in red blood cells significantly affected the peak value of the ORF1a gene ($p = 0.027$), while for the N gene, there was no significant difference ($p = 0.059$). In multivariate analysis, no related factors that significantly affected the Ct peak were found (see Appendix Table A2).

The total positive rate of nucleic acid detection from sputum was the highest (67.2%), followed by oropharyngeal swabs (53.1%) and saliva (36%). According to the number of weeks after hospitalization, the positive rate of nucleic acid detection from the three sample types gradually decreased, the positive rate of nucleic acid detection from saliva was 83.33% in the second week, 48% in the third week, and 0% in the seventh week (see Appendix Fig. A1). While the positive rates of nucleic acid detection from saliva, sputum, and oropharyngeal swab samples were significantly different at 3 and 6 weeks (see Appendix Table A3).

The average time for nucleic acid detection results to become negative was 27.29±7.73 days for sputum samples, 27.82±12.09 days for oropharyngeal swab samples, and 24.53±13.59 days for saliva samples (see Appendix Table A4). Univariate analysis revealed that the clinical classification had a significant impact on both the time of the positive to negative conversion of sputum, oropharyngeal swab and saliva samples ($p = 0.001$, $p = 0.001$, $p = 0.012$), while only red blood cell reduction had a significant effect on the positive to negative conversion time of saliva samples ($p = 0.032$). Multivariate analysis found that clinical classification had a significant impact on the time of sputum and oropharyngeal swab samples to become negative ($p = 0.007$, $p = 0.002$) (see Appendix Table A5). Taking sputum specimens as an example, the average time for test results to become negative in asymptomatic patients was 14 days, while the average times for patients with mild and moderate disease were 25 days and 32 days, respectively.

Using the sputum-oropharyngeal swab test results as a reference, that is, a negative result was when the nucleic acid results of both specimen types were negative, and if one of the samples had a positive test result, it is considered a positive result. The efficiency of saliva single detection method and saliva-sputum combined detection method was tested. The results showed that the total sensitivity, efficiency and specificity of saliva single detection method were 74.10%, 83.90% and 94.40%, respectively. The overall sensitivity, efficiency and specificity of saliva-sputum combined detection method were 93.40%, 94.00% and 95.20%, respectively (see Appendix Table A6). Studies have conducted research on the effectiveness of saliva to diagnosis COVID-19, and the overall efficiency rate differs, ranging from 30.7% to 100%.[1,5–9] total efficiency and specificity of the saliva detection method in this study were higher than those of the sputum and oropharyngeal swab detection methods (83.90% and 94.40%, respectively). The saliva-sputum combined diagnosis is more effective, with a total efficiency and specificity of 94.00% and 95.20%, respectively. In addition, to verify the specificity of saliva testing, the saliva and oropharyngeal swab samples of 50 patients were tested, and the results of all of these patients were negative.

However, only 34 patients were included and it was not possible to collect all three sample types from every patient at the same time. We also fails to obtain the true copy of the virus, that is, the viral copies per ml of sample. Nonetheless, our results show that combined sputum-saliva detection is a reliable method for monitoring the viral load of patients recovering from COVID-19.

Table 1
Patient characteristics by severity of disease.

	Asymptomatic disease ($n=6$)	Mild disease ($n=6$)	Moderate disease ($n=22$)	p value
Age, years	37 (28–48)	38.7 (21–57)	44.4 (21–64)	0.354
Sex				
Female	2 (33.3%)	0	12 (54.5%)	0.764
Male	4 (66.7%)	6 (100%)	10 (45.5%)	0.821
Presenting symptoms				
Fever	0	5 (83.3%)	21 (95.5%)	0.683
Chills	0	1 (16.7%)	3 (13.6%)	0.898
Dyspnea	0	0	4 (18.2%)	0.853
Cough	0	4 (66.7%)	11 (50%)	0.638
Runny nose	0	0	1 (4.5%)	0.792
Blocked nose	0	2 (33.3%)	3 (13.6%)	0.483
Sore throat	0	1 (16.7%)	5 (22.7%)	0.606
Chest discomfort	0	0	0	—
Nausea	0	0	0	—
Diarrhea	0	1 (16.7%)	1 (4.5%)	0.64
Myalgia	0	0	2 (9.1%)	0.898
Malaise	0	0	2 (9.1%)	0.443
Loss of taste	0	1 (16.7%)	4 (18.2%)	0.81
Loss of smell	0	2 (33.3%)	4 (18.2%)	0.316
Antibody				
IgM	0	2 (33.3%)	10 (45.5%)	0.947
IgG	0	1 (16.7%)	9 (40.9%)	0.537
Blood tests on admission				
Total white blood cell count, $\times 10^9$ per L	4.26 (3.39–5.33)	5.85 (3.16–8.91)	4.91 (2.83–10.98)	0.21
Total white blood cells $<4 \times 10^9$ per L	2 (33.3%)	1 (16.7%)	5 (22.7%)	0.777
Lymphocyte count, $\times 10^9$ per L	1.69 (1.26–2.27)	1.87 (1.23–3.21)	1.65 (0.58–3.38)	0.091
Lymphocytes $<1{\bullet}0 \times 10^9$ per L	0	0	4 (18.2)	0.762
red blood cell count, $\times 10^9$ per L	4.45 (3.95–5.07)	4.54 (2.54–5.28)	4.89 (3.90–5.87)	0.184
red blood cell count, 4×10^9 per L	1 (16. 7%)	1 (16.7%)	1 (4.5%)	0.251
Platelet count, $\times 10^9$ per L	202.67 (162–282)	221.67 (147–364)	190.74 (118–296)	0.536
Platelets $<100 \times 10^9$ per L	0	0	0	—

Data are n (%) or median (range), unless otherwise stated. For statistical analyses, ANOVA was performed for continuous variables, and chi-squared test was performed for categorical variables.

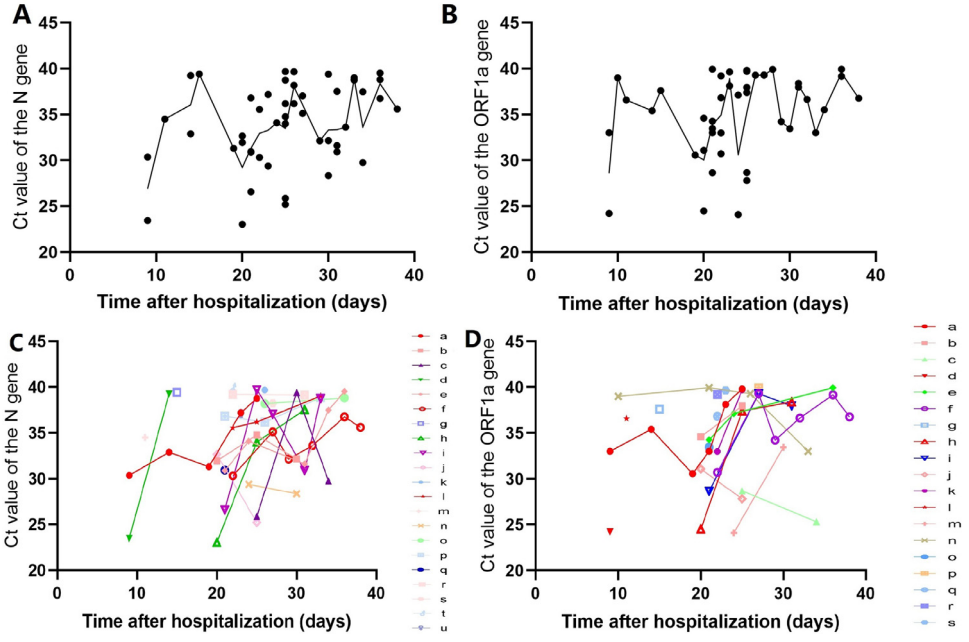

Fig. 1. Ct value from serial semiquantitative detection of SARS-CoV-2 for all 34 patients(A-B); Fig. 1A shows the N gene, and Fig. 1B shows the ORF1a gene. Datapoints denote the Ct value, and the curve indicates the median value.
Ct value of each patient after hospitalization(C-D). Fig. 1C shows the N gene, and Fig. 1D shows the ORF1a gene.

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Data availability

Data directly supporting the study results can be found in Beijing Ditan Hospital in paper form.

Declaration of Competing Interest

None.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.jinf.2020.10.002.

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Discovery of mcr-3.1 gene carried by a prophage located in a conjugative IncA/C2 plasmid from a Salmonella Choleraesuis clinical isolate

Dear Editor,

The emergence of *mcr*-gene posts a serious threat to the use of colistin which is one of the last-resort treatments against human infections caused by MDR Gram-negative bacteria. In this Journal, the discovery of plasmid-mediated colistin resistance gene *mcr-3* in poultry fecal samples from China live poultry markets (LPMs) has been recently reported (1, 2). Here, to the best of our knowledge, we report the first case of plasmid-borne *mcr-3.1* present in an MDR *Salmonella enterica* serovar Choleraesuis isolate from human.

Salmonella Choleraesuis is a highly invasive zoonotic pathogen that causes a serious systemic infection such as bloodstream infections in humans, and the emergence and increase of MDR S. Choleraesuis has become a serious global therapeutic problem (3). In this study, S. Choleraesuis XXB210 was isolated from blood sample of a 52-year-old female patient with blood poisoning in November 2014 in China, who did not receive colistin treatment before.

Susceptibility testing by Vitek-2 system showed that isolate XXB210 was resistant to most of the antimicrobials tested (Table 1), including members of the Carbapenem class of β-lactam antibiotics [imipenem (MIC 4 mg/L)]. The MIC of colistin for this isolate was determined to be 4 mg/L according to CLSI guidelines. The transferability of plasmids was investigated by conducting conjugation experiments. The *mcr-3.1* could be transferred into Sodium azide resistant *Escherichia coli* J53, *Pseudomonas aeruginosa* ATCC 9027 and cefepime-resistant *Acinetobacter baumannii* ATCC 19606 at the frequencies of 10^{-3}, 10^{-1} and 10^{-2} respectively, suggesting that the *mcr-3.1*-carrying plasmid has the potential to transfer to other clinically relevant pathogens.

The genomic DNA of *S.* Choleraesuis XXB210 was sequenced using the combination of Nanopore GridION and Illumina HiSeq platforms, followed by assembling with Unicycler. The sequence of pXXB210 revealed a circular plasmid of 193,865 bp in length with 50.26% GC-content. BLASTn analysis showed that pXXB210 was most similar (63% query coverage and 99.99% identity) to plasmid p08–5333.1 (CP039562.1) from a clinical *Salmonella enterica* isolate in Canada.

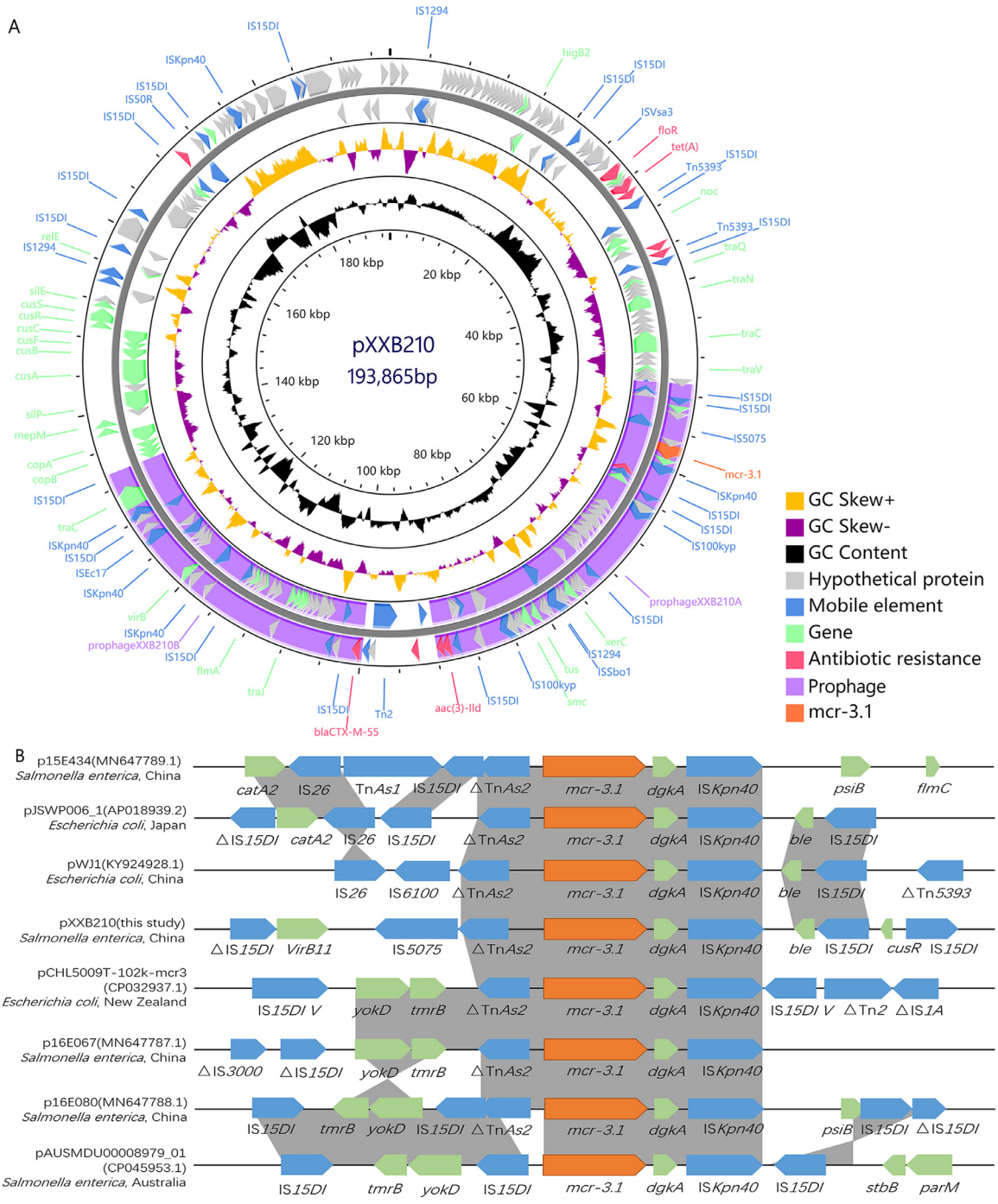

Fig. 1. The genetic structure of *mcr-3.1*-carrying plasmid. (A) Scheme for *mcr-3.1*-harbouring plasmid pXXB210. Circles from inside to outside indicate the GC content, GC screw and the open-reading frames in different DNA strands. (B) Comparison of the genetic environments of *mcr-3.1* gene. Genetic environment of seven *mcr-3.1*-carrying plasmids (KY924928, CP032937, CP045953, AP018939, MN647787, MN647788 and MN647789) were extracted from the GenBank according to the Accession ID described in reference papers. Arrows indicate the positions and directions of the genes. △indicates the truncated gene. Regions with >99% homology are indicated in the light gray shadow.

PlasmidFinder showed that pXXB210 had 100% identity with the IncA/C2 replicon. Incompatibility group IncA/C plasmids are large, low copy, theta-replicating plasmids, and associated with the emergence of multidrug resistance in enteric pathogens of humans and animals (4). Consistent with its multidrug resistance phenotype (Table S1), except *mcr-3.1*, pXXB210 harbored additional five classes of antimicrobial resistance genes for β-lactams ($bla_{CTX-M-55}$), aminoglycosides [*aph(6)-Id*, *aac(3)-IId*], phenicols (*floR*), sulfonamide-trimethoprim (*sul2*), and tetracyclines (*tetA*) according to CARD database annotation.

Interestingly, two regions in length of 41.2 kb and 33.6 kb, respectively, in pXXB210 was predicted as two intact prophage sequences by PHASTER. As shown in Fig. 1A, prophage_XXB210A and prophage_XXB210B were separated by a transposition unit of IS*15DI*-$bla_{CTX-M-55}$-Tn*2*-*aac(3)-IId*-IS*15DI*. However, analysis of the target site duplication (TSD) at the boundary of the transposition unit did not support the existence of a larger prophage that was truncated by the insertion of this transposition unit. Both the two regions had lower GC-content (49.45% and 47.66%, respectively,) than that of the rest part of the plasmid (51.27%).

Table 1
Antimicrobial susceptibility profiles of *Salmonella* Choleraesuis XXB210.

Antimicrobials	MIC (mg/L)	Antimicrobials	MIC (mg/L)	Antimicrobials	MIC (mg/L)
Ampicillin	32	Amikacin	8	Ceftazidime	64
Ampicillin-Sulbactam	32	Tobramycin	8	Ceftriaxone	64
Gentamycin	16	Aztreonam	64	Nitrofurantoin	64
Ciprofloxacin	4	Cefazolin	64	Piperacillin-Tazobactam	128
Ertapenem	0.5 (S)	Cefepime	64	Trimethoprim-Sulfamethoxazole	80
Imipenem	4	Cefotetan	4	Levofloxacin	8
Colistin	4				

The *mcr-3.1* gene was just located in prophage_XXB210A which contained a total of 39 CDS including transposase, recombinase and capsid protein coding sequence. BLAST search in NCBI showed that prophage_XXB210A had only 18% coverage (99.64% nucleotide identity) of *Escherichia* P1 virus (MH445380.1) and 16% coverage (99.52% identity) of a 115.1-kb *Escherichia* phage RCS47 (NC_042128.1) which was described as a P1-like bacteriophage carrying an SHV-2 extended-spectrum β-lactamase from an *E. coli* Strain (5).

The role of phages in disseminating *mcr-3.1* is still a matter of debate. Nonetheless, some studies have found that phages are able to transfer ARGs (Antimicrobial Resistance Genes) conferring resistance to aminoglycosides (6), β-lactams (7), chloramphenicol (8), or tetracycline (6) via transduction. Two *E. coli* phages promoting the transfer of ARG-carrying plasmids by transformation were also reported (9). And, a *mcr*-1-carrying P7 phage-like plasmid from a *Klebsiella pneumoniae* clinical isolate was identified and characterized, and reported to be circulated in China (10).

In addition, IS elements were annotated by ISfinder, and alignments were performed against published *mcr-3.1*-carrying plasmid genomes (KY924928, CP032937, CP045953, AP018939, MN647787, MN647788 and MN647789). All *mcr-3.1*-carrying plasmids showed 100% homology to the backbone that was composed by △Tn*As2-mcr-3.1-gdkA*-IS*Kpn40*, except in pAUSMDU00008979_01, where △Tn*As2* on the upstream of *mcr-3.1* was absent (Fig. 1B). The mechanism responsible for generating the △Tn*As2-mcr-3.1-gdkA*-IS*Kpn40* structure is unclear but might have involved recombination. Also, other insertion elements such as the IS*15DI*, IS*bo1*, IS*5075* and IS*100kyp* were found in prophage_XXB210A, which may indicate the recombinational activity of this prophage. A further blastn search against the NCBI database (Access on 30 March 2020) identified 14 *mcr-3* sequences in 11,103 genomes of *Salmonella* spp. and 11 in 17,003 completed bacterial genomes (data not shown). However, none of them was located in prophage sequence on plasmid.

In conclusion, we report the first case of a *mcr-3.1*-positive zoonotic *S. Choleraesuis* isolate from human with blood poisoning. Moreover, the first complete genome of a transferable plasmid with *mcr-3.1* contained in a prophage sequence was identified and characterized. This *mcr-3.1*-harboring IncA/C2 plasmid may represent a new type of vehicle to mediate the spread of *mcr-3.1* in *S.* Choleraesuis, highlights the urgent need for more extensive surveillance and effective action to control its further dissemination.

Authors' contributions

BLZ and YFH conceived the study. YLP, YF, YQF, NL, JL carried out the analysis. LPC and XBX performed the experiments. YLP, YFH and BLZ drafted the manuscript. All authors read, revised the manuscript, and gave final approval for publication.

Data availability

Genome sequence of *S.* Choleraesuis XXB210 and plasmid pXXB210 has been deposited into GenBank under the accession number PRJNA633322.

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Declaration of Competing Interest

The authors declare that there is no conflict of interest.

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