Lower respiratory tract samples are reliable for severe acute respiratory syndrome coronavirus 2 nucleic acid diagnosis and animal model study

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ABSTRACT

Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) and coronavirus disease 2019 (COVID-19) continue to impact countries worldwide. At present, inadequate diagnosis and unreliable evaluation systems hinder the implementation and development of effective prevention and treatment strategies. Here, we conducted a horizontal and longitudinal study comparing the detection rates of SARS-CoV-2 nucleic acid in different types of samples collected from COVID-19 patients and SARS-CoV-2-infected monkeys. We also detected anti-SARS-CoV-2 antibodies in the above clinical and animal model samples to identify a reliable approach for the accurate diagnosis of SARS-CoV-2 infection. Results showed that, regardless of clinical symptoms, the highest detection levels of viral nucleic acid were found in sputum and tracheal brush samples, resulting in a high and stable diagnosis rate. Anti-SARS-CoV-2 immunoglobulin M (IgM) and G (IgG) antibodies were not detected in 6.90% of COVID-19 patients. Furthermore, integration of nucleic acid detection results from the various sample types did not improve the diagnosis rate. Moreover, dynamic changes in SARS-CoV-2 viral load were more obvious in sputum and tracheal brushes than in nasal and throat swabs. Thus, SARS-CoV-2 nucleic acid detection in sputum and tracheal brushes was the least affected by infection route, disease progression, and individual differences. Therefore, SARS-CoV-2 nucleic acid detection using lower respiratory tract samples alone is reliable for COVID-19 diagnosis and study.

Keywords: COVID-19; SARS-CoV-2; Diagnosis; Animal model

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INTRODUCTION

In early December 2019, a pneumonia of unknown etiology was first recognized in patients from Wuhan, China (Huang et al., 2020). Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2, 2019-nCoV) was quickly identified as the cause of this disease (Wang et al., 2020; Zhu et al., 2020), which was subsequently named coronavirus disease 2019 (COVID-19) by the World Health Organization (WHO). Since the first reported case, COVID-19 has spread rapidly throughout the world. As of 23 January 2021, there have been 98,713,370 confirmed cases of COVID-19, including 2,114,643 deaths, reported by the WHO. This disease has impacted the global economy, social and health care systems, personal interactions, and, indeed, our way of life (McKechnie & Blish, 2020). SARS-CoV-2 continues to spread rapidly due to its strong transmission ability, relatively long incubation period (Chakraborty et al., 2020; Lan et al., 2020; Jiang et al., 2020; Shang et al., 2020), inadequate diagnosis and preventive measures, and absence of effective vaccines and therapeutic drugs (Li et al., 2020a).

Following its initial identification and isolation, the SARS-CoV-2 genome was rapidly sequenced and released, which improved disease diagnosis based on nucleic acid detection. Although antibody diagnosis is developing rapidly, and chest radiography and computed tomography (CT) imaging are applied as assistant diagnostic methods, nucleic acid detection remains the primary method for COVID-19 diagnosis and gold standard for confirmation of SARS-CoV-2 infection (Al-Tawfiq & Memish, 2020; Feng et al., 2020). Sputum and respiratory samples, e.g., from the oropharynx and nasopharynx regions, collected from patients with clinical symptoms or from direct contacts of SARS-CoV-2-infected patients, have been used for nucleic acid detection via reverse-transcriptase polymerase chain reaction (RT-PCR), real-time RT-PCR, or droplet digital PCR (ddPCR) (Chakraborty et al., 2020; Huang et al., 2020; Yu et al., 2020). Furthermore, anal swabs, fecal samples, and saliva specimens have been used for analysis of viral persistence (Kipkorir et al., 2020; Zhang et al., 2020). However, negative and false-negative nucleic acid detection results for SARS-CoV-2-infected patients have been reported in various clinical specimens (Xie et al., 2020). These results are likely impacted by factors such as virus gene mutation, infection route, disease progression status, specimen type, and collection time and method (Yu et al., 2020). This has not only resulted in misdiagnosis and poor treatment but has also impacted accurate evaluation of disease progression and prognosis and potential effects of vaccines and drugs (Yu et al., 2020). Thus, it is now suggested that different types of samples be collected at the same time for nucleic acid detection to improve diagnosis (Xie et al., 2020). However, such protocols can be time consuming, laborious, and expensive, which are critical factors given the urgency of COVID-19 diagnosis and study. Moreover, complementary methods in conjunction with nucleic acid detection, such as chest radiography and CT imaging, are also suggested to achieve more reliable diagnosis in clinical practice (Ai et al., 2020).

To improve SARS-CoV-2 detection and reduce time and labor costs in diagnosis, we used 394 samples from COVID-19-confirmed patients and 420 samples from SARS-CoV-2-infected monkeys for nucleic acid detection and analysis. We compared the nucleic acid detection rate in different sample types, as well as the diagnosis rate based on integration of the nucleic acid results from various samples, longitudinal SARS-CoV-2 nucleic acid detection rate, dynamic changes in SARS-CoV-2 load in different specimens, and detection rate of immunoglobulin M (IgM) and G (IgG) antibodies against SARS-CoV-2. We found that sputum and tracheal brush specimens produced the most stable and reliable results. Thus, lower respiratory tract samples appear to be reliable for COVID-19 diagnosis and animal model study.

MATERIALS AND METHODS

Clinical samples

For the current study, we used data obtained from the medical records of 29 COVID-19 patients from the Yunnan Infectious Diseases Hospital in Kunming, China. Data from the first positive test to before three consecutive negative tests were included in statistical analysis. Among the patients, 10.34% (3/29) were asymptomatic, 24.14% (7/29) were mild, 48.28% (14/29) were moderate, 13.79% (4/29) were severe, and 3.45% (1/29) were critical (Supplementary Table S1). Data on SARS-CoV-2 nucleic acid detection from nasopharyngeal swabs (89, 22.59%), pharyngeal swabs (146, 37.06%), sputum samples (72, 18.27%), anal swabs (28, 7.11%), fecal samples (38, 9.64%), urine specimens (10, 2.54%), serum samples (9, 2.28%), and saliva samples (2, 0.51%) were included for analysis (Figure 1A). Additional information on samples is shown in Supplementary Table S1. Clinical samples were processed in the P2 Laboratory of the Yunnan Infectious Diseases Hospital in accordance with the Laboratory Biosafety Guide for the Novel Coronavirus (2nd edition) issued by the National Health Commission of China.

Samples from SARS-CoV-2-infected monkeys

Eleven Chinese rhesus macaques (Macaca mulatta) and four northern-pigtail macaques (Macaca leonina) were challenged with 10^7 TCID50 (median tissue culture infectious dose) of SARS-CoV-2 (NMDC accession No.: NMDCN000HUI) through the bronchus (eight rhesus macaques and four northern-pigtail macaques) or both the nose and bronchus (three rhesus macaques) (Song et al., 2020, 2021). Nasal swabs (124, 29.52%), pharyngeal swabs (124, 29.52%), anal swabs (124, 29.52%), and tracheal brushes (48, 11.43%) were collected over 15 days after infection (DAI). After collection, samples were immediately placed on dry ice and stored at −80 °C until nucleic acid detection (Figure 1B). Additional information on monkey samples is shown in Supplementary Table S2. During the study, the animals were housed at the Animal Biosafety Level 3 Laboratory of the
Kunming Institute of Zoology, Chinese Academy of Sciences. All sample manipulations were performed in the same laboratory. All procedures were approved by the Institutional Committee for Animal Care and Biosafety of the Kunming Institute of Zoology, Chinese Academy of Sciences (approval No.: IACUC20005; IACUC20016).

Nucleic acid detection in clinical samples

Nucleic acid was extracted from nasal swabs, pharyngeal swabs, sputum specimens, anal swabs, fecal samples, serum samples, urine specimens, and saliva samples using an RNA/DNA Purification Kit (DAAN Gene, China) in accordance with the manufacturer-provided instruction manual. The SARS-CoV-2 ORF1ab and N genes were detected using a 2019-nCOV Real-Time PCR Kit (Bai’Jie, Shanghai BioGerm Medical Technology, China) according to the instruction manual. Amplification was performed using the ABI Prism 7500 Sequence Detector system (Applied Biosystems, Thermo Fisher, USA). A positive control consisting of SARS-CoV-2 RNA reference material (virus-like particles containing ORF1ab- and N-specific fragments and RNase P-specific fragments) and a negative control (saline solution) were included.

Figure 1 Sample distribution, distribution, and frequency of SARS-CoV-2 nucleic acid-positive samples

In total, 394 samples from COVID-19 patients (A) and 420 samples from SARS-CoV-2-infected monkeys (B) were included in this study. Nucleic acid was purified from COVID-19 patient samples (C, E) and SARS-CoV-2-infected monkey samples (D, F). SARS-CoV-2 was detected by real-time RT-PCR with a TaqMan probe. Distributions of SARS-CoV-2 nucleic acid-positive samples (C, D) and frequency of positive samples (E, F) are shown.
Nucleic acid detection in SARS-CoV-2-infected monkeys

Nucleic acid was extracted from nasal swabs, pharyngeal swabs, anal swabs, and tracheal brushes using a High Pure Viral RNA Kit (Roche, Germany) according to the manufacturer’s instructions. The SARS-CoV-2 NP gene was detected using a Probe One-Step qRT-PCR Kit (Toyobo, Japan) according to the provided instruction manual. Primer and probe sequences for quantification of the SARS-CoV-2 NP gene included: forward primer, 5'-GGGGAACCTTCTCC TGCTAGAAT-3'; reverse primer, 5'-CAGACATTGTGCTCT CAAGCTG-3'; and probe, 5'-FAM-AGCCGCCGCTGGTCAACTCG-TAMARD-3'. Amplification was performed using an ABI Prism ViA 7 Sequence Detector system (Applied Biosystems, Thermo Fisher, USA). A positive control consisting of SARS-CoV-2 RNA reference material (National Institute of Metrology, China; GBW(E)091089) and a negative control consisting of blank elution buffer were included in each reaction (Xu et al., 2020).

Anti-SARS-CoV-2 antibody assays for clinical samples

Magnetic particle chemiluminescence-based Novel Coronavirus (2019-nCOV) IgM and IgG Detection Kits (Bioscience Diagnostic, Tianjin, China) were used to detect anti-SARS-CoV-2 IgM and IgG antibodies, respectively. All tests were performed on an Axcceed 260 (Bioscience Diagnostic, Tianjin, China) per the manufacturer’s protocols.

Statistical analyses

All data were analyzed using GraphPad 6.0 software. Welch's t-test was used to compare differences between two groups.

RESULTS

High SARS-CoV-2 nucleic acid detection in sputum and tracheal brush samples

As shown in Figure 1C, D, a total of 198 samples from COVID-19 patients and 139 samples from SARS-CoV-2-infected monkeys were SARS-CoV-2 nucleic acid-positive, representing 50.25% (198/394) and 33.10% (139/420) of total samples, respectively. For nucleic acid-positive clinical samples, sputum samples accounted for the largest proportion (33.33%; 66/198), followed by nasopharyngeal and pharyngeal swabs (28.79% (57/198) and 27.27% (54/198), respectively), then fecal samples, anal swabs, and saliva samples (8.08% (16/198), 2.02% (4/198), and 0.51% (1/198), respectively). We did not detect SARS-CoV-2 nucleic acid in serum or urine samples of COVID-19 patients. Different from the clinical samples, a similar number of nasal swabs, pharynx swabs, anal swabs, and tracheal brushes were positive for SARS-CoV-2 nucleic acid, with positive proportions of 20.86% (29/139), 23.74% (33/139), 25.18% (35/139), and 30.22% (42/139), respectively (Figure 1D). Furthermore, regardless of the clinical manifestation of infection in COVID-19 patients, sputum samples exhibited the highest nucleic acid-positive rate compared to other samples, followed by nasopharyngeal swabs (Figure 1E; Supplementary Figure S1).

High longitudinal SARS-CoV-2 nucleic acid detection in sputum and tracheal brush samples

To test diagnosis stability based on different sample types and individuals, we compared the SARS-CoV-2 nucleic acid detection rate in individuals longitudinally. Data from three or more time points of the same sample type from the same individual were used for analysis. This included 72 nasopharyngeal swabs, 132 pharyngeal swabs, 21 fecal samples, and 61 sputum samples from 14, 20, 6, and 14 individuals, respectively. Based on sample type, the detection rate for an individual was the ratio of the number of positive detections to the total number of tests performed on this individual. Based on analysis, SARS-CoV-2 was only detected continuously in a few individuals based on nasopharyngeal (4/14), pharyngeal (2/20), and fecal/anal swabs (1/6) obtained at different time points. Notably, for some individuals, the virus was not detected in any nasopharyngeal swab (3/14), pharyngeal swab (4/14), or fecal/anal swab (2/20) sample (Figure 2A). In contrast, SARS-CoV-2 was detected continuously in the sputum of most COVID-19 patients (10/14), with only occasional non-detection in a small number of individuals (4/14). Thus, sputum samples showed a significantly higher diagnosis rate than that of the other sample types (P=0.001 8; P<0.000 1; P<0.000 1). There were no significant differences in the longitudinal SARS-CoV-2 nucleic acid detection rates among patients with different clinical manifestations (Supplementary Figure S3). The diagnosis rate in SARS-CoV-2-infected monkeys was similar to that observed in COVID-19 patients, with the highest diagnosis rate based on tracheal brush samples (P<0.000 1; P<0.000 1; P<0.000 1) (Figure 2B). Thus, regardless of the clinical manifestation observed in infected patients and monkeys, SARS-CoV-2 nucleic acid detection and diagnosis were effective and stable when based on sputum and tracheal brush samples.
Anti-SARS-CoV-2 antibody detection is unsuitable for early diagnosis of SARS-CoV-2 infection

No detectable anti-SARS-CoV-2 antibodies (i.e., IgM and IgG) were found in the serum of COVID-19 patients on day 1 of hospitalization (Figure 2C, D). It is worth noting that two asymptomatic-infected patients (20# and 21#) were negative for IgG and IgM (IgG-IgM-) on day 12 of hospitalization and only weakly positive for IgG on day 18 of hospitalization; in addition, two patients (23# and 26#) were negative for anti-SARS-CoV-2 antibodies (IgM and IgG) during their 18 days of hospitalization (Figure 2C). Moreover, only 31.58%, 25.00%, 50.00%, and 36.36% of SARS-CoV-2-infected patients were anti-SARS-CoV-2 IgM-positive on day 3, 7, 12, and 18 of hospitalization, while 42.1%, 40.00%, 70.00%, and 81.82% of patients were anti-SARS-CoV-2 IgG-positive, respectively (Figure 2D). We also detected anti-SARS-CoV-2 IgM longitudinally in virus-infected monkeys. Unexpectedly, the first appearance of anti-IgM occurred later than 11 DAI. Of the six monkeys observed at 15 DAI, only three were positive for anti-SARS-CoV-2 IgM antibodies (Supplementary Figure S4). These results indicate that SARS-CoV-2 detection based on anti-SARS-CoV-2 antibodies is insufficient and occurs too late for early and effective diagnosis.

SARS-CoV-2 nucleic acid detection in sputum and tracheal brush samples alone can successfully diagnose SARS-CoV-2 infection

To improve the diagnosis rate of SARS-CoV-2-infected patients, we integrated the detection results of different sample types from the same individual at the same time. If any sample was SARS-CoV-2 nucleic acid-positive, the individual was diagnosed as SARS-CoV-2 infected. A total of 183 sampling time points for 28 COVID-19 patients and 124 sampling time points for 15 SARS-CoV-2-infected monkeys were included. As shown in Figure 3A, compared to sputum samples alone, combining the detection results from nasopharynx swabs, pharynx swabs, and anal swabs/fecal samples did not significantly improve the diagnosis rate. This was also true when anal swabs or fecal tests were combined with nasopharynx swabs or pharynx swabs (Figure 3A). However, when the pharyngeal swabs were combined with the nasopharyngeal swabs, the diagnosis rate increased significantly from 36.99% to 61.87%, equal to that obtained using nasopharyngeal swabs alone. Thus, combining the results of different sample types improved the diagnosis rate of low detection samples, but had no impact on the diagnosis rate of high detection samples (Figure 3A). In the SARS-CoV-
2-infected monkey model, the influence of tracheal brush samples was similar to that of sputum samples, i.e., improved the diagnosis outcome for low detection samples but did not improve the diagnosis results for high detection samples (Figure 3B). Except for tracheal brushes, the integrated detection rate of other types of samples only slightly improved the detection rate of SARS-CoV-2 (Figure 3B). Thus, these results imply that sputum and tracheal brush samples can stably and reliably detect SARS-CoV-2 nucleic acid and therefore successfully diagnose infection.

Time-course changes in SARS-CoV-2 load in COVID-19 patients and infected monkeys

To study the dynamic changes in SARS-CoV-2 infection in patients and monkeys, we determined viral load based on cycle threshold (Ct) values. As displayed in Figure 4A, the Ct values of SARS-CoV-2 were mostly ≤30 in nasopharyngeal swabs at the early stage of hospitalization, then gradually decreased and were below the detection limit in most patients at 20 days after hospitalization (Figure 4A). Different from nasopharyngeal swabs, the viral loads in the pharyngeal swabs from some patients were below detection at the early stage of hospitalization and decreased soon after hospitalization. At 20 days after hospitalization, SARS-CoV-2 was undetectable in pharyngeal swabs from almost all individuals (Figure 4B). Similar to pharyngeal swabs, the viral loads in sputum samples were higher at the early stage of hospitalization, then gradually decreased, but were still higher than the detection limit at 20–30 days after infection (Figure 4C). In SARS-CoV-2-infected monkeys, the dynamic changes in viral load in nasal and pharyngeal swabs were influenced by their low virus detection rates. SARS-CoV-2 was only detected continuously in the nasal and pharyngeal swabs of a few monkeys, with a peak in viral load at 3–5 DAI, followed by a gradual decrease (Figure 4D, E). Dynamic changes in viral load in the tracheal brushes were more obvious than changes in the nasal and pharyngeal swabs.

Viral load in the tracheal brush samples of most subjects peaked at 3 DAI (or 5 to 7 DAI in others), then gradually decreased, and persisted in most subjects until 15 DAI (Figure 4F). Therefore, these results suggest that sputum and tracheal brush samples are the most suitable for dynamic assay of SARS-CoV-2.

DISCUSSION

SARS-CoV-2 and COVID-19 have severely impacted human society, including the global economy and health care systems. COVID-19 has spread to almost every country worldwide, not only due to the nature of SARS-CoV-2 itself (Chakraborty et al., 2020), but also inadequate analysis and treatment measures. Thus, a huge gap remains in accurate and timely diagnosis because of insufficient manpower and resources in many countries, as well as unreliable evaluation systems for drugs and vaccines, which have hindered the development of therapeutic strategies. Here, following horizontal and longitudinal analysis of the detection rates of SARS-CoV-2 nucleic acid in different types of clinical and animal model specimens, the highest rates were obtained from COVID-19 patient sputum samples and SARS-CoV-2-infected monkey tracheal brush samples, which also showed marked time course-dependent changes in viral load. We further determined that SARS-CoV-2 diagnosis based on antibodies was insufficient. In addition, integration of nucleic acid detection results from the various sample types did not improve the diagnosis rate of SARS-CoV-2 infection. Thus, our results suggest that detection based on lower respiratory tract samples alone is reliable and suitable for clinical SARS-CoV-2 diagnosis and animal model study.

It has been speculated that SARS-CoV-2 can infect lung type II pneumocytes, ileal absorptive enterocytes, and nasal goblet secretory cells (Sungnak et al., 2020; Ziegler et al., 2020). SARS-CoV-2 protein and nucleic acid have been detected in various tissues, such as the lungs, tracheas,
kidneys, gastrointestinal tracts, and brains of infected humans and monkeys (Munster et al., 2020; Song et al., 2021; Xiao et al., 2020). Furthermore, SARS-CoV-2 infection is known to cause multiple organ damage both directly and indirectly, with primary pulmonary symptoms (Yao et al., 2020). Nasal, pharyngeal, and sputum specimens have been suggested for diagnosis of SARS-CoV-2 infection, as used for other infectious respiratory diseases (Li, 2020); nevertheless, different infections may behave in different ways. As shown in Figure 1, the detection rates of SARS-CoV-2 nucleic acid in sputum samples and tracheal brushes from SARS-CoV-2-infected subjects reached 91.67%, significantly higher than that found in the other sample types. Although sampling procedures may cause false negatives in nasal, pharyngeal, fecal, and anal specimens (Tahamtan & Ardebili, 2020), this was avoided in our study due to experienced collection and quality control. Thus, the differences in the detection rates in different samples may reflect differences in viral replication site and dynamic changes in viral replication (Pan et al., 2020; Zou et al., 2020). The low detection rate of SARS-CoV-2 may also be due to individual differences in viral replication sites. This hypothesis was supported by the longitudinal SARS-CoV-2 detection rates found in the clinical samples and SARS-CoV-2-infected monkeys (Figure 2). Furthermore, we speculated that the differences observed in individuals in our study may come from different infection routes. Although this conjecture was difficult to verify with the clinical study, it was partly supported by our animal model. As shown in Figure 2B, at 7 DAI, 8.33% (1/12) of the bronchial-challenged group and 66.67% (2/3) of the nasal/bronchial-challenged group had 100% SARS-CoV-2 detection in the nasal and throat specimens. However, this needs to be further confirmed due to the small number of individuals used. In our study, the detection rate was also affected by sample collection time (Figure 4). As the disease progressed, the virus disappeared in some tissues and organs, while appearing in others (Figure 4). As shown in Figure 4A, B, with disease recovery, SARS-CoV-2 was not detectable in the nasal or pharyngeal swabs. Consistently, the virus in the sputum and tracheal brush specimens also decayed gradually but persisted for longer (Figure 4C). As such, viral replication was better reflected in the lower respiratory tract samples from infected subjects. The high and persistent SARS-CoV-2 detection rates in sputum and tracheal brush specimens thus allowed reliable time-course testing of the virus. However, the detection rate of SARS-CoV-2 in sputum and tracheal brush samples did not reach 100%, and it could not be improved by integration of multiple sample tests (Figure 3). Therefore, these results suggest that compensatory approaches are needed to improve diagnosis of nucleic acid-negative individuals (Ai et al., 2020). Although serological testing facilitated identification of asymptomatic SARS-CoV-2 infection in Wuhan, China (Wu et al., 2020), it does not appear to be suitable for early diagnosis of SARS-CoV-2 infection, as not all patients (41.37% and 13.80%) in the current study had detectable anti-SARS-CoV-2 IgM or IgG antibodies or antibodies were produced too late. Low positive rates of IgM and IgG have also been reported in previous SARS-CoV-2 research (Li et

Figure 4 Dynamic changes in SARS-CoV-2 load
Nucleic acid was purified from nasopharyngeal swabs (A), pharyngeal swabs (B), and sputum samples (C) obtained from COVID-19 patients or nasal swabs (D), pharyngeal swabs (E), and tracheal brushes (F) obtained from SARS-CoV-2-infected monkeys. SARS-CoV-2 was detected by real-time RT-PCR with a TaqMan probe. SARS-CoV-2 load is shown using cycle threshold (Ct). Each different colored dot and shape represent an individual.

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