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The N gene of SARS-CoV-2 was the main positive component in repositive samples from a cohort of COVID-19 patients in Wuhan, China

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ABSTRACT

Background: Repositivity of SARS-CoV-2 nucleic acid in discharged COVID-19 patients was reported recently. However, the characteristics of repositive results are still not well understood, leading to a lack of effective monitoring strategies.

Methods: In the present study, a total of 59 COVID-19 patients were enrolled, and the characteristics of the repositive samples were analyzed.

Results: The repositive rate in this cohort was 15.79%. The N gene was the main target gene that was positive in the repositive results as well as in the last positive results of all patients. The median duration from diagnosis to the last positive test was 20 days (IQR, 16–31 days), and the longest duration was 40 days. Repositivity was only observed in IgM single- or both IgM- and IgG-positive patients, instead of IgG single-positive patients.

Conclusions: There was a significant proportion of repositives in the recovered COVID-19 patients, and increasing the required number of negatives for consecutive nucleic acid tests may reduce the incidence of repositives. The recommended monitoring strategy for repositivity is monitoring the N gene in IgM-positive patients. This can ensure high sensitivity while reducing the time and cost of nucleic acid detection.

1. Introduction

Since the beginning of December 2019, a new coronavirus, named SARS-CoV-2 by the World Health Organization (WHO), emerged from Wuhan, China and rapidly expanded to more than 180 countries and regions throughout the world [1]. According to statistics from the Coronavirus Disease 2019 (COVID-19) global cases special website by the Center for Systems Science and Engineering at Johns Hopkins University, as of May 30, 2020, there have been 5,930,096 confirmed cases and 365,015 deaths worldwide [2]. COVID-19 has become a major threat to the health of people around the world and has been declared a pandemic by the WHO [3]. How to manage patients more accurately and safely, thereby reducing possible virus transmission, is of great significance for better control of the epidemic.

The diagnosis of COVID-9 is based on viral nucleic acid detection. The judgment of whether a COVID-19 patient is cured is also based on both clinical symptoms and nucleic acid test results. However, existing studies have suggested a significant proportion of false-negative results for nucleic acid testing [4]. A positive virus test in some COVID-19 patients may last for a relatively long time, even after the patient's clinical symptoms have been well relieved [5]. Some COVID-19 patients may still have a repositive nucleic acid test after being discharged [6–8] or even have reactivation and symptoms [9]. Currently, there is no analysis of the characteristics of nucleic acid test results in patients who are repositive after a negative nucleic acid test.

In the present study, we retrospectively analyzed COVID-19 patients from a critically ill ward. We collected their results of the nucleic acid tests for SARS-CoV-2 as well as the results of tests for antibodies against

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SARS-CoV-2, and performed a statistical analysis on these patients with a repositive nucleic acid test. We described the characteristics and related influencing factors of COVID-19 patients with a repositive nucleic acid test and believe this study will provide a valuable reference for monitoring repositivity.

2. Methods

2.1. Patients

Data from COVID-19 patients admitted to and managed by the Aid Hubei Medical Team from the Second Affiliated Hospital of Zhejiang University School of Medicine from February 15, 2020 to March 29, 2020, was evaluated. Among these, 59 patients who underwent at least 3 nucleic acid tests and had at least 1 negative nucleic acid test were included in this study. The COVID-19 case was confirmed, and the clinical classification was defined based on the New Coronavirus Pneumonia Prevention and Control Guideline published by the National Health Commission of China. All enrolled patients were confirmed to be positive for SARS-CoV-2 nucleic acid by real-time fluorescent RT-PCR on samples from the respiratory tract and they received arbidol as an antiviral treatment for no more than 10 days. The date of illness onset, SARS-CoV-2 nucleic acid testing results, anti-SARS-CoV-2 antibody results, and other clinical characteristics of the enrolled patients were obtained from the clinical records. This study was reviewed and approved by the Medical Ethical Committee of the Second Affiliated Hospital of Zhejiang University School of Medicine (2020–224). Written informed consent for this retrospective analysis was waived by the Ethical Committee because of the urgent need for infection control. All of the patients gave their oral consent to participate in this retrospective study.

2.2. Real-time reverse transcription polymerase chain reaction (RT-PCR) assay for SARS-CoV-2

Nasopharyngeal swab samples were collected and stored in special tubes with viral transport medium and then sent to the laboratory for SARS-CoV-2 RNA extraction and SARS-CoV-2 detection by real-time RT-PCR as previously described [10]. A cycle threshold value (Ct-value) < 37 was defined as a positive test result, while a Ct-value of 40 or more was defined as a negative test result. These diagnostic criteria were based on the recommendations of the National Institute for Viral Disease Control and Prevention (China) [11]. A Ct-value of 37 to < 40 required confirmation by retesting. The sampling time between the two test specimens was at least 24 h.

2.3. Definition of different repositive categories

If the nucleic acid test was repositive after one negative test, then these repositive tests were defined as the one-negative category. If the nucleic acid test was repositive after two or three consecutive negative tests, then these repositive tests were defined as two-negative or three-negative categories.

2.4. Detection of SARS-CoV-2 antibodies

Antibody tests for antibodies against SARS-CoV-2 in the data collected in this study included total antibody, IgM antibody and IgG antibody. All antibodies in the plasma samples were detected by enzyme-linked immunosorbent assay (ELISA) kits supplied by the Beijing Wantai Biological Pharmacy Enterprise Co., Ltd., according to the manufacturer's instructions.

2.5. Statistical analysis

Statistical analysis was performed using SPSS 20 (IBM, Chicago). All of the statistical tests were two-sided, and significant differences were considered at $p < 0.05$. Continuous variables were evaluated using the median and interquartile range (IQR) values. Chi-square or Fisher exact tests were utilized to compare the proportions of the categorical variables.

3. Results

3.1. Clinical characteristics of the included patients.

From February 15, 2020 to March 29, 2020, a total of 90 COVID-19 patients were managed by our medical team, 59 of whom (65.6%) were included in the present study. The median age of the included patients was 60 years (IRQ, 55–-71), and 39 were male (66.1%). According to the New Coronavirus Pneumonia Prevention and Control Guideline, all patients met the criteria for severe patients and received antiviral treatment. Forty-nine patients (83%) had comorbidities other than COVID-19. By March 29, 2020, a total of 43 patients (72.9%) had been discharged or transferred to mild wards due to comorbidities.

3.2. Repositive rates in different groups according to the number of negative SARS-CoV-2 nucleic acid tests.

In this study, a classification analysis of repositive rates was performed based on the different repositive categories. There was a significant difference among the repositive rates in the different repositive categories (Table 1, $p = 0.004$). The one-negative category had the highest repositive rate (19/59, 32.2%), followed by the two-negative category (9/57, 15.79%) and the three-negative category (3/43, 6.98%). The two- and three-negative categories showed significantly lower repositive rates than the one-negative categories ($p < 0.05$). However, there was no significant difference in the repositive rate between the two- and three-negative categories ($p = 0.179$), although the repositive rate in the three-negative categories was only approximately half of that of the two-negative categories.

3.3. The N gene of SARS-CoV-2 was the main positive component in repositive samples as well as the last positive results.

Currently, the detection of SARS-CoV-2 RNA is achieved by real-time RT-PCR detection of two target genes, including the open reading frame of 1ab (ORF1ab) and the nucleocapsid protein (N) [10]. In this study, statistical analysis of the distribution of the two genes among these repositive results was performed. When the same patient had multiple repositive tests, the statistics were counted according to the number of repositive tests instead of the number of patients. As shown in Fig. 1A, the highest proportion of positive results was the N gene in all groups, followed by both ORF1ab and N ($p < 0.001$ for one-

Table 1
Repositive rates based on different consecutive negative test of SARS-Cov-2 nucleic acid.

	One-negative category (n = 59)	Two-negative category (n = 57) *	Three-negative category (n = 43) *
Repositive n (%)	19/59 (32.20%)	9/57 (15.79)	3/43 (6.98)
Non-repositive n (%)	40/59 (67.80)	48/57 (84.21)	40/43 (93.02)

One-negative category means repositive after one result of both negative test for SARS-Cov-2 ORF1ab and N; two-negative category means repositive after two consecutive negative tests, both SARS-Cov-2 ORF1ab and N, performed more than 24 hours apart; three-negative category means repositive after three consecutive negative tests, both SARS-Cov-2 ORF1ab and N, performed more than 24 hours apart each other. *:$p < 0.05$

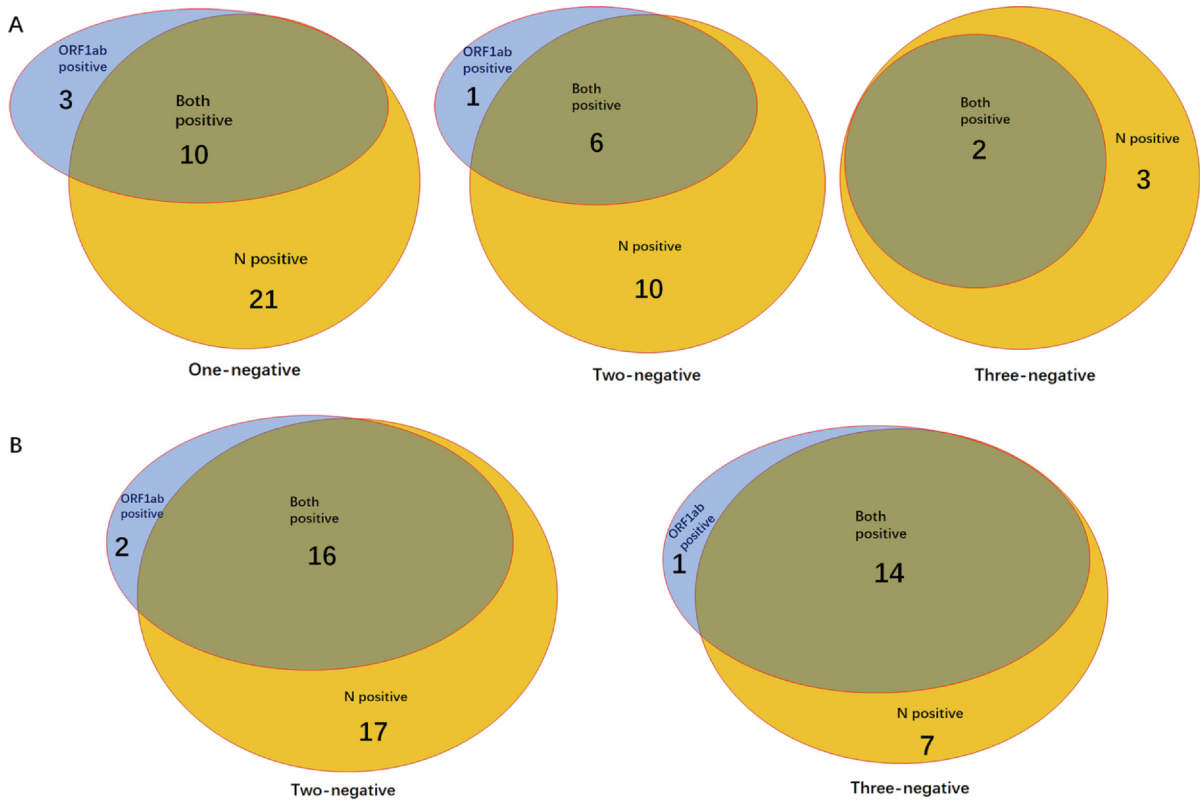

Fig. 1. Distribution of the two SARS-CoV-2 genes in repositive tests and in all of the last positive tests from twice or more negative test patients. A: Distribution of ORF1ab and N in repositive results of one-negative, two-negative and three-negative categories. B: Distribution of ORF1ab and N in the last positive results of two-negative and three-negative categories. N: number of ORF1ab or N positive results.

Table 2
Distribution of antibody types when antibodies and nucleic acids were both positive

	One-negative category	Two-negative category	Three-negative category
Total antibody n (%)	5/8 (62.5)	4/6 (66.67)	1/3 (33.3)
IgM n (%)	1/8 (12.5)	1/6 (16.7)	1/3 (33.3)
IgG n (%)	0/8 (0)	0/6 (0)	0/3 (0)
IgM + IgG n (%)	2/8 (25)	1/6 (0)	1/3 (33.3)
total n	8	6	3

negative category, $p = 0.001$ for two-negative category, and $p = 0.002$ for three-negative category), whether the cutoff value was defined as once or consecutive twice or thrice negatives (Table 2, $p = 0.942$). Therefore, if the proportions of N gene positive and double gene positive were calculated together, the proportion was higher than 90% in all three categories (91.8%, 94.12% and 100%). These results prove that the N gene was the main positive gene among the repositive samples. The repositive rate obtained by detecting the N gene alone and the repositive rate obtained by detecting both genes were almost the same (8/57 vs. 9/57, $p = 1.0$).

The gene distribution among all of the last positive tests was also analyzed. The result was similar to the gene distribution when the SARS-CoV-2 nucleic acid test was repositive. The proportions of N gene positive were also the highest, followed by that of double gene positive (Fig. 1B, $p < 0.001$ both for two-negative and three-negative categories). The proportion of combined N gene positive and double gene positive was also higher than 90% (94.29% and 95.45%). These results suggest that the N gene was still the most important gene when monitoring nucleic acids.

3.4. Repositivity was only observed in IgM single- or both IgM- and IgG-positive patients, instead of IgG single-positive patients.

It is still unclear how to monitor antibodies and viral nucleic acids at appropriate points in the course of this disease. Proper arrangements for the detection of antibodies and viral nucleic acids are important to reduce patient sampling and monitoring costs. For this reason, the present study also analyzed the situation of viral nucleic acid positivity when different antibodies appear. As Table 2 shows, nucleic acid positivity mainly occurred in the total antibody group, and both the IgM and IgG positive groups and the IgM single positive group also had some nucleic acid positive cases. However, no nucleic acid-positive cases were observed in the IgG single-positive group. The median duration for SARS-CoV-2 clearance in the total antibody-positive group was 4 days (range 3–9 days). The duration of virus clearance for the only IgM-positive case was 6 days, and for the two IgM- and IgG-positive cases, those durations were 2 and 8 days. However, due to the limited number of patients subjected to antibody monitoring, these data cannot be statistically analyzed, so whether these data are truly meaningful still needs further study.

3.5. The median duration from diagnosis to the last positive test was 20 days (IQR, 16–31 days), and the longest duration was 40 days.

Nine repositive patients after two consecutive negative tests are shown in Fig. 2. Among the patients enrolled in this study, the median duration from diagnosis to the last positive test was 20 days (IQR, 16–31 days) for patients who had reached two consecutive negative tests, and the longest duration was 40 days. The median duration from diagnosis to the last positive test was 17 days (IQR, 15.5–22.5 days) for patients who had reached three consecutive negative tests, and the longest duration was 35 days.

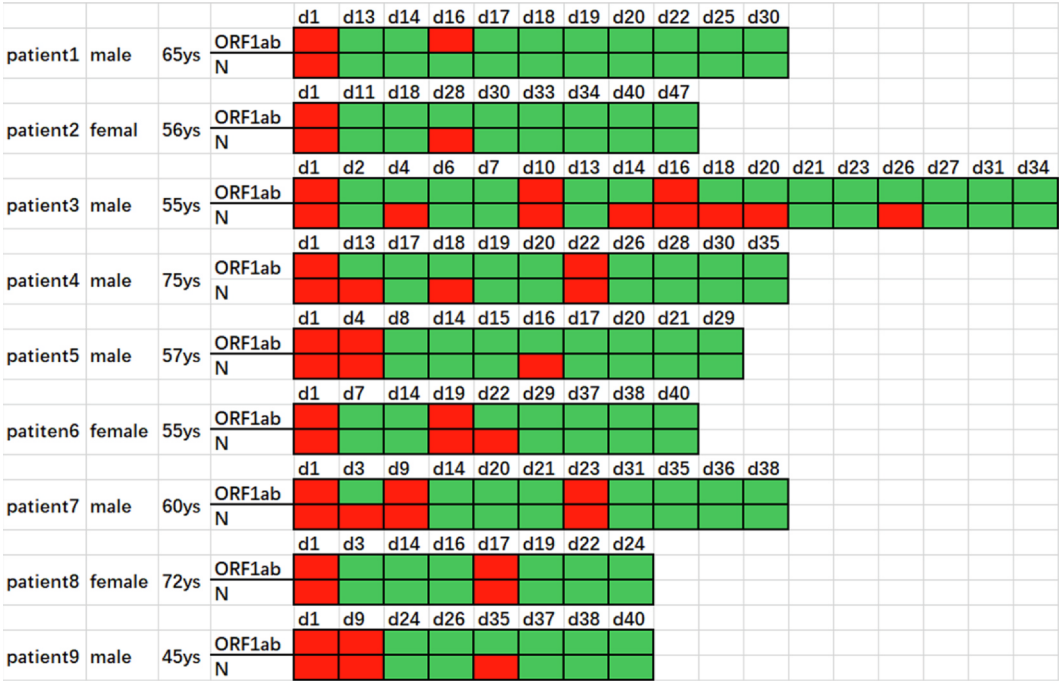

Fig. 2. SARS-CoV-2 nucleic acid detection results of 9 repositive patients. Red indicates positive, green indicates negative, and d(n) indicates the day after diagnosis. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

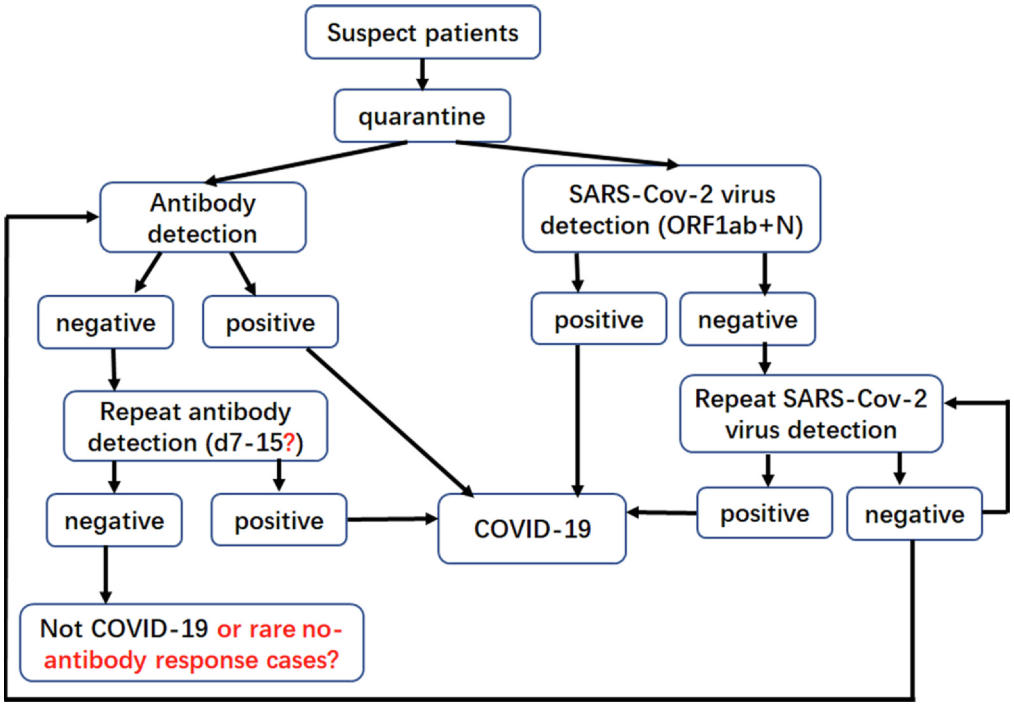

Fig. 3. Recommended process for the combined application of nucleic acid and antibody detection for the diagnosis of COVID-19.

4. Discussion

The present study has provided some novel detailed features of SARS-CoV-2 repositive samples and patients. According to China's New Coronavirus Pneumonia Prevention and Control Guideline, patients with relieved clinical symptoms and two negative viral nucleic acid tests more than 24 h apart meet the discharge criteria. However, in this study, it was found that even patients who met the above discharge criteria could still be repositive in subsequent viral nucleic acid monitoring. This proportion could reach 15.79% among the severe patients included in this study. The proportion of repositive patients after three consecutive negative tests was only 6.98%. Most of these patients continued to be hospitalized because of comorbidities other than COVID-19 and continued to be monitored for SARS-CoV-2 nucleic acids during treatment. The results of this study suggest that if the required number of consecutive negative nucleic acid tests is increased from 2 to 3, the proportion of repositive patients may be reduced, although there was no significant difference due to the limited number of samples.

The repositive rate in the present study was similar to that previously reported [8], which showed repositivity mainly among young

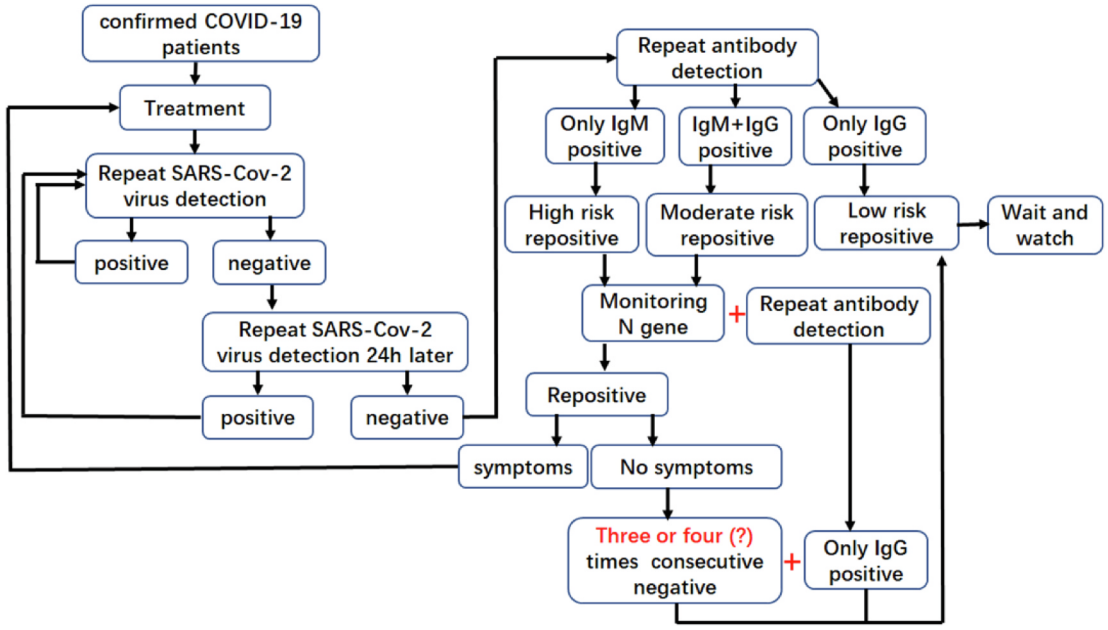

Fig. 4. Recommended process for the combined application of nucleic acid and antibody detection for monitoring SARS-CoV-2.

patients. However, it was higher than the previously reported proportion of symptomatic reactivation in patients with recurring symptoms [9]. None of the repositive patients in this study experienced COVID-19-related symptoms again, which is consistent with previously published reports [6–8]. The patients included in this study were all severe COVID-19 patients with lung lesions. Although the lung lesions were significantly reduced after treatment, these residual lesions may increase the possibility of repositivity. There are also reports suggesting that the conventional RT-PCR method has a certain false negative rate [12,13], and it seems that it can be overcome by detecting different types of specimens [14] or using more sensitive methods [15]. However, this study also found that even in patients with two consecutive negative nucleic acid tests, similar to patient No. 3, multiple repositives could also be detected by the same method. This phenomenon suggested that in some patients, the virus still persists or could be reactivated.

Recently, people with asymptomatic infections have received increasing attention. However, it is still unclear whether these repositive patients are still infectious and whether there is a possibility that the virus escapes immunity. Therefore, further research is needed on whether infectious viruses are present in the repositive specimens and the repositives or reactivation the mechanisms.

The results of this study also suggest that among the repositive specimens, the main positive fragment is the N gene, and the proportion of those positive for single ORF1ab was very low. By detecting the N gene alone, a repositive rate similar to that of detecting both genes can be obtained. During the viral monitoring process, it seemed that the detection of both the ORF1ab and N genes could be replaced by just detection of the N gene. The results of the last nucleic acid positive test was the same in all patients who met the discharge criteria. Recently, Wang C et al. reported that nucleotide variation was more frequent in 1ab than in N, and the hot spot nucleotide variation rate was higher in 1a than in N. Additionally, nucleotide variations between the published primer/probe sequences and reference sequences were more frequent in ORF1ab than in N [16]. Thus, the fact that the N gene has less nucleotide variation than ORF1ab may make the detection of the N gene more stable than that of ORF1ab, which could partially explain why the N gene, rather than ORF1ab, was detected more often in the repositive and last-positive test results.

The time and cost of nucleic acid testing for COVID-19 patients is currently a challenge for doctors and medical administrations in various countries around the world. In the follow-up viral nucleic acid monitoring of diagnosed patients, if the cost and time of testing can be selectively reduced, it will greatly alleviate the pressure currently placed on countries around the world. The results of this study suggest that in subsequent viral nucleic acid monitoring of COVID-19 patients, the detection of the N gene alone can cover more than 90% of repositive specimens. Therefore, it may be possible to more conveniently, effectively and economically monitor repositive COVID-19 patients. Of course, the number of specimens in this study was not large enough, and this conclusion still needs to be verified in a larger population.

The relationship between repositivity of SARS-CoV-2 nucleic acid and antibody types was also analyzed in the present study. As expected, the emergence of IgG antibodies means that the likelihood of repositivity was greatly reduced, and patients who were only IgM positive or IgM and IgG double-positive were the main populations for repositivity. In this study, we also included a group of patients who were only tested for total antibodies in the early stages of the virus epidemic without further distinguishing the types of antibodies, and this group of patients was also a high-risk group that was positive again. However, previous studies have confirmed that total antibodies appear earlier than IgM [17], so it is speculated that the majority of these patients should be positive for IgM antibodies. Therefore, the results of this study suggested that for COVID-19 patients who were only positive for IgM antibodies or positive for both IgM and IgG antibodies, more attention should be paid to the monitoring of SARS-CoV-2 nucleic acids to avoid missing repositive patients. In particular, the monitoring of the N gene is more meaningful. For some patients who were only tested for total antibodies and were positive, the monitoring of SARS-CoV-2 nucleic acids should also be prioritized. For patients who had been serologically converted to positive IgG antibodies alone, the risk of repositives was very low.

Therefore, our antibody detection recommendations are to start monitoring them only after the nucleic acid tests switch to negative in confirmed patients. Then, according to the characteristics of the antibody at this time, selective nucleic acid monitoring should be performed to detect high-risk repositive patients. This conclusion has potential value for how to select patients more specifically for SARS-CoV-2 nucleic acid and antibody monitoring, thereby reducing the cost of testing.

Relying on nucleic acid test results to diagnose COVID-19 is often affected by a low sensitivity [13]. Antibody detection is simpler and more efficient than nucleic acid detection. Consistent with the results of other studies [17,18], this study also confirmed once again that COIVD-19 patients have a very high positive rate (100% in the present study) of SARS-CoV-2 antibodies. This also suggests that a diagnostic method combining viral nucleic acid detection and viral antibody detection will greatly improve the diagnostic sensitivity of COVID-19 patients, especially in the case of a shortage of nucleic acid detection reagents and a low positive rate. Of course, the diagnosis of patients in the early stages of infection before the appearance of antibodies is still highly dependent on nucleic acid test results. To date, no antibody-negative cases have been reported after infection. If suspected patients continue to be negative for antibodies and nucleic acids, the diagnosis of COVID-19 may be ruled out.

In addition, this study also found that the viral nucleic acid tests can continue to be positive for up to 40 days in some patients. The duration of SARS-CoV-2 nucleic acid positivity lasted longer than previously reported [5]. This also confirmed that the virus may persist for a long time in some patients, but whether the long-lasting virus is still infectious needs to be studied further.

There are several possible causes of repositive nucleic acid phenomena in COVID-19 patients who meet the discharge criteria. First, SARS-CoV-2 nucleic acid tests have a significant rate of false negatives. This is why one of the criteria recommended by the current guidelines for discharging or releasing COVID-19 patients from quarantine is two consecutive negative nucleic acid tests at a 24-hour interval. Existing studies have confirmed a certain false negative rate of nucleic acid test results [4], which has led to the fact that some patients who actually still have SARS-CoV-2 in their body are mistakenly considered cured under these criteria. As observed in this study, increasing the number of consecutive nucleic acid negatives greatly reduces the probability of a repositive rate, but this still needs to be confirmed by further research.

Second, there may be differences in the rate of repositivity among patients with different clinical characteristics. The available research evidence suggests that factors influencing repositivity include age [19] and immune status, such as NK cell counts [20]. These results suggest that differences in immune status between patients may affect the clearance of residual virus in the body, leading to differences in repositivity rates.

Third, differences in test specimens can also affect the positive rate of nucleic acid testing. Studies have found differences in positive nucleic acid testing rates between different specimens, such as feces, sputum, nasopharyngeal swabs and oropharyngeal swabs [21], which may also lead to a repositive test if different sources are used.

Finally, although there is no evidence supporting this hypothesis at this time, there is also the possibility that a discharged patient could be reinfected and result in a repositive test, although this seems unlikely. In conclusion, the mechanisms and significance of repositivity remain to be clarified by further research.

There were some limitations of this study. First, the detection of nucleic acids and antibodies was not carried out according to a fixed schedule. This leads to the possibility that certain nucleic acid and antibody conversion time points may have been missed, thus having a certain impact on the results. Second, this study used upper respiratory tract samples for nucleic acid detection, lacking deep respiratory specimens, among which the positive rate may be higher. Third, the sample size of this study was not large enough, and all of the patients were severe patients. A larger cohort study and longer follow-up times are needed.

Based on the findings of this study combined with existing research conclusions, we also established a recommended process for the combined application of nucleic acid and antibody detection for the diagnosis (Fig. 3) and monitoring (Fig. 4) of SARS-CoV-2.

5. Conclusion

In conclusion, the present study demonstrates that there was indeed a significant proportion of repositives in patients who were negative on two consecutive nucleic acid tests, and increasing the number of negatives for consecutive nucleic acid tests to three may reduce the incidence of repositives. The N gene could be detected separately for monitoring viral nucleic acids. Nucleic acid monitoring may be selectively performed on IgM-positive patients, thereby increasing the efficiency of repositive detection and reducing costs. On this basis, we have established a recommended process for the combined application of nucleic acid and antibody detection for the diagnosis and monitoring of COVID-19.

CRediT authorship contribution statement

Xuzhao Zhang: Conceptualization, Methodology, Software, Writing - original draft, Funding acquisition. **Min Li:** Conceptualization, Methodology, Writing - original draft. **Bin Zhang:** Investigation, Data curation. **Tao Chen:** Validation. **Dong Lv:** Investigation, Data curation. **Pengfei Xia:** Investigation, Data curation, Investigation, Data curation. **Zhuanyi Sun:** Resources. **Xiaoyan Shentu:** Resources. **Haiyan Chen:** Resources. **Libin Li:** Writing - review & editing. **Wenbin Qian:** Supervision.

Declaration of Competing Interest

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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