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Commentary

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Future MICROBIOLOGY

N-acetylcysteine as a potential treatment for COVID-19

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"Oral administration of NAC (600 mg/day) could function as a preventive measure, particularly in those repeatedly exposed to possible SARS-CoV-2 carriers (e.g., health workers)."

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Keywords: COVID-19 • N-acetylcysteine • SARS-CoV-2

The novel coronavirus (CoV) disease 2019 (COVID-19), which first appeared in Wuhan, China, in December 2019, spreads efficiently from person to person. After it had reached over 100 countries, on 11 March 2020 the WHO declared it a pandemic [1]. COVID-19 is caused by SARS-CoV-2, and by 9 June 2020 had been responsible for 7,039,918 confirmed cases and 404,396 deaths worldwide [2]. At the time of writing, the five countries with the highest number of cases are the USA (1,933,560 cases), Brazil (691,758 cases), the Russian Federation (485,253 cases), the UK (287,403 cases) and India (266,598 cases) [2].

The scientific community's rapid response has allowed description of the complete SARS-CoV-2 genome, which is currently available on bioinformatics platforms. Analysis of the genome has found an 88% identity with two bat-derived SARS-like CoVs, bat-SL-CoVZC45 and bat-SL-CoVZXC21, both collected in 2018 in Zhoushan, Eastern China; it also has approximately 79% identity with 2002 SARS-CoV [3]. It is no surprise therefore that SARS-CoV-2 shares host cell infection mechanisms with SARS-CoV. Angiotensin-converting enzyme 2 (ACE2) has been shown to be the receptor in which the SARS-CoV-2 spike (S) glycoprotein allows membrane fusion and internalization [4]. The SARS-CoV-2 S glycoprotein bonds to ACE2 resulting in reduced expression of the enzyme; this generates angiotensin II accumulation generated by ACE. The depleted ACE2 is unable to convert angiotensin I into the vasodilator heptapeptide angiotensin 1–7, thus generating pulmonary injury; also, angiotensin II type-1 receptor overstimulation results in increased lung vascularity which contributes to the overall pathology. Human ACE2 and the SARS-CoV-2 S glycoprotein have consequently been identified as the therapeutic targets for development of new treatments such as antivirals and monoclonal antibodies, or for identification of existing drugs capable of blocking interaction between the virus and the host cell.

The SARS-CoV-2 S glycoprotein consists of two subunits, S1, which facilitates viral bond to the host cell, and S2, which assists viral membrane fusion [5]. The fusion process depends on S glycoprotein cleavage at the S1/S2 multibasic site, mainly by the human protease furin [6]. *In vitro* results demonstrate the essential role of this cleavage site to promote viral entrance into lung cells [6]. Thus, direct inhibition of furin or disruption of the interactions between the S1/S2 complex and this protease are potential therapeutic approaches.

We propose N-acetylcysteine (NAC) as a potential treatment, preventive and/or adjuvant against SARS-CoV-2. It has two principal activities: NAC exhibits a mucolytic effect due to its free sulfhydryl group which reduces disulfide bonds in the cross-linked mucus glycoproteins matrix, thereby lowering mucus viscosity [7]; and NAC is a potent antioxidant with a direct effect on certain oxidant species, an indirect effect because it acts as a precursor to cysteine (required for glutathione synthesis), and the ability to restore thiol pools which in turn regulate redox state [7].

Considering these properties, we hypothesize that NAC could negatively affect SARS-CoV-2 activity for the following reasons:

- The E protein of SARS-CoV (genetically related to SARS-CoV-2) consists of 76–109 amino acids, ranging in size from 8.4 to 12 kDa. Its primary and secondary structures have a short, hydrophilic amine terminus group of 7–12 amino acids followed by a hydrophobic 25 amino acid transmembrane domain which ends in a hydrophilic carboxyl group terminus [8]. The SARS-CoV-2 E protein includes a triple cysteine motif (NH_2- . . . L-Cys-A-Y-Cys-Cys-N . . . -COOH) after the transmembrane domain which interacts with a similar motif from S protein terminal C- (NH_2- . . . S-Cys-G-S-Cys-Cys-K . . . -COOH) [8]. Both motifs interact through disulfide bonds [8], and NAC may cleave them. This would decrease SARS-CoV-2 infectivity;
- *In vitro* studies have shown NAC to decrease angiotensin II bonds to angiotensin II type 1 receptor in a dose-dependent manner [9]. In the COVID-19 context, NAC could block excessive production of angiotensin II, which cannot be cleaved to angiotensin 1–7 by ACE2. This may decrease pulmonary disease severity;
- *In vitro* and clinical studies have shown NAC to block ACE. In one experiment isosorbide dinitrate (vasodilator activity) was administered to six male participants for 48 h, but at 24 h NAC was added (2 g intravenously [iv.] followed by 5 mg/kg/h). Angiotensin II plasma concentrations increased during the first 24 h of isosorbide dinitrate administration but just 2 h after NAC initiation they had decreased from 28 ± 4 to 14 ± 2 ng/l ($p < 0.05$) [10]. This suggests that, by blocking ACE, NAC may provide protection from the deleterious effects of angiotensin II, a potentially useful activity in a SARS-CoV-2 infection scenario;
- The oxidative stress environment created by cytokine storm syndrome and production of reactive oxygen species (ROS) may be attenuated by NAC's antioxidant effect [11]. Also, the SARS-CoV-2 immunopathology may be similar to that of SARS-CoV, which generates an immune response involving diverse pro-inflammatory cytokines (IL-1, IL-2, IL-4, TNF and IFNs). The IFNs are classified in type-I (IFN-α and β), -II (IFN-γ) and -III. Type-I IFNs are suppressed during SARS-CoV infection due to impairment of signal transducer and activator of transcription 1, which ultimately antagonizes IFN. This complex mechanism may also generate delayed IFN response due to cytokine storm syndrome during SARS-CoV-2 infection, possibly explaining COVID-19 pathology. NAC may amplify the signaling functions of toll-like receptor 7 and mitochondrial antiviral signaling protein in restoring type-I IFN production during SARS-CoV-2 infection [11];
- NAC has been shown to restore platelet GSH reserves (in a murine model) which in turn can prevent protein glycosylation by methylglyoxal, a pathologic mechanism in diabetic patients [12]. The SARS-CoV-2 S glycoprotein differs from that of SARS-CoV in that it gains new glycosylation sites (NGTK, NFTI, NLTT and NTSN), allowing SARS-CoV-2 to enter the host cell [5]. Administration of NAC could prevent additional glycosylation events in SARS-CoV-2, thus inhibiting its infectivity and any associated pathologies;
- In a recent study the NF-κB was described as a mediator of SARS-CoV-2 pulmonary pathology since it triggers the production of numerous pro-inflammatory cytokines. This process generates macrophage and neutrophil infiltration, both of which cause irreparable damage to pulmonary epithelium cells. NAC was shown to inhibit NF-κB activation in an *in vitro* influenza (A and B) model [13]; the proposed mechanism is restoration of thiol pools, which may allow ROS scavenging. This is relevant because recent clinical experience has shown that severity of COVID-19 clinical manifestations might be associated with decreased GSH levels and the consequent increased ROS production. Severe COVID-19 cases would therefore probably manifest lower GSH levels, higher ROS levels and greater redox status (ROS/GSH ratio) than milder cases [14];
- In the context of influenza virus infection, NAC administration (100 mg/kg continuous iv. infusion daily for 3 days) was reported to promote clinical improvement in a woman with H1N1 influenza pneumonia; oseltamivir was also employed during treatment [15]. However, other studies have found no beneficial *in vitro* or *vivo* effects with NAC administration [16]. NAC (600 mg twice daily) has also been reported to attenuate influenza symptoms in patients ≥65-years old with chronic-degenerative diseases [17].

Given this pandemic's immense health risk, several drugs have been employed with and without clinical evidence for the treatment of COVID-19, NAC among them [18]. Administration of NAC (oral, iv. or inhaled) as an adjuvant treatment in patients with mild–severe COVID-19 symptoms is worth considering as a cost–effective clinical strategy. Currently, there are some clinical trials assessing the potential use of NAC against COVID-19; for example, the 'Efficacy and Safety of Nebulized Heparin-N-acetylcysteine in COVID-19 Patients by Evaluation of Pulmonary Function Improvement (HOPE)' clinical trial is aimed at determining the efficacy of nebulized NAC and heparin in ventilated COVID-19 patients [19]. The aim is to increase ventilator-free days in hospitalized patients with moderate–severe COVID-19 symptoms. Another recent study is 'A Study of N-acetylcysteine in Patients With COVID-19 Infection', a clinical trial aimed at quantifying: the number of patients successfully extubated and/or

transferred from critical care unit due to clinical improvement; and the number of patients discharged due to clinical improvement. Patients are receiving NAC iv. 6 g/day in addition to other treatments prescribed for COVID-19 [20].

Oral administration of NAC (600 mg/day) could function as a preventive measure, particularly in those repeatedly exposed to possible SARS-CoV-2 carriers (e.g., health workers). This application could be a particularly urgent approach since, despite the use of personal protective equipment, healthcare workers in the USA, Italy, China, Mexico, etc., have become infected while caring for hospitalized patient. Other workers who, due to their job requirements, cannot work at home and/or ensure self-isolation might also benefit from preventive use of NAC administration. If deemed effective, this latter use could potentially help to flatten the exponential contagion curve in several countries. More clinical trials would clearly be needed to validate this application.

Basic laboratory and clinical studies are required to confirm possible use of NAC as an element in combating the disease caused by SARS-CoV-2. This would need to be one of myriad efforts to identify additional treatments (novel or not) aimed at halting the current COVID-19 pandemic, or at the very least slowing person-to-person contagion.

Author contributions

Both authors equally contributed to this manuscript.

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Case Series

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Future MICROBIOLOGY

Anal swab as the potentially optimal specimen for SARS-CoV-2 detection to evaluate the hospital discharge of COVID-19 patients

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Since December 2019, an outbreak of SARS coronavirus 2 (SARS-CoV-2) began in Wuhan, and has rapidly spread worldwide. Previously, discharged patients with coronavirus disease 2019 (COVID-19) patients met the criteria of China's pneumonia diagnosis and treatment program of novel coronavirus infection (trial version 7) for cure of viral infection. Nevertheless, positive detection of SARS-CoV-2 has been found again in several cured COVID-19 patients, leading to conflicts with current criteria. Here, we report clinically cured cases with positive results only in anal swabs, and investigate the clinical value of anal swabs for SARS-CoV-2 detection.

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Keywords: anal swab • COVID-19 • RT-PCR • SARS-CoV-2

Since December 2019, an outbreak of atypical pneumonia caused by SARS coronavirus 2 (SARS-CoV-2) has led to a serious epidemic in China and other countries. Phylogenetic analyses of the coronavirus genomes revealed that SARS-CoV-2 belongs to the *Betacoronavirus* genus, a class of positive-sense, ssRNA viruses that can cause respiratory, intestinal, liver and nervous system infections in animals and humans [1]. SARS-CoV-2 is composed of four structural proteins, known as the S (spike), E (envelope), M (membrane) and N (nucleocapsid) proteins, and possesses 82% identity to SARS-CoV and 50% identity to Middle East respiratory syndrome coronavirus (MERS-CoV) based on genome sequencing [2]. Moreover, it spreads by human-to-human transmission via droplets or direct contact, and infection has been estimated to have a mean incubation period of 6.4 days and a basic reproduction number of 2.24–3.58 [3].

Numerous retrospective studies have indicated that prevalent clinical manifestations of COVID-19 patients are fever, dry cough and dyspnea [4]; less common symptoms present as the production of sputum, headache and some gastrointestinal symptoms; moreover, an increasing number of patients with asymptomatic infection patients have been discovered [5–7]. According to the latest guidelines of the diagnosis and treatment of pneumonitis caused by 2019-nCoV (trial version 7) published by the National Health Commission of the People's Republic of China [8], the diagnosis of COVID-19 must be confirmed by reverse transcriptase-PCR (RT-PCR) or gene sequencing. At present, various biological samples of COVID-19 are used in the detection of SARS-CoV-2, and upper respiratory tract nasopharyngeal swabs are the most common sample type. However, growing evidence has revealed positive detection of nucleic acids in anal swabs of patients with COVID-19, although the positive rate is low [9,10].

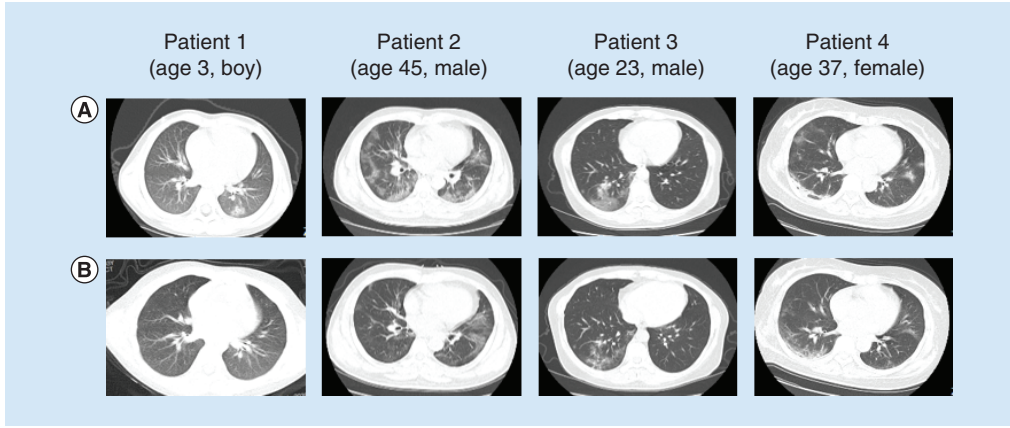

Figure 1. CT scans of patients. (A) CT scans performed on admission to the hospital; **(B)** CT scans performed after quarantine.

A previous study showed a positive RT-PCR test on throat swabs of patients recovered from COVID-19 [11], leading to conflicts with current criteria [8]. Here, we reported clinically cured cases with only positive results in anal swabs, which conflicts with current criteria for releasing people from quarantine, and further investigated the clinical value of anal swabs for SARS-CoV-2 detection. We propose anal swabs as the potentially optimal specimen for SARS-CoV-2 detection for evaluation of hospital discharge of COVID-19 patients.

Materials & methods

Sample collection

Throat swab and anal swab samples were collected using the standard process as previously described [9], and sputum swab samples were induced by inhalation of isotonic saline with salbutamol [12]. All swabs were immediately placed into a sterile tube containing 2–3 ml of transport media [13] and transported to the laboratory within 30 min.

SARS-CoV-2 nucleic acid detection by RT-PCR

RNA was extracted with protease K-magnetic beads (BioPerfectus Technologies, Jiangsu, China). Then, the sequences of SARS-CoV-2 were amplified by targeting three genes (*ORF1ab, N* and *E* genes) (Liferiver Bio-Tech, China). The RT-PCR assay was performed on a 7500 thermal cycler (ABI, US) under the following conditions: 50°C for 10 min for the reverse transcription reaction, initial denaturation at 95°C for 5 min, followed by 45 cycles of denaturation at 95°C for 10 s, followed by extension and capture of the fluorescence signal at 55°C for 40 s. Both internal and negative controls were routinely performed with each batch of tests.

Demographic information, laboratory findings and radiological features were collected from electronic medical records. This study was approved by the Weihai Municipal Hospital review board, and the need for informed consent was waived.

Case presentation

Four patients presented to the local fever clinic with fever, cough or both occurring at onset from 2 February 2020 to 20 February 2020. There were three adults and one child, and the age ranged from 3 to 45 years. SARS-CoV-2 detection was positive in throat swab samples, and CT manifestations showed single or multiple patchy areas of ground-glass opacity (Figure 1A). Combined with laboratory examination, these patients were diagnosed with mild COVID-19 infection according to the criteria of China's pneumonia diagnosis and treatment program of novel coronavirus infection. Noticeably, the 3-year old boy was diagnosed with mild COVID-19 based on both clinical criteria and radiological criteria according to the clinically based classification of disease severity forpediatric COVID-19 [14].

After appropriate supportive care and active treatment with antiviral therapy for less than 9 days, mainly including oral lopinavir and ritonavir tablets, aerosol inhalation of interferon-α2b at a dose of 5×10^6 U/day and oral administration of acetylcysteine tablets and Chinese medicine Lianhua Qingwen capsule. SARS-CoV-2 detection was successively negative twice for these patients, in addition to normal body temperature for 3 days as

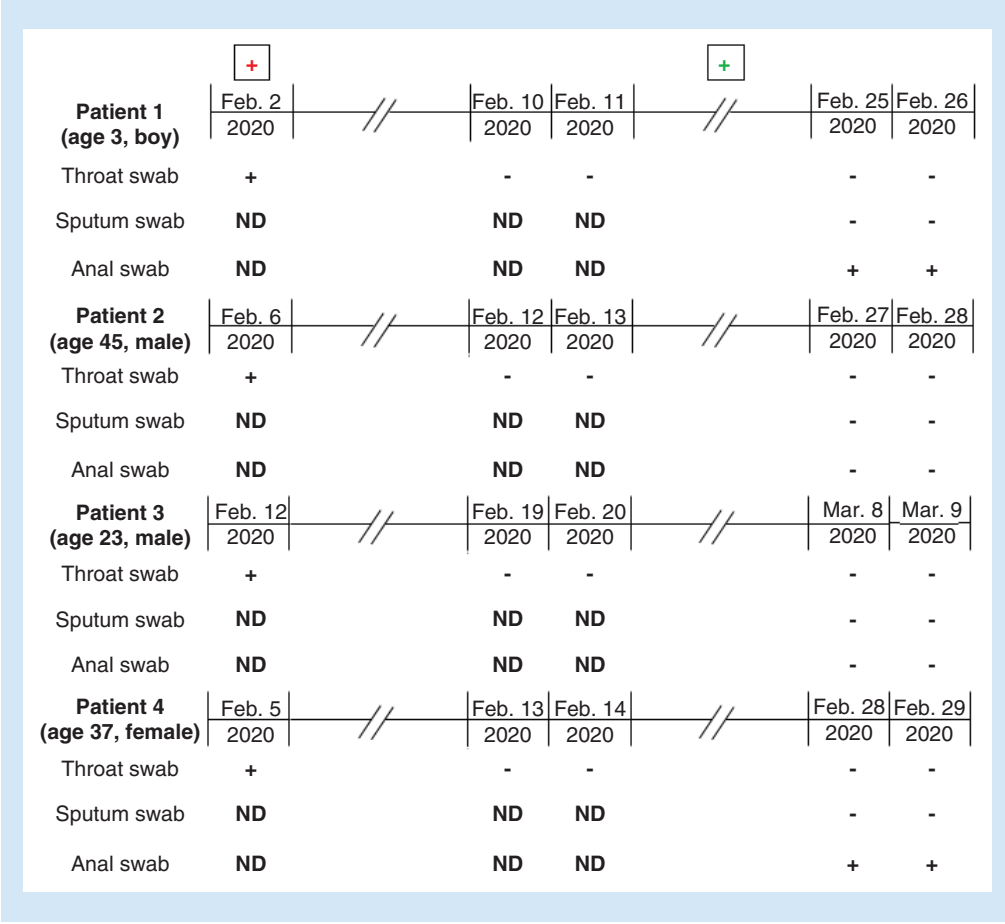

Figure 2. Chronology of treatment and detection of reverse transcriptase-PCR on throat swabs, sputum swabs and anal swabs. The box with an internal red cross means admission to the hospital; the box with an internal green cross means quarantined in the hotel or in the hospital for 2 weeks.
+: Positive result; -: Negative result; ND: No detection.

well as obvious improvement in respiratory symptoms and CT scan. Therefore, the patients were determined to be clinically cured at discharge [8]. One discharged patient was quarantined in a hotel, and the other three patients were quarantined in the hospital for 2 weeks.

To determine whether the abovementioned patients could discontinue quarantine, throat, sputum and anal swab samples were collected for SARS-CoV-2 detection at 2 weeks after quarantine. Intriguingly, SARS-CoV-2 detection was positive in the anal swab of two patients and negative in throat swab and sputum samples. RT-PCR was performed again for all patients the following day, and positive detection was confirmed, including in the 3-year old boy (Figure 2). Further clinical manifestations, laboratory characteristics and chest CT findings (Figure 1B) showed obvious improvement in all patients.

Discussion

Since December 2019, the outbreak of COVID-2019 caused by SARA-CoV-2 has become a global health concern [15]. SARS-CoV-2 has quickly spread across China and all continents except Antarctica [16–19] and caused more than 3.5 million confirmed cases with 24,000 deaths (https://www.who.int/emergencies/diseases/novel-coronavirus-2019/situation-reports). Thus far, the origin of the coronavirus remains unclear. The latest report discovered that the pangolin-CoV genome showed 91.02% nucleotide identity with the SARS-CoV genome, which suggested that pangolin species are a natural reservoir of SARS-CoV-2-like CoVs [20].

Molecular detection remains the gold standard for diagnosis. As a recommended method, RT-PCR is widely used to detect SARS-CoV-2. To date, throat, sputum and anal swabs have been considered applicable for RT-PCR

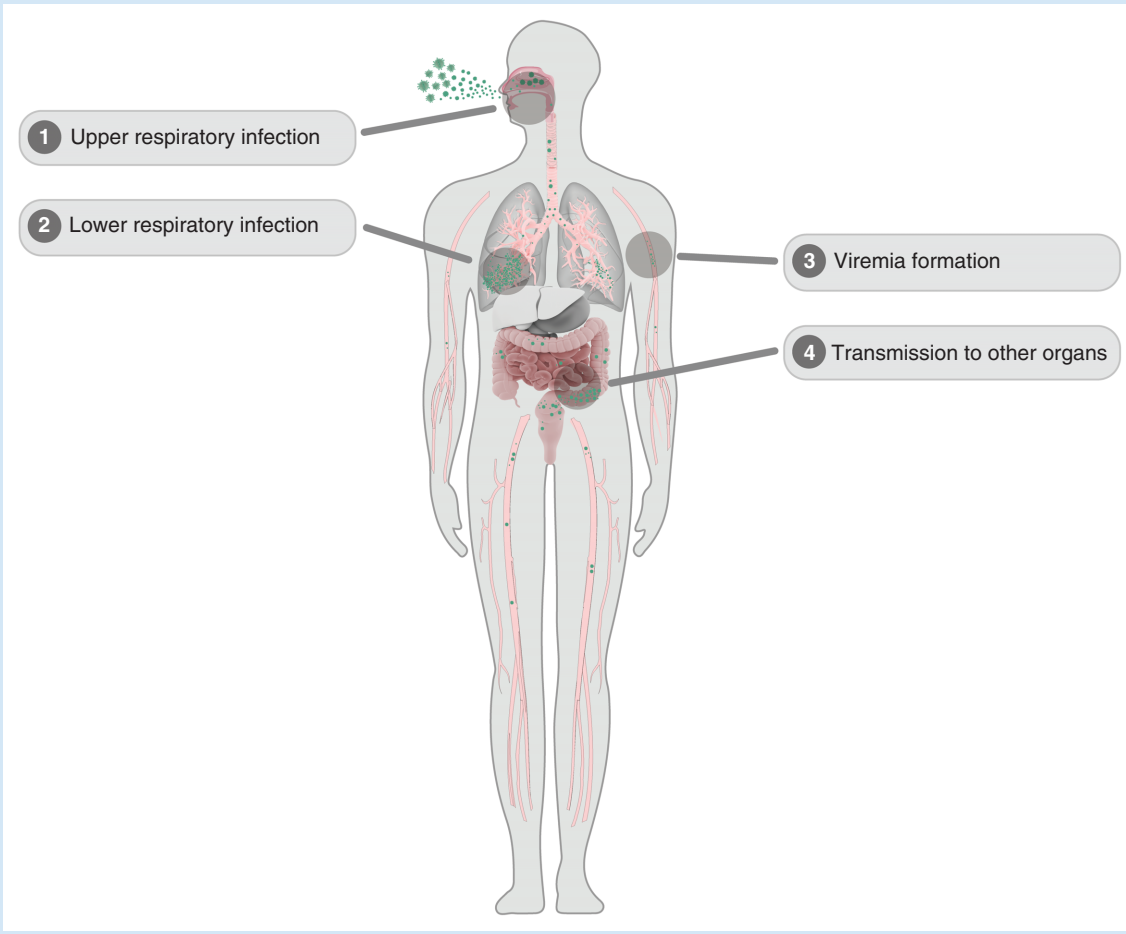

Figure 3. Potential infection course of SARS-CoV-2 and the different specimens for SARS-CoV-2 detection. First, SARS-CoV-2 infects the upper respiratory system mainly by respiratory droplets (asymptomatic or fever, dry cough, fatigue, myalgia and dyspnoea; high positive RT-PCR results in throat swabs). Subsequently, it infects the lower respiratory tract and massively replicates (mainly presented as pulmonary infection; high positive results of RT-PCR in throat swabs and sputum). Furthermore, virus is released into blood, leading to the formation of viremia (low copy number detected in blood by RT-PCR). Finally, it is transmitted to other organs, including the GI tract, and colonizes via ACE2 (higher positive detection rate in anal swabs).
ACE2: Angiotensin-converting enzyme 2; GI : Gastrointestinal; RT-PCR: Reverse transcriptase-PCR.

detection. Evidence has shown that sample type plays a critical role in SARS-CoV-2 detection. Viral RNA can be easily detected in nasopharyngeal, sputum and stool specimens [21], and the highest positivity rates were detected in sputum and bronchoalveolar lavage specimens [22]. However, the course of SARS-CoV-2 infection remains unclear.

As the cellular receptor for SARS-CoV-2, angiotensin-converting enzyme 2 (ACE2) is the key for SARS-CoV to enter target cells during the course of viral infection [23–25]. Expression of ACE2 protein in human organs showed that ACE2 is most abundantly expressed on the surface of alveolar epithelial cells and small intestine epithelial cells [26], which are involved in the progression of pneumonia [27]. Intriguingly, a connection may exist between the lungs and GI tract [28], and SARS-CoV-2 may be shed through multiple routes in the different phases of viral infection.

In this study, we found that SARS-CoV-2 detection was positive in anal swabs but negative in other sample types of a few cured patients, which challenges the current standards for discharge and termination of compulsory isolation for COVID-19 patients. Similar to SARS-CoV and MERS-CoV patients [29,30], intestinal infection was observed in the later stages of infection, indicating that the clearance time of SARS-CoV-2 in the digestive tract was later than that in the respiratory tract. In particular, gastrointestinal symptoms were found in children with COVID-19 [31]. However, the burden of novel coronavirus infections is still underestimated; only approximately

1% of all confirmed SARS-CoV-2 cases involve children according to the current estimates [32], so more biological samples and methods (e.g., serologic detection) for SARS-CoV-2 infection in children must be studied. Notably, live SARS-CoV-2 virus was isolated from fecal samples in three of 11 adult patients [33]. Therefore, anal swabs might be the optimal specimen for SARS-CoV-2 detection to evaluate hospital discharge of COVID-19 patients. Patients with positive stool results require further isolation until the virus is completely eliminated.

Based on the knowledge about this specific viral infection and considering the prolonged viral RNA detection in anal swabs [34] and detectable viral RNA in the blood cohort progressing to a severe symptom stage [35], we proposed the potential infection course of SARS-CoV-2 as follows (Figure 3): upper respiratory infection (mainly by respiratory droplets); lower respiratory infection (mainly presented as pulmonary infection); viremia formation; and transmission to other organs (including the GI tract) and colonization via ACE2. Therefore, different sample types should be chosen for SARS-CoV-2 detection in various infection phases. Fortunately, Sethuraman *et al.* devised a clinically useful timeline of diagnostic markers for the detection of COVID-19 [36].

In summary, we found that SARS-CoV-2 detection was positive in anal swabs but negative in other sample types of several cured patients. Our findings greatly contribute to a comprehensive understanding of COVID-19. Although the study was limited to a small number of patients, and further longitudinal studies on a larger cohort would help to understand the prognosis of the disease.

Summary points

- The COVID-19 outbreak caused by SARS-CoV-2 has become a global health concern.
- SARS-CoV-2 detection is positive in anal swabs but negative in throat swabs and sputum swabs of a few discharged patients.
- Anal swabs might be the optimal specimen for SARS-CoV-2 detection to evaluate the hospital discharge of COVID-19 patients.

Author contributions

M-Y Wang conceived and designed the study. M Sun, D Guo, J Zhang and J Zhang performed the literature survey. D Guo, H-F Teng, Q-X Ge and J Xia performed the experiments and analyzed the data. M Sun, D Guo and P Liu contributed to the writing and checking of the letter. All authors read and approved the final manuscript.

Financial & competing interests disclosure

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Ethical conduct of research

This study was approved by the Weihai Municipal Hospital review board, and the need for informed consent was waived.

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Research Article

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Possible SARS-coronavirus 2 inhibitor revealed by simulated molecular docking to viral main protease and host toll-like receptor

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Aim: SARS-coronavirus 2 main protease (Mpro) and host toll-like receptors (TLRs) were targeted to screen potential inhibitors among traditional antiviral medicinal plants. **Materials & methods:** LeDock software was adopted to determine the binding energy between candidate molecules and selected protein pockets. Enrichment analyses were applied to illustrate potential pharmacology networks of active molecules. **Results:** The citrus flavonoid rutin was identified to fit snugly into the Mpro substrate-binding pocket and to present a strong interaction with TLRs TLR2, TLR6 and TLR7. One-carbon metabolic process and nitrogen metabolism ranked high as potential targets toward rutin. **Conclusion:** Rutin may influence viral functional protein assembly and host inflammatory suppression. Its affinity for Mpro and TLRs render rutin a potential novel therapeutic anti-coronavirus strategy.

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Keywords: COVID-19 • GO and KEGG enrichment analysis • molecular docking • rutin • SARS-CoV-2 main protease • toll-like receptors • traditional antiviral medicinal plants

SARS coronavirus 2 (SARS-CoV-2) first emerged in the city of Wuhan, China and progressively evolved into a severe pandemic [1]. The WHO proclaimed the outbreak of coronavirus disease-2019 (COVID-19) to be a Public Health Emergency of International Concern (PHEIC), which was the highest level of epidemic prevention in the world, suggesting its gravity [2]. Through international transportation, SARS-CoV-2 spread globally with more than four million reported cases and over 290,000 fatalities by 14 May 2020. The high morbidity and mortality rates were ascribed to the lack of effective drug treatment. COVID-19, for which SARS-CoV-2 is the etiological agent, poses a serious threat to human life during the continuation of the global outbreak.

Currently, the two main strategies for developing anti-CoV therapeutics have focused on virus-based or immunomodulatory treatments [3]. Numerous compounds directly targeting the virus inhibit the entry and/or replication of CoV *in vivo* or *in vitro*. For example, remdesivir and chloroquine target the RNA polymerase of CoV to exert a significantly strong inhibition [4]. Immunomodulators, such as either glucocorticoids to relieve symptoms of pulmonary inflammation by delaying the inflammatory cytokine storm, or interferon treatments to enhance the innate antiviral response, have been thought as excellent anti-CoV remedies [5,6].

In addition, numerous natural products have been suggested and tested for their antiviral effects. Augmentation of the interferon response by the administration of natural products has been reported [7–9]. In the past 20 years, a total of 109 natural constituents with antiviral or immunoregulation functions also have been reported and reviewed in [8]. Those 109 constituents were mainly isolated and purified from heat-clearing and detoxifying herbs and were classified as various kinds of alkaloids, terpenes, flavonoids or saponins. To screen potential SARS-CoV-2 inhibitors more effectively, the 109 constituents were selected as candidate molecules to dock with the crystal

structure of SARS-CoV-2 main protease (Mpro) [10], which we tested herein via molecular docking software (Supplementary Table 1).

Materials & methods

Acquisition of chemical structure

The structures of 109 compounds [8] obtained from PubChem were saved as spatial data files, input into ChemBio3D Ultra 14.0 to minimize energy for the force field of the structure, and then saved in MOL2 molecular structure format.

Docking method

The 3D structure of Mpro and a series of host toll-like receptors (TLRs) were obtained from the Research Collaboratory for Structural Bioinformatics protein data bank database. Protein data bank IDs of these molecules are as follows: Mpro (6lu7), TLR1 (6NIH), TLR2 (5d3i), TLR3 (1ziw), TLR4 (2z62), TLR5 (3v44), TLR6 (3a79), TLR7 (5gmf), TLR8 (4qc0) and TLR9 (3wpf). Inhibitor N3 was used as a ligand while analyzing the crystal structure of SARS-CoV-2 Mpro [10]. LeDock software was used to calculate the binding energy between ligands and targeted proteins because LeDock software presents significant reliability and accuracy compared with other docking software [11]. First, the input protein structure was provided with an added hydrogen for the sake of being charged electrically. Then, compound structures were input as ligands. Subsequently, the site of the grid box was identified according to the coordinates of the positive ligands in the target protein complex [10]. After the active pocket was well placed, LeDock calculations were performed for molecular docking. For each chemical structure, several docking poses were recommended through LeDock in addition to generate the binding energy. The optimum docking poses of each structure were applied for ranking, and the visualization of docking was performed with PyMOL 1.8 v4.4.0 (www.pymol.org) and LigPlot^{+} v.2.2 (www.ebi.ac.uk/thornton-srv/software/LigPlus/) software, respectively.

Heatmap

The binding energy between 11 representative compounds and TLRs were visualized as a heatmap by MeV 4.9.0 based on the results presented in Table 2.

Prediction for molecular mechanisms of rutin

The structure of rutin was loaded into Swiss Target Prediction (www.swisstargetprediction.ch/) to screen the potential target gene [12]. The functional annotation of the Database for Annotation, Visualization and Integrated Discovery (DAVID) v6.8 (https://david.ncifcrf.gov/) was applied for target gene annotation, and the Official Gene Symbol was chosen as the identifier in DAVID v6.8. Each target gene was analyzed via gene ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) [13]. The KEGG pathway enrichment bubble map was formed by R program v3.5.0.

Results

Docking between candidate molecular & Mpro

LeDock results depicted that flavonoid compounds (Table 1, Figure 1) displayed lower binding energy with Mpro compared with other structure types such as alkaloids, terpenes and saponins. Eleven compounds were identified with binding energies <-6.5 kcal/mol (Table 1), most of which were flavonoids. Of these, rutin demonstrated the lowest predicted binding energy in the active pocket of Mpro (-8.67 kcal/mol), even lower than the reported positive inhibitor (Table 1). Remdesivir was also regarded as the positive inhibitor toward Mpro with the lowest binding energy (-9.00 kcal/mol).

The affinity between flavonoids and targeted protein was much stronger compared with other types of compounds. This may be because the abundant phenolic hydroxyl group in flavonoids, especially the hydroxyl group in the sugar group of flavonoids, bind more easily with the heteroatoms of amino acids from Mpro (Figure 3). Rutin forms multiple hydrogen bonds with the main chain of residues like Phe-140, Glu-166, Thr-26, Leu-141, Ser-144, Cys-145 and His-163. In particular, Asn-142 and Gln-189 were thought to contribute to the hydrophobic interactions with rutin (Figure 3).

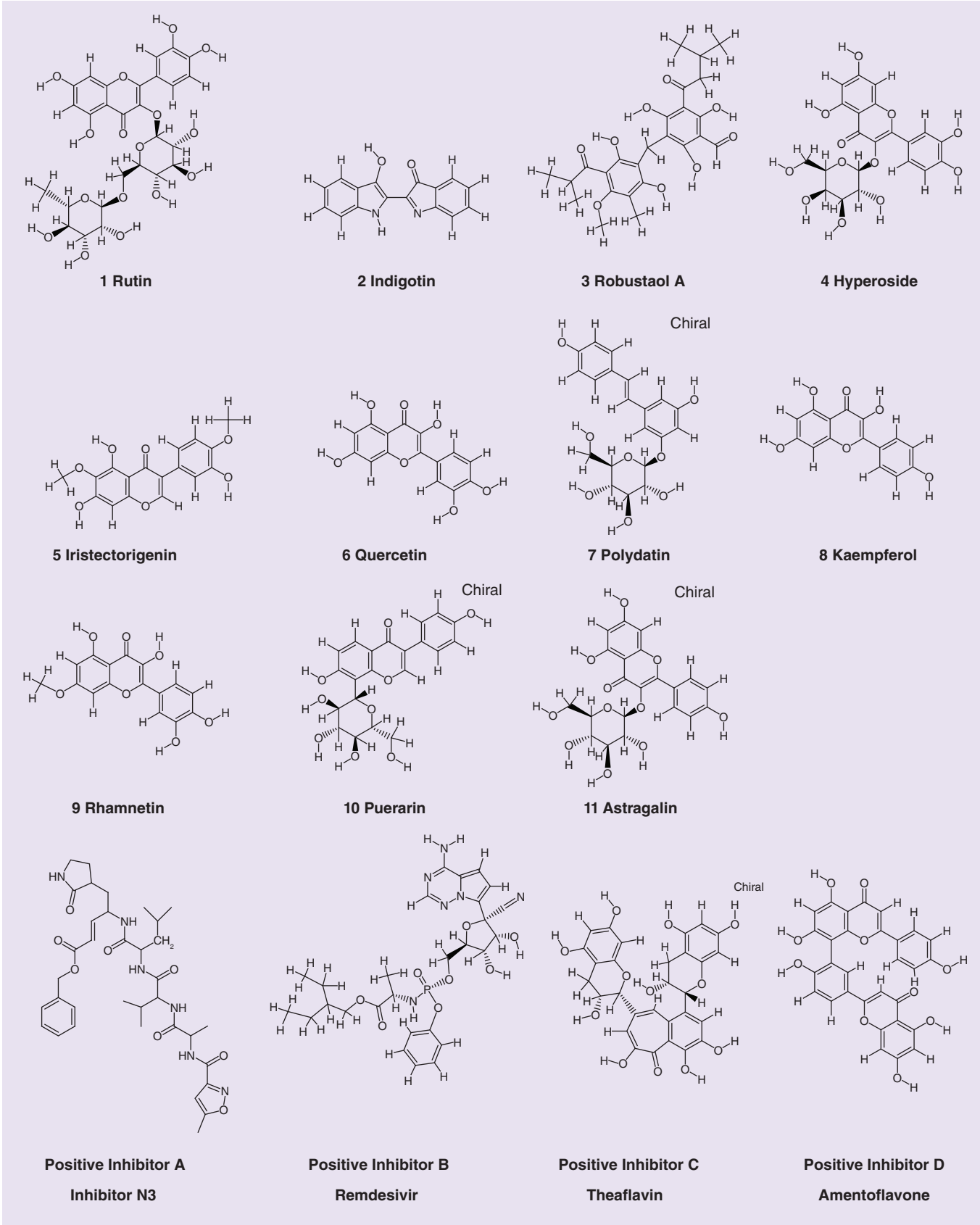

Figure 1. The chemical structure of representative compounds.

Table 1. Docking results of representative compounds toward SARS-coronavirus 2 main protease (Mpro).

Active Order	Compound	Molecular weight	Binding energy (kcal/mol)	Original plant	Ref.
1	Rutin	610	-8.67	*Forsythia suspense* (Thunb.) Vahl. *Houttuynia cordata* Thunb. *Prunella vulgaris* Linn. *Morus alba* L.	
2	Indigotin	262	-6.99	*Polygonum tinctorium* Ait. *Isatisin digotica* Fort.	
3	Robustaol A	474	-6.85	*Eucalyptus robusta* Smith.	
4	Hyperoside	464	-6.82	*Prunella vulgaris* Linn.	
5	Iristectorigenin	330	-6.8	*Belamcanda chinensis* (L.) DC.	
6	quercetin	302	-6.78	*Houttuynia cordata* Thunb. *Astragalus membranaceus* (Fisch.) *Pyrrosia lingua*(Thunb.)Farw. *Polygonum porfoliatum* L. *Patrinia villosa* (Thunb.) *Lonicera japonica* Thunb.	
7	Polydatin	390	-6.74	*Polygonum cuspidatum* Sieb. Et Zucc.	
8	Kaempferol	286	-6.68	*Polygonum tinctorium* Ait.	
9	Rhamnetin	316	-6.65	*Coptis chinensis* Franch.	
10	Puerarin	416	-6.63	*Pueraria lobata* Ohwi	
11	Astragalin	448	-6.51	Glycyrrhiza uralensis Fisch.	
Positve Inhibitor A	Inhibitor N3	680	-7.05	—	[10]
Positve Inhibitor B	Remdesivir	603	-9.00	—	[14]
Positve Inhibitor C	Theaflavin	564	-6.21	—	[15]
Positve Inhibitor D	Amentoflavone	538	-6.06	*Forsythia suspensa* (Thunb.) Vahl.	[16]

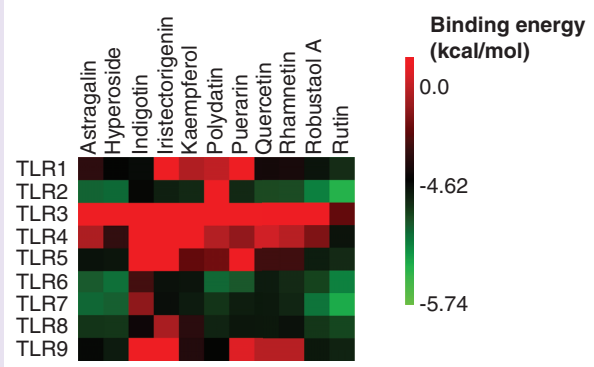

Figure 2. The hot map of docking between representative compounds and toll-like receptors. The greener square represents lower binding energy between TLR and compounds, indicating the potential interactions. In contrast, the red square means the interactions between molecules and targets are extremely impossible.
TLR: Toll-like receptor.

Docking between 11 selected compounds & TLRs

TLRs play an important role in mediating the inflammatory response and host-based anti-CoV activity. The pocket site of TLR2, TLR6 and TLR7 presented potential combinations between rutin with binding energies of <-8 kcal/mol (Table 2, Figure 2). TLRs generally stimulate pro-inflammatory and antiviral host pathways. These potential bindings indicate two possible activities: antagonistic or stimulatory. For patients with COVID-19, this may provide a dual benefit, both preventing over-inflammation and restoring innate antiviral immunity [3].

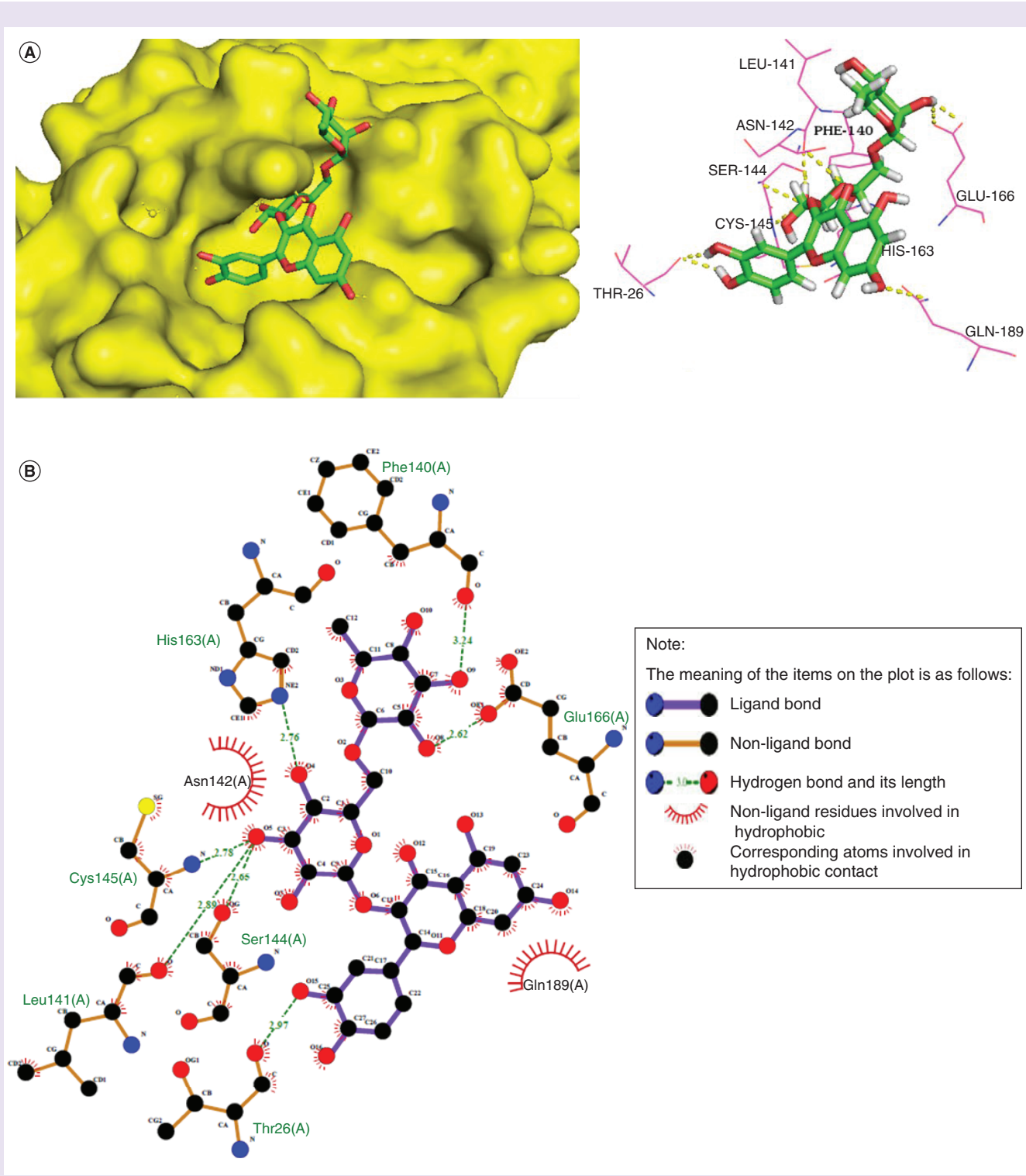

Figure 3. The docking model between rutin and SARS-coronavirus 2 main protease (Mpro) is exhibited as 3D interaction diagram through the LeDock server. The yellow dash lines represented potential interactions between the amino acid residues of Mpro and rutin. The name of binding amino acid residues are labeled with abbreviations.

Table 2. Docking result of representative compounds toward toll-like receptors.

Binding energy (kcal/mol)	Astragalin	Hyperoside	Indigotin	Iristectorigenin	Kaempferol	Polydatin	Puerarin	Quercetin	Rhamnetin	Robustaol A	Rutin
TLR1	-5.51	-5.74	-5.89	-4.55	-4.95	-4.89	-4.51	-5.65	-5.63	-6.11	-6.79
TLR2	-7.99	-8.12	-5.83	-6.45	-6.65	0	-6.65	-7.42	-7.43	-8.63	-9.76
TLR3	-4.26	-4.4	-3.37	-3.9	-3.54	-4.62	-3.84	-4.34	-4.43	-4.48	-5.29
TLR4	-4.97	-5.49	-3.6	-4.43	-4.1	-4.94	-5.09	-4.82	-4.92	-5.17	-6.1
TLR5	-6.07	-6.13	-4.58	-4.58	-5.29	-5.23	-4.67	-5.42	-5.43	-6.34	-6.72
TLR6	-7.79	-8.28	-5.41	-6.04	-6.11	-8.1	-7.71	-6.29	-6.7	-7.28	-8.66
TLR7	-8.09	-7.88	-5.1	-5.95	-6.29	-6.94	-6.37	-6.24	-6.55	-8.33	-9.58
TLR8	-6.93	-6.96	-5.68	-4.99	-5.53	-6.51	-6.18	-6.2	-6.02	-7	-7.31
TLR9	-5.84	-6.26	-3.94	-4.37	-5.57	-5.74	-4.76	-4.93	-4.93	-6.21	-6.51

TLR: Toll-like receptor.

GO & KEGG enrichment analysis of potential targets toward rutin

The Swiss Target Prediction yielded more than 100 target genes for rutin. GO annotation output was classified into three enrichment branches: biological process (BP), cellular component and molecular function (Figure 4). Carbonate dehydratase and protein kinase C activity were of greater significance in rutin mediating BP. As for cellular component, the rutin-predicted target mainly participated in the cytosol and troponin complex. The one-carbon metabolic process and peptidyl-serine phosphorylation were thought to be closer interrelated with rutin-predicted targets during molecular function.

The KEGG pathway showed potential rutin targets in pathways such as nitrogen metabolism, proteoglycans in cancer, Rap1 signaling and VEGF signaling (Figure 5). These pathways are closely related with lung inflammation, suggesting that the application of rutin may exert suppression of inflammation during CoV infection [17].

Discussion

Virally induced pneumonia has been associated with the secretion of pro-inflammatory cytokines. Cytokine storms are thought to be the main cause of progressive respiratory failure via induction of inflammatory cell infiltration and alveolar damage [18]. Pro-inflammatory cytokines IL-1 and IL-6 are believed to play catalytic roles in viral inflammation [19]. Recent studies have shown the potential of therapeutic anti-inflammatory cytokines, including IL-37 or IL-38, to demonstrate immunosuppressive activity and alleviate lung inflammation, fever and fibrosis [20], suggesting the possibility that viral inflammation may be inhibited by anti-inflammatory cytokines. Cytokine signaling is highly associated with the activation of TLRs [21]. A series of studies reveal that blocking TLR signaling also prevents cytokine storms, indicating a potential therapeutic target for SARS-CoV-2-induced inflammation. Interestingly, men seem to be more vulnerable than women to SARS-CoV-2 infection due to the differences in immune responses to innate immunity. Triggering TLR7 to produce interferon appears to occur more readily in women than in men [22].

The antiviral properties of natural compounds via regulation of the innate antiviral response provides a promising therapy for the clinical treatment of SARS-CoV-2 infection. Mpro, a coronavirus main protease, is a critical enzyme mediating the production of CoV functional proteins [23,24]. Recently, a high-resolution crystal structure of Mpro was identified, making it an attractive target for drug discovery [10]. This enabled us to use LeDock to determine the binding capacity between SARS-CoV-2 Mpro and the 109 compounds previously identified in natural products.

The potential immunomodulatory effects of 11 of these compounds were determined via docking with TLR1 through TLR9 (Table 2). TLRs are pattern recognition receptors that recognize pathogen-associated molecular patterns [21]. When TLRs recognize an exogenous ligand, the innate immune response is activated and begins activation of the adaptive response, causing antiviral immunity or even excessive inflammatory response. In this study, we discovered that rutin not only binds tightly to Mpro, but also acts as a regulator of TLR2, TLR6 and TLR7 (Figure 2).

In terms of the source of rutin, traditional Chinese medicines such as *Forsythia suspense*, *Houttuynia cordata*, *Prunella vulgaris* or *Morus alba*, possess rutin as an active constituent (Table 1). Apart from traditional Chinese medicines, tea leaves and apples also contain ample rutin [25]. *Fagopyrum* species such as buckwheat are the richest source of the flavonoid rutin [26]. Rutin, also known as vitamin P, has been widely used as an antioxidant in the food

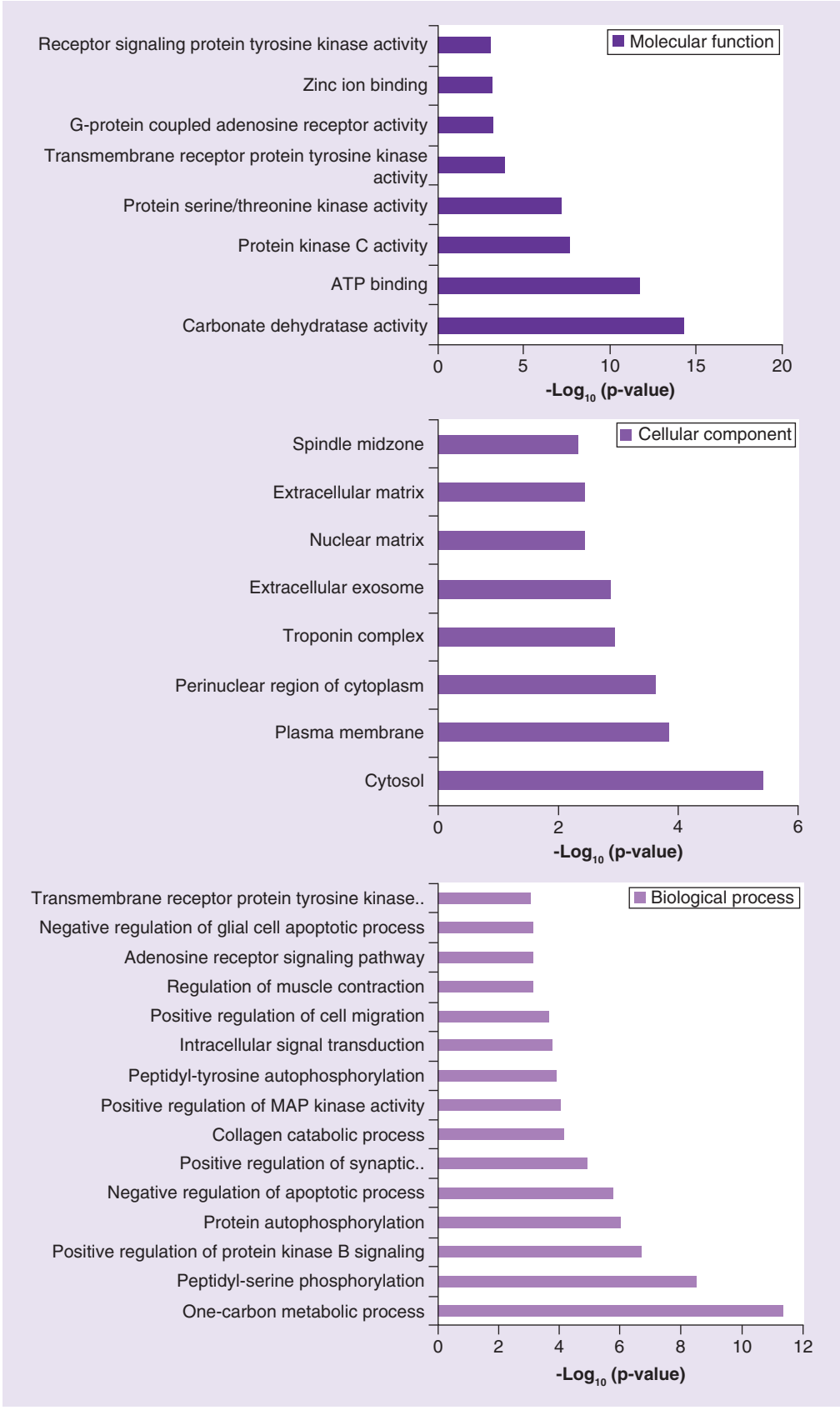

Figure 4. Gene ontology enrichment analysis of the targets toward rutin. In term of molecular function, the predicted targets mainly participate into the carbonate dehydratase activity. As for cellular component, the predicted targets mainly occurred in cytosol. During biological process, one-carbon metabolic is thought to be the major process.

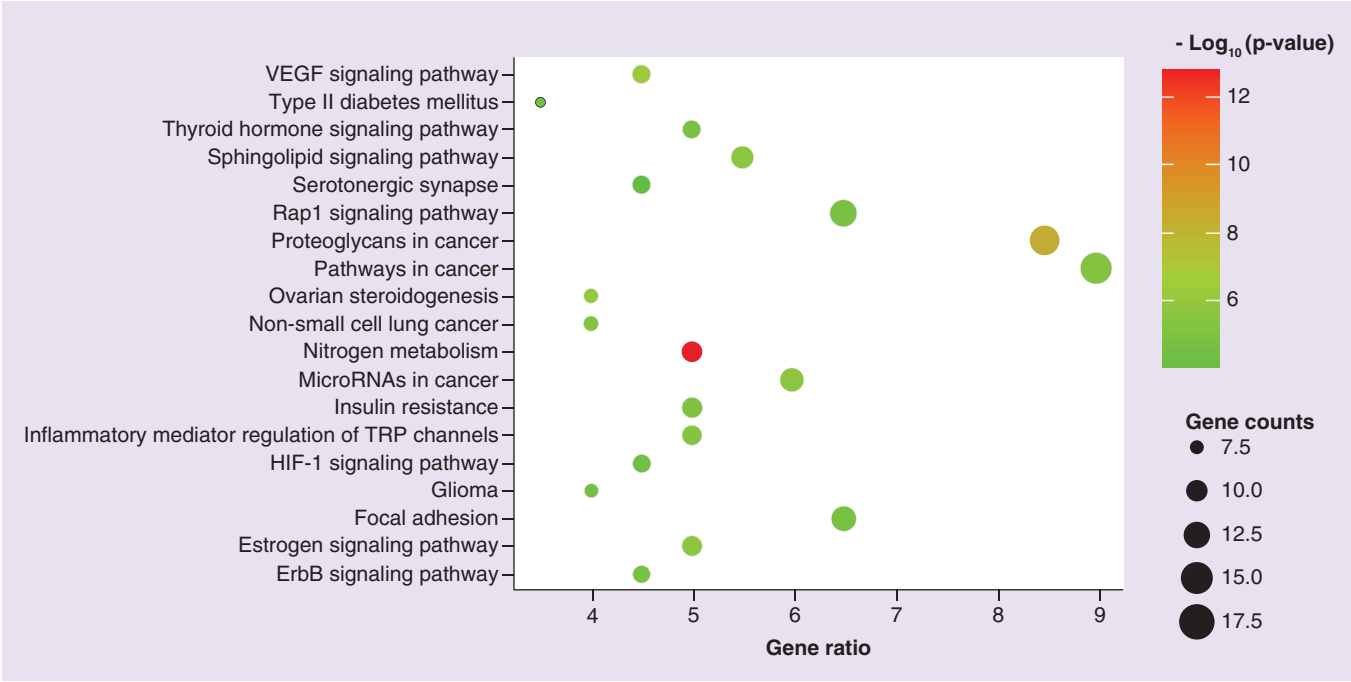

Figure 5. Analysis of Kyoto Encyclopedia of Genes and Genome enrichment in related pathways as targets of rutin. The diameter of the circle represented the accounts of rutin target gene. The deeper shadow of orange represents the greater difference in significance. Rutin-related target genes (CA14, CA9, CA13, CA7, CA12, CA6, CA4, CA3, CA2 and CA1) were assigned to nitrogen metabolism signaling pathway with significant differences.

processing industry. Therefore, it would be easy to ingest rutin in daily meals. In addition, many supplementary complex vitamins contain rutin [27]. Therefore, it would be beneficial for our body to ingest complex vitamins, especially those containing rutin, during the outbreak of COVID-19.

The possible involvement of cellular BPs and pathways of rutin were briefly discussed based on bioinformatics analysis. We found that the one-carbon metabolic process ranked high as a potential target of rutin (Figure 4). A previous study showed that carbohydrates may serve as receptor determinants when SARS-CoV-2 attaches to host cells [28]. Therefore, the effect of rutin-related one-carbon metabolic processes deserves further research during viral infection. The functional annotation of GO and the enrichment analysis indicated that related pathways (nitrogen metabolism, proteoglycans in cancer, Rap1 signaling pathway, VEGF signaling pathway) may play critical roles in the anti-inflammatory response with rutin (Figure 5). Interestingly, nitric oxide (NO) had been reported to inhibit the SARS-CoV-2 viral RNA production [29]. Besides this, NO or its derivatives may also influence palmitoylation of the nascently expressed viral spike (S) protein, blocking the reorganization process of angiotensin converting enzyme 2. Thus, anti-CoV medicine may be developed by targeting NO-related enzyme proteins. However, the role of rutin in mediating nitrogen metabolism during SARS-CoV-2 infection requires further studies.

All of the data provided in this paper are based on pure bioinformatic analyses. Therefore, the results should not be applied clinically without further evaluation of the potential inhibitors via experimental confirmation *in vitro* and *in vivo*.

These docking results must be validated by a process first involving expression and purification of the SARS-CoV-2 main protease via recombinant gene expression *in vitro*. Then a tryptophan-based fluorescence method reported recently could be used to confirm the interaction between Mpro and rutin, or other potential inhibitors [30]. This process would be direct and simple and available in biosafety level 1 laboratories. Furthermore, biochemical and cell-based assays must be applied to evaluate the solubility, toxicity and pharmacodynamic properties of rutin toward SARS-CoV-2 Mpro. If the EC_{50}/IC_{50} are high at indicated dosages, the antiviral activity of rutin or other potential inhibitors would be further studied *in vivo*, especially in animal models that express human ACE2, the putative cellular receptor for SARS-CoV-2 [31]. To clarify the anti-inflammation mechanism of rutin toward SARS-CoV-2, knockout mice with deficiencies in T cells, B cells and/or natural killer (NK) cells could also be utilized [3]. To

date, however, evaluation of anti-CoV activity is only available in biosafety level 3 laboratories, where experiments are highly technically demanding.

Conclusion

Flavonoid compounds, particularly rutin, exhibited good characteristic of binding with SARS-CoV-2 Mpro and TLRs, indicating it as a novel therapeutic option via virus-based and host-based anti-CoV strategies.

Summary points

- 11 compounds (Table 1) with lower binding energy were identified as SARS-coronavirus 2 potential inhibitors.
- Rutin was highlighted not only because it fits snugly into the substrate-binding pocket of Mpro, but also because it presents a strong interaction with TLR2, TLR6 and TLR7.
- Gene ontology suggested that carbonate dehydratase and protein kinase C activity are of greater significance in rutin-mediating biological processes. The rutin-predicted target mainly participates in the troponin complex of the cellular component category. One-carbon metabolic process and peptidyl-serine phosphorylation are more closely interrelated with rutin in the molecular function category.
- Kyoto Encyclopedia of Genes and Genome pathway analysis showed that rutin exerts anti-inflammatory activity via nitrogen metabolism.

Supplementary data

To view the supplementary data that accompany this paper please visit the journal website at: www.futuremedicine.com/doi/suppl/10.2217/fvl-2020-0099

Author contributions

X Hu implemented the virtual experiments and preparing original draft. Z He conceived the idea. X Song designed experiments and provided the training of docking. C Li, Q Zhang and IO Ekumi revised the English writing of the manuscript, X Cai, J Zhao and W Luo collected and analyzed the data.

Financial & competing interests disclosure

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No writing assistance was utilized in the production of this manuscript.

Data availability statement

All data and materials are contained and described within the manuscript.

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Short Communication

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The divergence between SARS-CoV-2 and RaTG13 might be overestimated due to the extensive RNA modification

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Aim: Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) has spread throughout the world. There is urgent need to understand the phylogeny, divergence and origin of SARS-CoV-2. **Materials & methods:** A recent study claimed that there was 17% divergence between SARS-CoV-2 and RaTG13 (a SARS-related coronaviruses) on synonymous sites by using sequence alignment. We re-analyzed the sequences of the two coronaviruses with the same methodology. **Results:** We found that 87% of the synonymous substitutions between the two coronaviruses could be potentially explained by the RNA modification system in hosts, with 65% contributed by deamination on cytidines (C-T mismatches) and 22% contributed by deamination on adenosines (A-G mismatches). **Conclusion:** Our results demonstrate that the divergence between SARS-CoV-2 and RaTG13 has been overestimated.

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Keywords: divergence • overestimate • RaTG13 • RNA modification • SARS-CoV-2

The spread of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) needs to be controlled [1–3], and meanwhile its outbreak provides an opportunity for evolutionary biologists to investigate the viruses from the angle of evolution. The ultimate ambition might be finding out the origin and evolving patterns of SARS-CoV-2.

With or without much knowledge of virology, the evolutionary formula or algorithms could be easily applied to the virus sequences by using software or manual calculation. A previous study focusing on the origin and continuous evolution of SARS-CoV-2 (Tang *et al.* 2020 [4]) has an interesting finding that the synonymous substitution rate (dS) between SARS-CoV-2 and RaTG13 (one of the bat SARS-related coronaviruses) is 17%, which is 14-times the divergence between human and chimpanzee. This divergence as high as 17% is much greater than the estimation of earlier studies. The authors commented that the difference between SARS-CoV-2 and RaTG13 has been underestimated by earlier papers.

The authors' opinion is that only the silent mutations should be used to calculate the divergence between SARS-CoV-2 and RaTG13, because these neutral sites are not affected by selection forces. By using the formula dS = 2ut, where dS represents substitution rate and u is the mutation rate, one could estimate the divergent time (t) between the two species.

Despite the terminology 'mutation' widely being used by evolutionary biologists, in many cases 'mutation' has been used in broad-sense, which represents all kinds of mismatches observed in the sequence alignment, no matter these mismatches are caused by natural mutation (such as replication errors) or other factors.

The cellular organisms have multiple RNA modification systems, which could modify any types of RNAs in the cell. Since SARS-CoV-2 and RaTG13 are coronaviruses (RNA viruses), when they infect the human cell, the RNA modification enzymes might act on the viral RNAs as they usually do to the host RNAs. Modified viral RNAs such as the methylated adenosines have been commonly observed [5–7]. Apart from the minor decorations such as methylation, two major deamination enzymes, ADAR [8] and APOBEC [9,10], are responsible for adenosine-to-inosine deamination and cytidine-to-uracil deamination, leading to an observed A-to-G and C-to-T change in the sequencing results. No matter which of SARS-CoV-2 and RaTG13 is modified, it will produce an A-G

Table 1. The length and aligned length of each ORF of SARS-CoV-2.

ORF ID	Length (amino acids)	Aligned length in RaTG13
E	75	75
M	222	222
N	419	419
ORF10	38	38
ORF1AB	7095	7093
ORF3A	275	275
ORF6	61	61
ORF7A	121	121
ORF7B	43	43
ORF8	121	121
S	1273	1273

or C-T mismatch in the alignment between two viruses. In mammals, ADAR is required to fight against the infected hepatitis C virus (HCV) [11–13]. Similar to coronavirus, the HCV is a positive-strand RNA virus, and the case of ADAR acting on HCV means that the deamination on viral RNAs (thus inducing mismatches against the reference sequence) is prevalent. In other invertebrate organisms, the mismatches induced from ADAR deamination is observed in sigma virus, a negative sense RNA virus [14–16]. Evidence shows that the ADAR-modified viral RNAs are not rapidly degraded so that the 'offspring' of the deaminated RNA would permanently carry this mutation [11].

In the dS calculation, any observed mismatches in the sequence alignment are regarded as mutations. Of course, the software would not automatically tell the users whether a mismatch is a natural mutation caused by replication error or an RNA modification site.

However, for DNA organisms like humans, the classic definition of mutation rate should mainly (perhaps not absolutely) refer to the replication error rate of DNA. For SARS-CoV-2, the mutation rate should mainly refer to the RNA replication error rate. Accordingly, the calculation of dS should only include the natural mutations introduced during RNA replication rather than the RNA-to-RNA mismatch sites caused by RNA modification system. The replication error rate should be very low while the occurrence of RNA modification could appear in any virus RNA which is exposed to the host's deamination enzymes. The RNA modification rate could be higher than RNA replication rate for orders of magnitude. The phenomenon that the viral RNAs or even proteins are modified by host cells is not rare at all [13,17] so that this issue should be considered when studying the divergence of RNA viruses.

Our idea is that when checking the sequence alignment between SARS-CoV-2 and RaTG13, if one found that plenty of the synonymous substitutions could be potentially explained by C-to-T deamination or A-to-G deamination then the actual divergence between SARS-CoV-2 and RaTG13 might have been overestimated by many times. We re-emphasize that we only say the C-T and A-G mismatches could be potentially explained by RNA modification but not definitely caused by RNA modification. The aim is to rationally estimate the real divergence between the two RNA viruses.

Materials & methods

We downloaded the sequences of SARS-CoV-2 and RaTG13 from GeneBank and aligned the coding sequences with MUSCLE [13]. The 11 nonredundant ORFs are annotated with names (such as *M*, *N*, *ORF1AB*) so that we put the two ortholog genes into a file and run the sequence alignment. The length of each ORF (number of amino acids) and the aligned length of each ORF are given in Table 1. For example, we put the two sequences of SARS-CoV-2 ORF10 and RaTG13 ORF10 into one file and run MUSCLE with default parameter. Then the output file would give us the aligned sequences of these two ORFs. From Table 1, we could see that the ORFs in two virus species are almost of the same length so that the parameters hardly affect the alignment results. We manually extract each codon in the alignment file using our own python script. The unaligned regions are gaps. As shown in Table 1, only ORF1AB have two triplets (codons) unaligned, and the other regions and other ORFs are well aligned. Next, most of the aligned regions are identical. The nonidentical regions are either missense or synonymous mutations. To help readers understand the process of extracting mutations (mismatches) from the alignment, we listed the first ten missense and synonymous mutations of ORF1AB in Tables 2 & 3, respectively.

Table 2. The first ten missense mutations in ORF1AB.

Position	SARS-CoV-2	RaTG13	Amino acid (SARS-CoV-2)	Amino acid (RaTG13)	Mismatch (nondirectional)
38	GTC	GCT	Val	Ala	C-T
110	CAT	TAT	His	Tyr	C-T
114	ATA	ACA	Ile	Thr	C-T
117	GCT	GTT	Ala	Val	C-T
172	GAA	GAT	Glu	Asp	A-T
280	ATA	ACA	Ile	Thr	C-T
376	TCA	CCA	Ser	Pro	C-T
395	ACC	CCC	Thr	Pro	A-C
417	CAT	TAC	His	Tyr	C-T
424	GTT	ATT	Val	Ile	A-G

Table 3. The first ten synonymous mutations in ORF1AB.

Position	SARS-CoV-2	RaTG13	Amino acid (SARS-CoV-2)	Amino acid (RaTG13)	Mismatch (nondirectional)
20	GTT	GTC	Val	Val	C-T
59	GGC	GGT	Gly	Gly	C-T
74	TCG	TCT	Ser	Ser	G-T
82	GGT	GGC	Gly	Gly	C-T
92	CTC	CTT	Leu	Leu	C-T
97	TAC	TAT	Tyr	Tyr	C-T
104	CTT	CTC	Leu	Leu	C-T
138	GCC	GCT	Ala	Ala	C-T
142	TCA	TCG	Ser	Ser	A-G
169	GTT	GTC	Val	Val	C-T

From Tables 2 & 3, we already see prevalent C-T mismatches.

It is possible that sometimes the mutation may be lethal, producing shortened protein if TAA is produced instead of CAA. We scanned the 11 nonredundant ORFs in SARS-CoV-2 and RaTG13. We did not find any internal stop codons in these ORFs.

For the multiple alignment incorporating other virus species ZXC21, ZC45 and BM48-31, we aligned the ORFs with the same method. Together with SARS-CoV-2 and RaTG13, we put the orthologous ORF of the five species into one file and run MUSCLE. The output alignment file was manually inspected. Each codon located in the ORFs were simply extracted by our own python scripts. The results of aligning SARS-CoV-2 and RaTG13 and the results calculated from aligning five species were compared. The relative alignment and mismatch profiles between SARS-CoV-2 and RaTG13 were found to be identical under two sets of strategies.

The ID of SARS-CoV-2 is NC_045512. The link of SARS-CoV-2 ORF1AB (coding sequence) is: https://www.ncbi.nlm.nih.gov/nuccore/NC_045512.2?from=266&to=21555&report=fasta

The ID of RaTG13 is MN996532. The link of RaTG13 genome is: https://www.ncbi.nlm.nih.gov/nuccore/MN996532.1/?report=fasta

The beginning of SARS-CoV-2 ORF1AB is 'ATG|GAG|AGC|CTT|GTC', the end of SARS-CoV-2 ORF1AB is 'GAT|GTT|CTT|GTT|AAC|AAC|TAA'. By manually searching 'ATGGAGAGCCTTGTC' and 'GATGTTCTTGTTAACAACTAA' in the RaTG13 genome sequence, we can anchor and extract the ORF1AB in the RaTG13 genome. The ORF1AB CDS alignment between SARS-CoV-2 and RaTG13 is provided in Supplementary Table 1. As we can see, most codons are identical. The nonidentical codons mostly have synonymous mutations.

Results

Substitutions between SARS-CoV-2 & RaTG13

We aligned the ORFs of SARS-CoV-2 and RaTG13 and manually extracted the codons in the alignment file (see Materials and methods). The statistics of the alignment results (Table 1) show that most of the ORFs are well

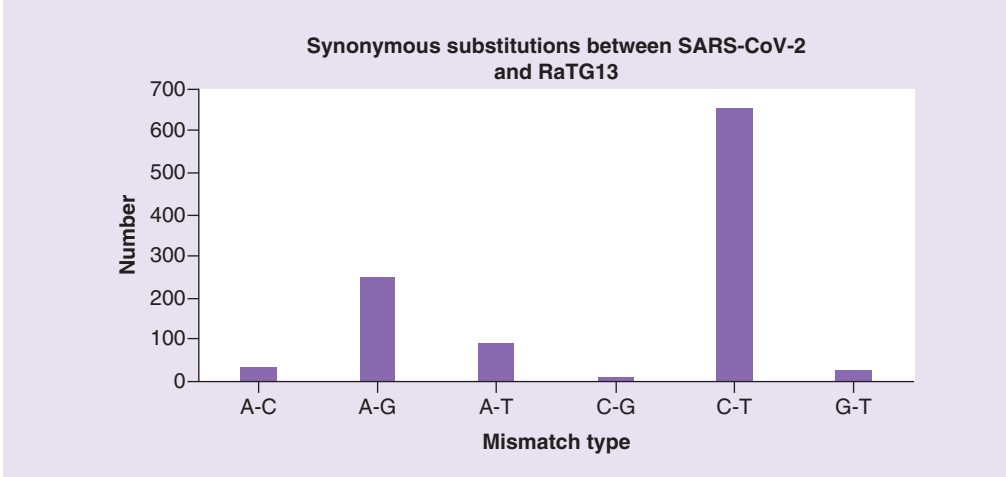

Figure 1. The numbers of mismatch types on synonymous substitution sites between SARS-CoV-2 and RaTG13.

aligned and only ORF1AB has two gaps. From the 9.7 thousand codons in the ORFs, we totally obtained 1076 nonidentical codon positions between SARS-CoV-2 and RaTG13, 931 of which encode the same amino acid (synonymous) and 145 of which encode different amino acids (missense). That is to say, there are 931 synonymous substitutions and 145 missense substitutions between SARS-CoV-2 and RaTG13. The other ORF regions (90%) are identical between SARS-CoV-2 and RaTG13.

Among the 9.7 thousand codons in the 11 nonredundant SARS-CoV-2 ORFs, the content of C and T is 51.2%. However, among the 931 codons with synonymous substitutions, the content of C and T is 56.1%, and the difference is significant using Chi-square test (p = 5.7E-3). It proves that the occurrence of synonymous substitutions is nonrandom and it tends to take place on codons containing C or T.

87% of the synonymous substitutions are C-T or A-G mismatches

We checked the 1076 substitution sites between SARS-CoV-2 and RaTG13, 84.4% of the mutations are A-G or C-T mismatches (61.0% C-T mismatches and 23.4% A-G mismatches). Among the 931 synonymous substitution sites (Figure 1), 86.7% of them are A-G or C-T mismatches (64.9% C-T mismatches and 21.8% A-G mismatches). This mismatch spectrum resembles the enrichment of C-to-T(U) deamination and A-to-G(I) deamination. Nearly 87% of the observed synonymous 'mutations' between SARS-CoV-2 and RaTG13 could be potentially explained by the RNA modification systems in host cells.

To help readers understand how the mismatches were extracted from the alignment file, we listed the first ten missense and synonymous mutations in ORF1AB, respectively (Tables 2 & 3). We have said that 90% of the aligned regions is identical and the nonidentical codons usually differ with a single nucleotide. In Table 2, seven out of the ten missense substitutions were C-T mismatches. In Table 3, eight out of the ten synonymous substitutions were C-T mismatches. Given the high similarity of the SARS-CoV-2 and RaTG13 sequences, these mismatches may not be caused by mis-alignment.

One may also be concerned whether the alignment and mismatch profile is different when using multiple virus species to run the alignment. We downloaded the ORFs of other SARS-related coronaviruses ZXC21, ZC45, and BM48-31 (see Materials and methods). We found that using multiple species does not affect the aligned regions between SARS-CoV-2 and RaTG13. Although additional gaps are introduced in the alignment, the relative position between SARS-CoV-2 and RaTG13 remains the same. So, the mismatches between SARS-CoV-2 and RaTG13 are not affected by different alignment strategies. The prevalence of C-T and A-G mismatches is robust.

Mismatch profile excluding the protease digestion sites in ORF1AB

The ORF1AB (pp1AB) would be cleaved into multiple proteins (nsp1-16) by protease. The cleavage sites are LQS and LQA sequences [18,19]. We could not simply call ORF1AB as one gene, so it is rational to exclude the mutations in digestion sites in the divergence analyses. We checked the mutations in the LQS and LQA regions. We only found one case. Amino acids 4252-4254 is Leu-Gln-Ala, and the Leu codon is CTA in SARS-CoV-2 and TTA in

Table 4. dS values and the fold of overestimation.

ORF	dS (Tang *et al.*)	C-T mismatch	A-G mismatch	Explained by modification (upper bound)	Fold of overestimation of dS (upper bound)
All	0.17	65%	22%	87%	7.7
ORF1AB	0.152	67%	22%	89%	9.2
S	0.321	59%	19%	78%	4.5
Other	Not provided	64%	27%	91%	10.9

RaTG13. This single C-T mismatch in digestion regions does not affect the overall mismatch profile. This also proves that the amino acid sequences of digestion sites might be highly conserved to avoid the loss of protease recognition. Again, our finding of prevalent C-T and A-G mismatches is robust.

ORF1AB & S contribute most of the mismatches

It is necessary to provide the influence of the tested number of genes on the estimated divergence. As seen in Table 1, ORF1AB and S are the longest ORFs. They contribute most of the mismatches if we look at the mismatch profile in all the ORFs. Here we list the dS values calculated by Tang *et al.* [4] and the percent of mismatches potentially explained by RNA modification (Table 4). ORF1AB, S, and the other ORFs are listed separately. Clearly, the choice of tested genes does not severely affect the pattern. In all genes, 87% mutations could be (potentially) explained by modified RNA. In ORF1AB, 89% mutations could be (potentially) explained by RNA modification. In S, 78% mutations could be (potentially) explained by modified RNA. In the remaining ORFs, 91% mutations could be (potentially) explained by modified RNA. Presume that 91% of the mismatches are caused by RNA modification, then the dS value is overestimated for more than tenfolds. The S ORF has a pretty high dS value, so it is especially necessary to question if the modification system contributes to the divergence.

Discussion

One argument is that in the alignment between SARS-CoV-2 and RaTG13 we did not use an outgroup species so that the direction of the mutation is uncertain. Yes, that is true. We do not worry about the ancestral state. SARS-CoV-2 and RaTG13 are RNA viruses. As long as we observe a C-T or A-G mismatch in the sequence alignment between them, we could speculate that the C-to-T or A-to-G deamination might have occurred in one of the two virus species.

Note that we only say 87% of the mutations could be potentially explained by RNA modification, rather than 87% of them are definitely caused by RNA modification. From the sequence alignment alone, it is impossible to know whether the mismatch is a '*de novo*' mutation or an RNA modification site. The software would not tell users what has caused this mismatch since it is technically indistinguishable. Improving the parameters only makes alignment more accurate but does not tell us the origin of the mismatch.

As understood by common researchers, the definition of dS between RNA viruses mainly (but not absolutely) refers to the natural mutations introduced by RNA replication error rather than the RNA modification sites caused by host cells. The RNA modification rate is many times higher than the replication error rate. This fact is consistent with our notion that the divergence between RNA viruses is overestimated.

According to our results, potentially 87% of the synonymous substitutions between SARS-CoV-2 and RaTG13 could be caused by RNA modification system in hosts. The remaining 13% of the substitutions should be genuine interspecific mutations as they could not be explained by known RNA modification types. The claimed dS = 0.17 should have been overestimated. The upper bound of overestimation is 1/0.13 = 7.7-times so that the lower bound of the dS value is 0.17/7.7 = 0.022.

Indeed, if the authors argue that the definition of dS itself already included any mutation types such as those RNA modification sites then the dS value of 17% would be valid. However, this definition of dS is not what we commonly understand, and the authors should have pointed this out in their article. Again, adjusting the parameters of any software only makes the alignment more accurate but is not helpful in determining whether the observed mismatches are modified RNA or the natural mutation introduced during RNA replication. A rational way to avoid a wrong and misleading conclusion is to calculate the upper bound and lower bound of the divergence value. Anyway, the currently proposed divergence (dS = 17%) between SARS-CoV-2 and RaTG13 has been severely

overestimated. We appeal that when calculating dN and dS between RNA viruses, the RNA modification should be taken into account.

The limitation of our study is that we were currently unable to provide experimental evidence for the modification on viral RNAs although this phenomenon is not new for virologists. At the same time, neither did Tang *et al.* [4] provide evidence to prove that the mismatches in the alignment are not caused by RNA modification. Since both sides lack experimental evidence, it is reasonable to think about this dilemma from the angle of maximum likelihood. That is, if the mismatch sites between SARS-CoV-2 and RaTG13 are really introduced by accumulation of RNA replication errors, they should not exhibit an excessive number of C-T and A-G mismatches (in that case the mutation types should be random).

Another limitation of our work is that we did not give an estimation of the real divergence value. As we have stated, the RNA modifications and normal mutation sites are technically indistinguishable. We only say that the proposed 17% divergence is higher than the real value but we still do not know what the real value is. Promisingly, experts in mutations could estimate the relative abundance of each type of mismatches and give a reasonable value of the divergence between SARS-CoV-2 and RaTG13.

Conclusion

Since we found 87% of the synonymous substitution sites between SARS-CoV-2 and RaTG13 could be potentially explained by RNA modification system in host cells, we are strongly concerned that the previously defined divergence between SARS-CoV-2 and RaTG13 has been overestimated.

Summary points

- The outbreak of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) has caused severe damage to the world.
- It is necessary to understand the origin and evolution patterns of SARS-CoV-2.
- A previous study claimed that SARS-CoV-2 and RaTG13 have 17% divergence on synonymous sites.
- We aligned the coding sequences of SARS-CoV-2 and RaTG13, and checked the substitution sites between them.
- The substitution sites are CT-enriched compared with background.
- Potentially 87% of the synonymous substitutions between SARS-CoV-2 and RaTG13 could be explained by RNA modification system in hosts.
- The divergence between SARS-CoV-2 and RaTG13 has been overestimated.
- The calculation of dN or dS between RNA viruses should take the RNA modification into consideration.

Supplementary data

To view the supplementary data that accompany this paper please visit the journal website at: www.futuremedicine.com/doi/suppl/10.2217/fvl-2020-0066

Author contributions

The corresponding author designed and supervised this research. All authors contributed to writing this article.

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Special Report

Future VIROLOGY

Transmission and clinical characteristics of asymptomatic patients with SARS-CoV-2 infection

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The 2019 novel coronavirus disease, SARS-CoV-2, is now spreading globally and is characterized by person-to-person transmission. However, it has recently been found that individuals infected with SARS-CoV-2 can be asymptomatic, and simultaneously a source of infection in others. The viral load detected in nasopharyngeal swabs of asymptomatic carriers is relatively high, with a great potential for transmission. More attention should be paid to the insidious spread of disease and harm contributed by asymptomatic SARS-CoV-2 carriers. To provide a theoretical basis for the accurate and early clinical identification of asymptomatic patients, this review objectively summarizes the epidemic status, transmission characteristics and clinical features of asymptomatic patients with SARS-CoV-2 infection.

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Keywords: asymptomatic infections • COVID-19 • epidemic • SARS-CoV-2

Background

COVID-19 caused by SARS-CoV-2, formerly 2019 novel coronavirus or 2019-nCoV [1], broke out in Wuhan, China in December 2019 [2]. Epidemics of COVID-19 are now occurring worldwide [3]. Since the first COVID-19 case in Wuhan was identified on 12 December 2019, in less than 4 months (1 April 2020), the number of cumulative confirmed cases in the world has exceeded 800,000 [4,5]. On 30 January 2020, the outbreak was declared an international public health emergency by the World Health Organization (WHO) [6].

SARS-CoV-2 is not the first coronavirus to threaten the life and welfare of humans. The years 2002 and 2012 saw the emergence of SARS-CoV and MERS-CoV [7]. Like SARS-CoV-2, the transmission of SARS-CoV or MERS-CoV is person-to-person [8–11], but they differ in crucial aspects. Most patients infected with SARS-CoV present with obvious clinical symptoms within a short period, the disease progresses rapidly and peak viral shedding occurs in the late stage [8]; few patients with SARS are asymptomatic [11]. MERS is primarily a zoonotic disease, and spread among humans was scattered and limited, the symptoms were obvious and infection rarely preceded symptom onset. Nosocomial transmission was more troublesome than community spread [8,12].

In contrast, onset of SARS-CoV-2 is insidious. When asymptomatic or the early symptoms are mild, patients can move freely and transmit the virus [13], with an incubation period that is long and infectious [14,15]. These characteristics allow for easy spread, and infection sources can be difficult to identify and isolate. In addition, the main routes of transmission are through respiratory droplets and contact [16], which is relatively easy to achieve [17].

At present, almost all countries in the world have recognized the seriousness of the COVID-19 pandemic and implemented various measures to curb its development, but asymptomatic patients are not always taken seriously by healthcare workers. Yet, asymptomatic infections of SARS-CoV-2 are probably an important source of transmission. The person with an asymptomatic confirmed case of infection has normal body temperature or is only slightly indisposed [18].

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These differences between COVID-19 and SARS or MERS require a change in epidemic response plan. Only by fully researching the various characteristics and mechanisms of asymptomatic infections can we lay a theoretical foundation for deployment of the next steps in its control. With that purpose, this review summarizes the epidemic status, transmission characteristics and clinical features of asymptomatic patients with SARS-CoV-2 infection.

Epidemic status & transmission characteristics of asymptomatic COVID-19 patients

Confirmed asymptomatic SARS-CoV-2 infections continue to increase

Multiple studies indicate that asymptomatic infections make up a large percentage of confirmed COVID-19 cases. A retrospective study in Beijing collected data for 262 individuals with diagnosed COVID-19 from 20 January to 10 February 2020, and 13 were asymptomatic (5.0%) [18]. In addition, 126 persons of German nationality were evacuated from Hubei Province to Frankfurt, Germany, on 1 February 2020. After strict screening and testing, two were confirmed to have SARS-CoV-2 infection, yet both patients were asymptomatic [19]. A further epidemiological investigation (28 January to 9 February 2020) was conducted in clinics and communities in Nanjing, Jiangsu Province, China. The survey screened the close contacts of patients with confirmed or suspected infections. The results of nucleic acid screening identified 24 confirmed SARS-CoV-2 carriers without any obvious symptoms. Of these, five patients developed typical symptoms during the subsequent hospitalization, while the other 19 patients remained asymptomatic [15]. Furthermore, the Ministry of Health, Labor and Welfare of Japan announced on 5 March 2020 that among 696 people on the 'Diamond Princess' cruise ship infected with SARS-CoV-2, 410 were asymptomatic [20]. All of the above indicates that in the community there may be a large number of unidentified asymptomatic people with contagious infections (Figure 1).

Asymptomatic patients with SARS-CoV-2 infection may carry high viral loads

SARS-CoV-2 has been detected in nasopharyngeal swabs and sputum samples from asymptomatic patients [11]. The viral load detected in asymptomatic individuals was similar to that of symptomatic patients suggesting that people without symptoms have a strong ability to transmit the virus to others [21]. In addition, SARS-CoV-2 has been detected in the blood and stool samples of seemingly well patients [22–24], and compared with the virus in respiratory secretions, the virus in feces may take longer to clear [25].

Complex incubation period in asymptomatic SARS-CoV-2 infection

In general, patients with symptoms of SARS-CoV-2 infection are admitted to hospital for detection and treatment under isolation. However, asymptomatic individuals may not be recognized by healthcare workers, and do not self-isolate or seek treatment. Bai *et al.* [14] showed that the incubation period of an asymptomatic patient was 19 days. What is more, Hu *et al.* [15] reported that the communicable period of asymptomatic COVID-19 patient may be as high as 29 days.

Transmission of SARS-CoV-2 by asymptomatic persons is implicated in crowd & family-clustered outbreaks

Multiple studies have found that there are asymptomatic SARS-CoV-2 infections in the process of crowds and family-clustered outbreaks. Among a family of six in Shenzhen who traveled to Wuhan from 29 December 2019 to 4 January 2020, five members were identified with COVID-19, including an asymptomatic 10-year-old boy [11]. A family of three who traveled on 22 January 2020 from Wuhan to Guangzhou, China, through the high-speed rail tested positive for SARS-CoV-2, but only one developed clinical symptoms, and the other two members had no signs or clinical symptoms [26]. Infants also are not spared from SARS-CoV-2 infection. The first pediatric case was confirmed asymptomatic in Singapore. The infant was part of a family transmission cluster, in which its parents and their live-in helper were symptomatic [22]. Furthermore, asymptomatic COVID-19 patients can even become the source of infection in contagious outbreaks among families. SARS-CoV-2 transmission from an asymptomatic infected person returning home from Wuhan on 10 January 2020 was suspected as the cause of a family cluster epidemic of five members in Anyang, China [14]. In fact, any infected person, symptomatic or asymptomatic, may be the first to transmit SARS-CoV-2 to other members in a clustered and family-clustered outbreak.

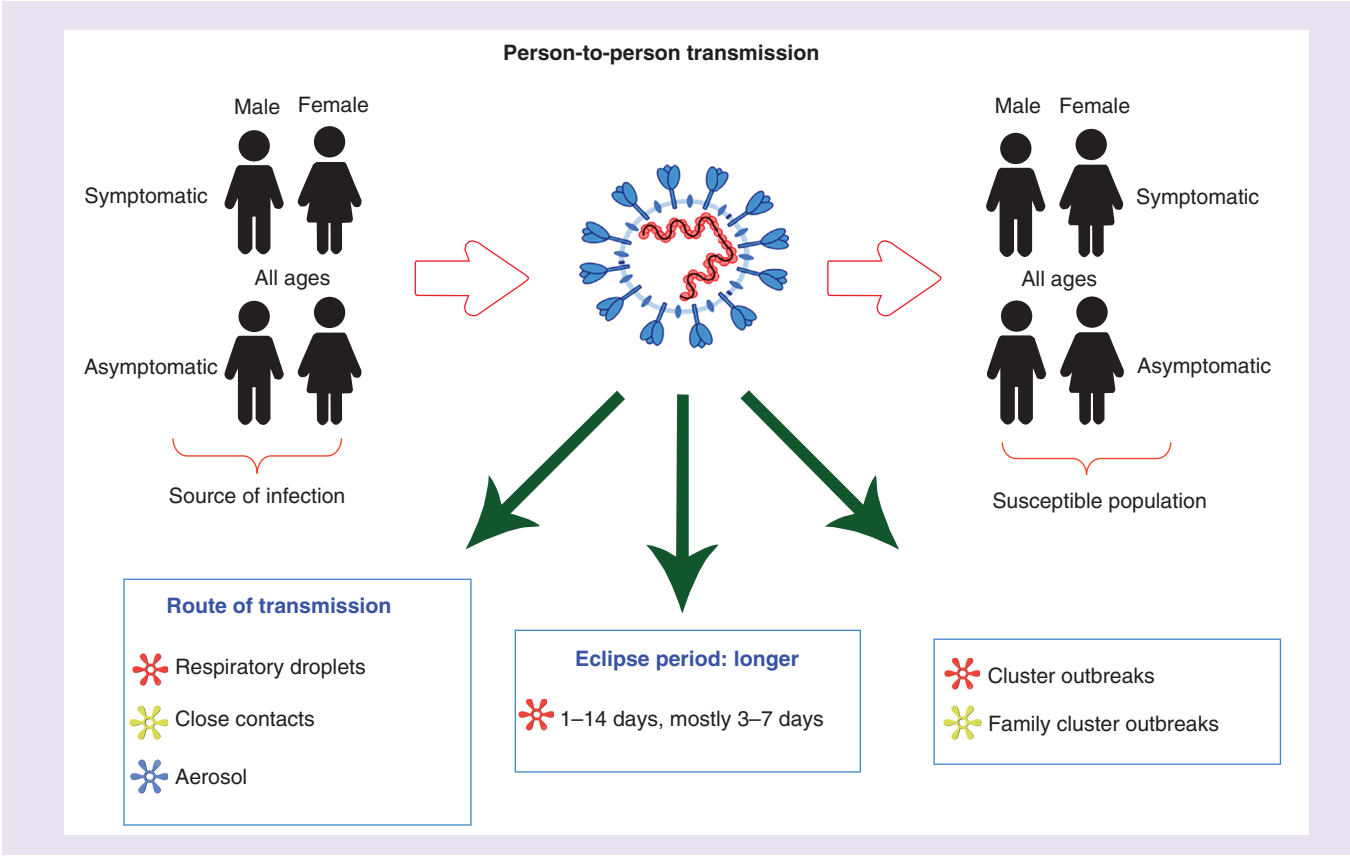

Figure 1. Transmission characteristics of SARS-CoV-2. The SARS-CoV-2 possesses the characteristics of person-to-person transmission. The source of infection and susceptible population exist in both the male and female of any age, and the disease performance is both symptomatic and asymptomatic. The SARS-CoV-2 is mainly transmitted through respiratory droplets and close contact; furthermore, there is a possibility of aerosol transmission when it is in a relatively closed environment and exposed to high concentrations of aerosol for a long time. The SARS-CoV-2 has an incubation period of 1–14 days, but mostly range 3–7 days. In addition, many studies have confirmed the existence of a large number of cluster outbreaks and family cluster outbreaks.

Clinical characteristics of asymptomatic patients with SARS-CoV-2 infection

Identification & diagnosis of asymptomatic SARS-CoV-2 infection

At present, cases of COVID-19 continue to occur around the world, so the rate of asymptomatic infections cannot be accurately determined. Identification and isolation of asymptomatic patients is essential to control virus outbreaks. Various studies have shown that asymptomatic persons with SARS-CoV-2 infection are generally not discovered until after their families, relatives, friends or close contacts have symptoms that are diagnosed [11,14,21,22,26–28]. Therefore, in order to not miss any infected patients, it is best to perform screening for all close contacts of patients with confirmed or suspected infections [15,19]. The main tests used to diagnosis COVID-19 are the SARS-CoV-2 nucleic acid test (NAT) of nasopharyngeal swab samples, the SARS-CoV-2 specific serological test and chest computed tomography (CT) scanning. NAT by reverse transcription-PCR (RT-PCR) is well established as the gold standard for the diagnosis of COVID-19 [29], but still the test is associated with false negatives due to problems with sample collection and the operating procedures [30,31]. Therefore, for cases that are highly suspicious of COVID-19 but test negatively by NAT, diagnosis via screening with a SARS-CoV-2 specific serological test and chest CT scan may be of great value [28,32,33]. In one study of 285 COVID-19 patients with acute antibody responses to SARS-CoV-2, in 19 days after the onset of symptoms, 100% of the patients were positive for antiviral IgG. Importantly, the seroconversion of IgG and IgM occurs simultaneously or sequentially, and the titers of IgG and IgM were found to be stable within 6 days after seroconversion [34]. However, if the NAT result is negative and the SARS-CoV-2 specific serological test is positive, the diagnosis still cannot be directly confirmed. It is necessary to continue to observe and conduct multiple NAT tests until either the NAT result is positive or the SARS-CoV-2 specific serological test is determined to be a false positive. A study conducted in the USA used 1020

serum specimens that were previously tested for HSV serology by western blotting in 2018 and 2019 (prior to SARS-CoV-2 circulation) and detected one false positive using the Abbott SARS-CoV-2 IgG test [35]. In addition, many studies on SARS-CoV-2 specific serological tests have shown that it is difficult to achieve 100% sensitivity and specificity [36,37]. Thus, for now, the diagnosis of COVID-19 remains a challenge globally. No test method is completely mature and reliable, but the combination of multiple testing methods can improve the effectiveness of screening [38] and avoid missed diagnoses and misdiagnoses as much as possible [33].

It is important to note that some patients with COVID-19 may experience only mild symptoms and signs. Kam *et al.* [22] reported a 6-month-old infant who developed a temperature of 38.5°C during hospitalization, although for only 1 h. Hoehl *et al.* [19] reported a 48-year-old German woman who experienced a mild rash and minimal pharyngitis after admission. Therefore, persons with COVID-19 may appear essentially asymptomatic, but do experience very mild symptoms and can enter the recovery period without being detected. Therefore, at the time of consultation healthcare workers should thoroughly interview the patient for any recollection of discomfort.

Variety of people with SARS-CoV-2 infection may be asymptomatic

Asymptomatic infection is not limited to young or middle-aged adults [21], but also children [11], infants [22] and even the elderly [27]. Hu *et al.* [15] showed that asymptomatic patients were relatively young, with a median age of 14 years in seven cases. In addition, asymptomatic infections were found in both males [19] and females [14].

Disease progression, changes in CT images & laboratory indicators in people with asymptomatic SARS-CoV-2 infection

In general, asymptomatic infected people do not suffer seriously, but the virus they transmit can cause others to develop severe disease [15]. Those with asymptomatic infections did not always show lung changes such as ground glass opacities after CT examination, and may appear normal [28]. Yet, in other cases the typical changes in CT examination may be observed [27]. Changes in laboratory test indicators typical of SARS-CoV-2 infection have been found in some asymptomatic patients [11,22,27], but for others, indicators are normal (Table 1) [14,19,26].

Conclusion

The number of people with COVID-19 continues to increase. Asymptomatic infections are hidden and easily overlooked. However, their potential to spread the virus cannot be underestimated, as the viral load they carried and their ability to infect close contacts may be similar to those of symptomatic individuals. In addition, asymptomatic infections can occur in any age range and either gender, and there may be no abnormalities in laboratory tests or CT examination. This, complete isolation of all sources of infection in the COVID-19 outbreak is a major problem. For this reason, measures of home quarantine and centralized isolation for observation over time have been and will continue to be necessary; otherwise, the pandemic will continue to cause great harm to the public and disease control will become even more complicated. Anyone who has had close contact with a confirmed or suspected case of COVID-19 should be closely monitored and screened; and therefore, the centralized isolation for medical observation and related tests of COVID-19 should be applied to the greatest extent possible, even if they have no symptoms. Healthcare workers should give close attention to screening consultations and collect detailed information, including the presence of even very slight symptoms. Overall, we have objectively summarized the current transmission and clinical characteristics of asymptomatic patients with COVID-19, which are deserve for further study and exploration in the future.

Future perspective

At present, the number of confirmed cases of COVID-19 continues to increase, and various prevention and control measures continue to be needed. With more in-depth research on COVID-19, systematic treatment plans and guidelines have been improved, so it is particularly important for patients to be diagnosed early and admitted to hospital for isolation treatment. Unfortunately, asymptomatic patients with SARS-CoV-2 infection are a silent source of infection, who can unknowingly place others at risk for infection. Therefore, as more research is conducted to understand the mechanisms of SARS-CoV-2 infection and to develop treatment methods, efforts to prevent the transmission of SARS-CoV-2 by asymptomatic individuals will be the key to reducing the spread of COVID-19. While the epidemics of SARS, MERS and COVID-19, were all caused by coronaviruses and shared other similarities, there are many differences among these diseases as well. The number of cases of COVID-19 far exceeds the case numbers for the other epidemics, and of these three diseases, COVID-19 is the only one to cause a global

Table 1. Clinical characteristics of asymptomatic patients with SARS-CoV-2 infection in recent studies.

Study	Country (patient)	Age (years)/sex	Chronic medical illness	Clinical characteristics			Collection site and viral load	Ref.
				Laboratory analysis	Computed tomography	Other situations		
Chan	China	10/Male	None	Alkaline phosphatase (↑)†	GGOs	NM	Nasopharyngeal swab (NF), throat swab (40)‡, sputum (27)‡	[11]
Bai	China	20/Female	NA	NOA	NOA	NOA	Nasopharyngeal swab (+)§	[14]
Hu	China	32.0¶ (15.0–57.0)	DMs (2)#	Blood leukocyte count (↓ 2)#,††	Normal (7)#	NOA	Pharyngeal swab (+)§	[15]
			Hypertension (2)#	Lymphocyte count (↓ 2)#,††	GGO or patchy shadowing (12)#			
		Male (8) Female (11)	CHD (1)#	C-reactive protein (↑ 2)†,#				
				Procalcitonin (↑ 4)†,#				
			CVD (1)#	Lactose dehydrogenase (↑ 3)†,#	stripe shadowing (5)#			
				Alanine aminotransferase (↑ 2)†,#				
				Creatinine (↑ 2)†,#				
				D-dimer (↑ 3)†,#				
Hoehl	Germany	58/Male	NA	Anemia	NM	NOA	Throat swab (24.39 and 30.25)‡	[19]
	Germany	48/Female	NA	NOA		Faint rash; Minimal pharyngitis		
Zou	China	26/Male	NA	NM	NOA	NOA	Nasal swab (22–28)‡ Throat swab (30–32)‡	[21]
Kam	Singapore	6-Month-old/male	NA	Neutropenia (day 8 of admission)	NP	Temperature rise (38.5°C in 1 h)	Nasopharyngeal swab (N gene 15.57; Orf1ab gene 13.73)‡ Blood sample and stool sample (+)§	[22]
Pan	China	33/Female	NA	NOA	NOA	NM	Nasopharyngeal swab: (+)§	[26]
		3/Male						
Lin	China	61/Male	None	C-reactive protein (↓)††	Multiple GGOs (day 1 of admission)	Only mild shortness of breath (1 day)	Throat swab (+)§	[27]
Bai SL	China	61/Male	CHD	NM	GGOs; lesion occupying lung field (different degree)	NOA	Throat swab (+)§	[28]
		53/Male	None					
		65/Female	DMs					
		34/Male	None					
		31/Female	None					

†The indicator increased.
‡CT value obtained by RT-PCR viral nucleic acid test.
§Positive by RT-PCR viral nucleic acid test but CT value was not shown.
¶Age, median-IQR.
#Number of cases with an indicator or performance.
††The indicator reduced.
CHD: Coronary heart disease; CoV: Coronavirus; CT: Computed tomography; Ct: Cycle threshold; CVD: Cerebrovascular disease; DM: Diabetes mellitus; GGO: Ground glass opacity; IQR: Interquartile range; NA: Not available; NF: No SARS-CoV-2 found; NM: Not mentioned; NOA: No obvious abnormality; NP: Not performed; RT: Reverse transcription.

pandemic. Notably, this, once insignificant and benign family of viruses, the coronavirus, has now generated three serious epidemics in the last two decades, indicating the importance of remaining alert to this class of emerging infectious diseases. This will be a long-term challenge, and we cannot yet predict when the next coronavirus outbreak will occur. What we can do is carry out more research and testing and develop better plans to handle such outbreaks in order to be better prepared when they emerge.

Executive summary

- The COVID-19 that originated in Wuhan, China in December 2019, is caused by SARS-CoV-2 infection. COVID-19 is now a global pandemic, and almost all countries in the world have recognized its seriousness and implemented various measures to curb its spread.
- The numbers of confirmed asymptomatic SARS-CoV-2 infections continue to increase, indicating that a large number of unidentified asymptomatic individuals with contagious infections may remain undetected in communities. In general, these patients do not know to self-isolate or seek treatment, and thus, are unlikely to be detected by healthcare workers. However, their potential to spread the virus cannot be underestimated, and emerging evidence indicates that they are an important source of transmission.
- SARS-CoV-2 has been detected in nasopharyngeal swabs, sputum samples, blood samples and stool samples from asymptomatic patients, and asymptomatic patients with SARS-CoV-2 infection can carry high viral loads.
- The incubation period of asymptomatic SARS-CoV-2 infections is complex and exists in the process of crowd and family-clustered outbreaks. In addition, asymptomatic patients are even the source of infection in contagious outbreaks among families.
- Asymptomatic infections can occur in patients of any age and either gender, and they may not exhibit any abnormalities on laboratory or computed tomography examinations.
- The identification and isolation of asymptomatic patients are essential to controlling virus outbreaks. To avoid missing any infected patients, anyone who has had close contact with a confirmed or suspected case of infection should be closely monitored and screened. Therefore, centralized isolation for medical observation and related tests for COVID-19 need to be applied to the greatest extent possible, even among contacts who have no symptoms. Healthcare workers should pay close attention to screening consultations and collect detailed information, including the presence of even very mild symptoms.
- Coronaviruses were once considered an insignificant, benign family of viral pathogens, but have now caused three major outbreaks of serious illness in the last two decades. Thus, we must all stay alert to such emerging infectious viral diseases, and this will be a long-term challenge.

Author contributions

Study concept and design was performed by Y Xin. Acquisition of the data was performed by J Tan, S Liu, L Zhuang, L Chen, M Dong and J Zhang. Analysis and interpretation of the data was performed by J Tan, S Liu and L Zhuang. Drafting of the manuscript was performed by J Tan and S Liu. Critical revision of the manuscript for important intellectual content was performed by Y Xin. Supervision was performed by Y Xin.

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Review

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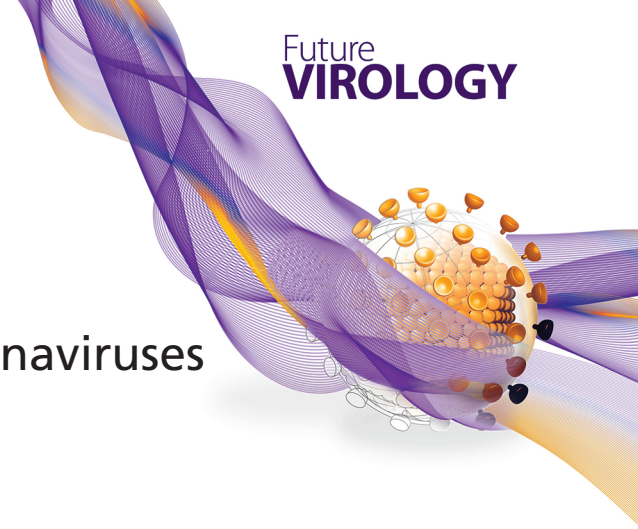

Membrane binding proteins of coronaviruses

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Coronaviruses (CoVs) infect many species causing a variety of diseases with a range of severities. Their members include zoonotic viruses with pandemic potential where therapeutic options are currently limited. Despite this diversity CoVs share some common features including the production, in infected cells, of elaborate membrane structures. Membranes represent both an obstacle and aid to CoV replication – and in consequence – virus-encoded structural and nonstructural proteins have membrane-binding properties. The structural proteins encounter cellular membranes at both entry and exit of the virus while the nonstructural proteins reorganize cellular membranes to benefit virus replication. Here, the role of each protein in membrane binding is described to provide a comprehensive picture of their role in the CoV replication cycle.

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Coronaviruses (CoVs) are enveloped positive sense RNA viruses causing a variety of diseases in man and animals and are considered to be the largest of the RNA viruses, with genomes ranging from 27–32 kb [1]. CoVs belong to the Coronaviridae family and contain two subfamilies Orthocoronavirinae and Letovirinae. Further, the Coronaviridae family is grouped into the suborder Cornidovirineae, which together with the suborders Abnidovirineae, Arnidovirineae, Mesnidovirineae, Monidovirineae, Ronidovirineae and Tornidovirineae forms the Nidovirales order [1,2], so named for the overlapping set of transcripts used by all members to encode viral proteins. The Coronaviridae are further subdivided phylogenetically into four genera, α, β, γ and δ [3]. The CoVs include members classified as emerging viruses, viruses that are able to cross the species barrier and cause pathology in a new target species. Two such recent events are the highly pathogenic severe acute respiratory syndrome-related CoV (SARS-CoV) that emerged in Southern China in 2003 and Middle East respiratory syndrome-related CoV (MERS-CoV), which appeared in Saudi Arabia in 2012 [4,5]. There is no effective treatment or licensed vaccine for either virus, which emphasizes the need to further understand CoV biology as a route to improved future intervention [6,7].

CoVs are structurally complex (Figure 1) with purified virus particles consisting of four or five structural proteins along with a variety of minor components including nonstructural and host cell-derived proteins [8]. All viruses have Nucleocapsid (N), Spike (S), Envelope (E) and Membrane (M) structural proteins and some also encode a hemagglutinin–esterase (HE) protein [1]. Despite their complexity and range of function however, [9,10] the structural proteins of CoVs occupy only about a third of the coding capacity of the genome. A much larger section of the genome, some two-thirds located at the 5′ end encode two long open reading frames 1a and 1b that together encode the nonstructural proteins of the virus. Each sequence is translated first as a polyprotein precursor, pp1a and pp1ab, the latter achieved by a frameshift event at the end of the 1a coding sequence. The polyproteins include several viral proteases that together process pp1a and pp1ab into 16 nonstructural proteins (nsp1–16), which are required at various stages of the virus replication cycle [1]. As an enveloped virus, the virus surface proteins, S, M and E encounter cellular membranes at the initiation of infection, again during the replication cycle when they are translated and incorporated into the endoplasmic reticulum and endoplasmic reticulum Golgi intermediate compartment (ERGIC) [11,12] and finally in the secretory pathway where budding of the mature virions occurs (Figure 2) [9,13]. In addition, many of the nonstructural proteins also interact with membranes as, in common with

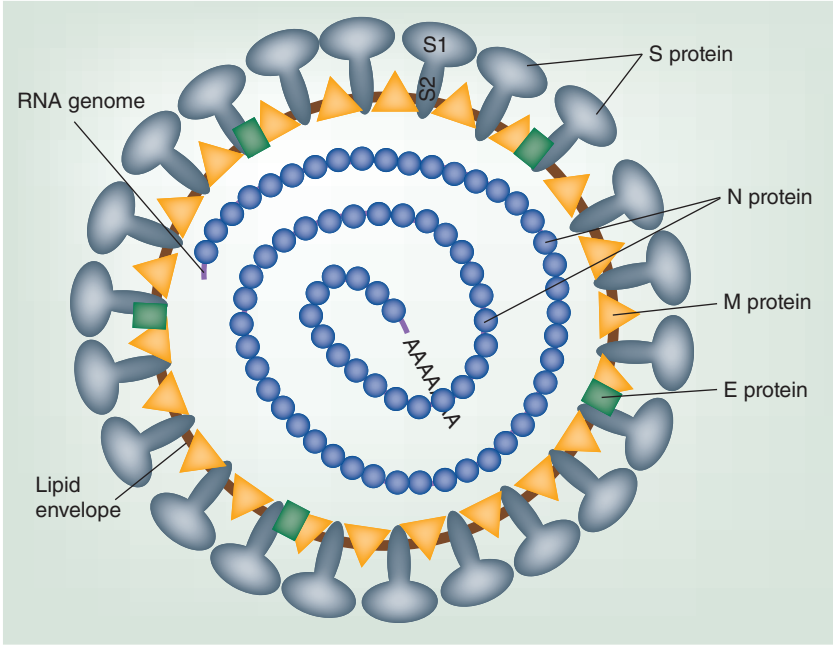

Figure 1. Schematic representation of a coronavirus particle. The structural components of the virus are indicated. Small amounts of host cell and virus nonstructural proteins, presumed to be captured nonspecifically during the budding process, are also found in virions but are not illustrated.

other positive strand RNA viruses, virus replication takes place in specialized cellular compartments induced by viral proteins which modify host membranes or organelles to set up sites for replication that are hidden from the cellular inducers of innate immunity [14]. The combination of multiple membrane interacting factors and multiple sites of membrane interaction make CoVs one of the more challenging virus-membrane interaction models available.

Structural protein interactions

The fusion process between viral and host membranes, mediated in CoVs by the S protein, is a crucial step in enveloped virus infection [15,16]. The S protein is a large class I fusion protein responsible for virus binding to target cells via cell surface receptors, which for CoVs can range from simple sugars to complex proteins (reviewed in [17,18]). For example the entry receptor for MERS-CoV infection has been identified as dipeptidyl peptidase-4 (DPP4) found on a variety of cell types including epithelial cells of the respiratory tract [19]. As a result, receptor distribution and the CoV–S-receptor interaction often defines tissue tropism and host range [18,19]. The S protein consists of two subunits, S1 and S2, with S1 at the N-terminus providing the receptor binding function and S2 at the C-terminus providing fusion activity [15]. The subunits are cleaved from the complete S by host cell proteases including members of the cathepsin family and transmembrane protease serine 2 (TMPRSS2) [20]. Following receptor binding by S1 and uptake into a vesicle the fusion mechanism of S2 acts to bring the viral and cellular membranes into such close proximity that fusion occurs [21,22]. The S2 sequence contains conserved regions that are necessary for function, notably a fusion peptide and two conserved heptad repeats (HR) [18]. Briefly, significant conformational change occurs in the late clathrin-coated endocytosed vesicle leading to release of the fusion peptide to interact with the vesicle membrane, provided that S has been cleaved into its requisite subdomains [23]. The collapse of S2, which is now bridging the virus and cellular membranes, pulls the two membranes together with HR1 and HR2 forming the canonical 6-helix bundle first described for CoVs in mouse hepatitis virus (MHV) [24].

In terms of sequence and location precise fusion peptides (FP) have yet to be defined for all CoVs [25] as recognition of the FP motif within the large spike protein can be difficult. However, bioinformatics analysis suggests that at least part of the fusion peptide is located near the N-terminus of S2 where a conserved motif with properties consistent with those expected of an FP, IEDLLF, occurs across the CoV family. This motif demonstrates very little variation and when substitutions are found, they are conservative replacements consistent with an essential function [26,27]. The motif is not located at the N-terminus of HR1 as suggested in some S protein cleavage maps (e.g., ref [21])

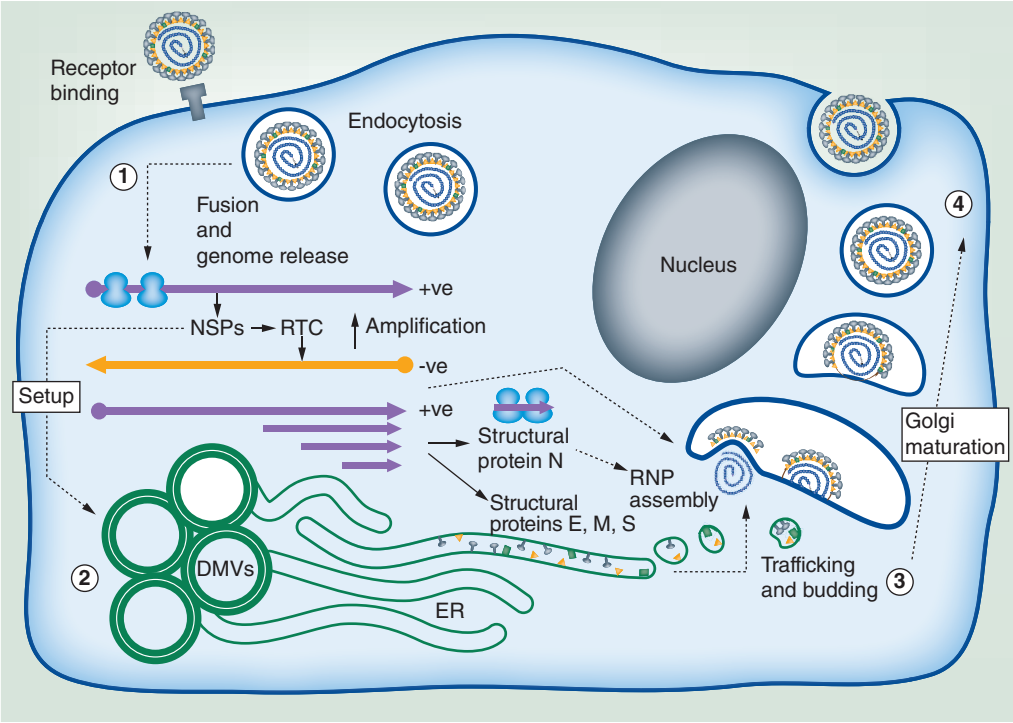

Figure 2. The coronavirus replication cycle highlighting areas where membrane interaction occurs. (1) Most Coronaviruses enter by receptor mediated endocytosis. The positive sense genomic RNA is released into the cytoplasm and translated into the initial virus polyproteins which encode the nsp. (2) The nsp stimulate the production of DMVs and establish the replication transcription complexes (RTC), which produce the -ve strand replicative intermediate from which more +ve strand genomes and mRNAs are produced. Translation of the N mRNA produces the N protein in the cytoplasm which combines with the new genomes to form RNPs while translation of the remaining structural proteins, M, E and S occurs in the ER where they accumulate in the ERGIC and cis-Golgi. (3) Virus assembly begins and completes as the protein cargos migrate through the Golgi stacks resulting in new virus particles in vesicles (4), which eventually fuse with the plasma membrane.
DMV: Double-membrane vesicle; ER: Endoplasmic reticulum; ERGIC: Endoplasmic reticulum Golgi intermediate compartment; nsp: Nonstructural protein; RTC: Replication transcription complex.

but immediately follows the second, S2′ cleavage site, originally mapped in SARS-CoV S and later in MERS CoV S [26,28,29]. A sequence which includes this motif has been shown directly for SARS-CoV to act as a fusion peptide when tested in an *in vitro* binding assay with multilamellar vesicles (MLVs) where it reorders membranes in a calcium-dependent manner [30].

The endodomain of S2 can been subdivided into two regions, a cysteine-rich region at the N-terminus and a carboxy-terminal region rich in charged residues [31–33]. It has been shown that clusters of cysteine residues are important for the palmitoylation of S. No particular cysteine residue is critical but in a study of fusion competence and replication in MHV a total of at least three cysteine residues was required [34] and other studies have confirmed that the cysteine-rich region is necessary for syncytium formation during viral infection [35,36]. While membrane binding and deformation is clearly a property of the FP sequence, propelled into the membrane by the conformational changes in S, palmitoylation of S may serve to stabilize the protein during its interactions with lipid rafts in the target membranes to allow time for fusion to occur. Recently, several CoV-S proteins including HCoV-HKU1, MHV, HCoV-NL63, SARS-CoV and MERS-CoV have had their structures solved at atomic resolution following imaging using cryo-electron microscopy [37–40]. All the confirmed structures of S are in their prefusion state and most have had their cleavage sites mutated to enhance S stability in order to enable the imaging process. As a result there is limited knowledge of the CoV FP within the fusion active conformation or of its structural characteristics when interacting with lipid bilayers after proteolytic processing at the S2` site [30,38,40].

By contrast with S, the CoV envelope protein (E) is a small hydrophobic integral membrane protein ranging from 76 to 109 amino acids. It has an N-terminal domain, a long α-helical transmembrane domain and a C-terminal hydrophilic domain and is found as a minor component in all CoV groups [41,42]. The E protein is also palmitoylated

at all three of its Cys residues [43] but the role of this secondary modification is debated. For MHV-CoV, single Cys residue changes do not significantly impair virus growth but modification of all three residues results in severe attenuation [44,45]. For SARS-CoV; however, triple mutation of the conserved Cys' does not impact secretion of virus antigen from expressing cells suggesting no particular dependence on palmitoylation [46]. Two membrane topologies have been demonstrated for E protein, hairpin or transmembrane, and it has been suggested that the level of palmitoylation may moderate their relative proportion, in turn allowing modified membrane curvature [45,47]. The E protein has demonstrated functions in virus assembly and release (below) and it appears to induce membrane curvature in the ERGIC leading to membrane scission of the budding virus particle and its release [48]. Envelope protein also interacts with the M protein and mutants of M that are unable to bud from cells can be complemented by mutated forms of E [49,50]. The membrane curving properties of E are such that co-expression of M and E is adequate for the efficient formation of virus-like particles [48,51], which can also incorporate S if it is co-expressed [52]. For many CoVs, including MHV, E protein has also been shown to have a role as an ion channel, a viroporin [53,54]. E function as a viroporin, including the trafficking of virions in the secretory pathways and membrane permeability, is essential for virus growth [55]. E also interacts with host cellular proteins including Proteins Associated with Lin Seven 1 (PALS1), which is known to maintain the epithelial cell junction, with clear implications for the virus assembly site in the Golgi [56,57]. While E function is critical for virus assembly, its viroporin activity in mobilizing calcium ions and its interactions with host tight junction cell proteins has been also implicated as a mediator of pathology in some CoV infections [55,57].

The CoV membrane protein (M) is a type III transmembrane glycoprotein and is the most abundant glycoprotein in the CoV particle. Despite variability in the primary M protein sequence the predicted secondary structures of M proteins are maintained [58]. The M protein is approximately 230 amino acids in length and is composed of three parts: a short N-terminal domain situated outside the virion membrane, three transmembrane domains and a carboxy-terminal domain situated inside the particle [59,60]. An amphipathic region situated at the end of the third transmembrane domain is well conserved in almost all Coronaviridae members [58]. CoV M proteins are characterized by N-linked glycosylation in the α and δ CoVs and O-linked glycosylation in the β CoVs [61,62] and study of chimeric M proteins has shown that the type of glycosylation is not critical for virus assembly or growth at 37°C [50]. It seems more likely that, as for many virus glycoproteins, glycosylation has a more general significance in maintaining bioactive conformation and antigenic character [63,64]. M is located among the S proteins in the virus envelope along with small amounts of E and is the primary driver of the virus budding process [51]. During assembly of the authentic virion M interacts with itself, with the nucleocapsid protein N, with E and with the S protein [44,58,65]. M protein is present as a dimer in the virion and high resolution imaging has suggested that it presents as two conformations, long and compact (M_{LONG} and $M_{COMPACT}$), which together induce membrane curvature as well as binding to the nucleocapsid [66,67].

Nonstructural protein interactions

CoV nsp 3, 4 and 6 (Figure 3) have fundamental functions in the rearrangement of host cell membranes that are required for the establishment of viral replication–transcription complexes (RTCs), also called replication organelles (RO) [68]. Indeed expression of just these proteins will induce the formation of the double-membrane vesicles (DMVs) and other structures that are characteristic of CoV-infected cells [69]. Replication complexes, intimately bound up with convoluted membrane structures, are a feature of all positive strand RNA viruses and serve at least three functions, probably connected. First, they serve to concentrate viral proteins in a microenvironment where all necessary replication factors are closely associated with the genomic RNA. Second, they exclude host factors so that the competition for resources can be focused on the virus and third they act to separate, as far as possible, the intermediates of replication, which are necessarily double stranded RNA molecules, from the host innate sensors such as TLR7 and MDA-5 [70,71].

Nsp3 has two transmembrane regions and approximately 10–16 identifiable domains (depending on the virus) within the approximately 200 kDa predicted primary translation product, eight of which are conserved [72]. It is co-translationally inserted into the endoplasmic reticulum resulting in the majority of the domains being tethered to the cytosolic side of the membrane (Figure 3). Nsp3 function is integral to CoV replication and its domains include many predicted or demonstrated to act as accessories in RNA replication such ssRNA binding and unwinding domains, as well as those for which no distinct function has yet been determined [72].

The 44 kDa CoV nsp4 protein is also a transmembrane protein, with four transmembrane helices and an internal C-terminal domain (Figure 3) and with nsp3 is an indispensable component required to produce DMVs [73,74]. All

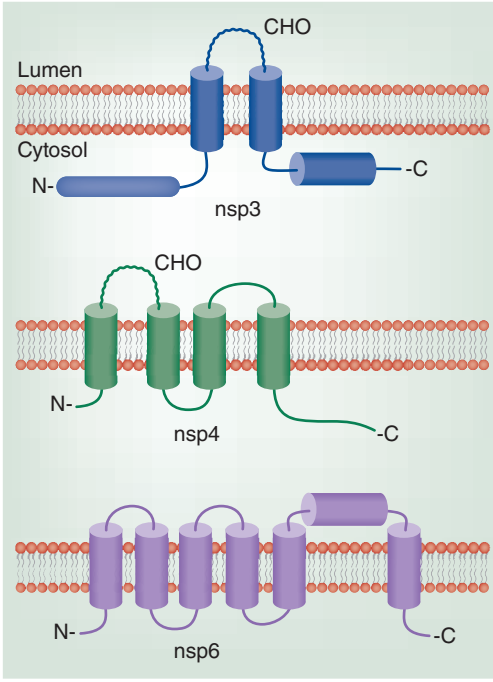

Figure 3. The suggested topology for the coronavirus nonstructural proteins associated with membrane deformation. Three proteins, nsp 3, 4 and 6 are implicated in membrane curvature and the formation of subcellular membrane organelles. The topologies suggested within the lipid bilayer (red) are supported by experimental evidence but may not be the same for all Coronaviruses. In all cases the white cylinders represent hydrophobic stretches of amino acids that are consistent with transmembrane domains although all may not be used as such (see nsp6). Only the region of the protein that interacts with the membrane is shown. N- the amino terminus of the protein, C- the carboxyl terminus of the protein, CHO- the addition of carbohydrate. The cartoon is not to scale.
Nsp: Nonstructural protein.

CoV-nsp4 molecules encode at least one predicted glycosylation site and in the case of MHV, it has been shown that mutation of the glycosylation site results in loss of virus fitness suggesting that nsp4 glycosylation is necessary for virus replication or the organization of the DMVs [75]. In an electron micrographic study, transfection of SARS-nsp3 and nsp4 alone caused considerable membrane deformation, producing a perinuclear double-walled maze-like body (MLB) [76] and the nsp3–nsp4 interaction was shown to be absolutely necessary for such membrane rearrangement. However the interaction of these two nsps was insufficient in itself to trigger membrane rearrangement and host factors such as EDEM1 and OS9 of the ER-associated degradation system have been shown to be co-factors [77,78]. Despite them being a universal feature of CoVs the size and number of DMVs does not appear to correlate directly with viral fitness, at least when virus is grown at reduced temperatures [79] nor are they a determinant of pathogenicity [80]. For the γ CoV Infectious Bronchitis Virus (IBV), nsp4 was essential and sufficient to induce membrane pairing, recognized as extensive areas of membrane accumulation or small regions of paired membrane, but expression of nsp3, nsp4 and nsp6 was required for DMV production which, even then, was poor for strain BeauR and not seen at all for strain M41. DMVs formed by IBV nsp3, nsp4, and nsp6 alone were poorly efficient when compared with DMVs formed by *Betacoronavirus* infection so supplementation of nsp4 with nsp6 is not sufficient for authentic IBV DMV production [81].

CoV nsp6 is a membrane protein of approximately 34 kDa predicted molecular mass with six transmembrane helices (Figure 3) including, in almost all viruses, a highly conserved C-terminus [82]. Although nsp6-stimulated internal cellular membrane rearrangement is observed with the addition of nsp3 and 4, nsp6 also causes membrane proliferation alone, including the formation of Atg5 and LC3II-positive vesicles classically observed in autophagy [83]. The autophagosomes produced are somewhat different from those induced by starvation; however, as although their number is higher and their size is reduced [84]. As noted above, along with nsp3 and 4, nsp6 functions to produce the canonical DMVs as well as many other types of intracellular vesicles observed in CoV infected cells such as convoluted membranes, vesicle packages, tubular bodies, large virion-containing vacuoles (LVCVs), cubic membrane structures (CMSs) and zippered ER spherules in the case of IBV [85,86]. An attenuated form of an IBV vaccine includes mutations in an nsp6 TM domain, confirming its role in virulence and replication [87].

Recruitment & modification of membranes by CoVs

As noted above, the membranous vesicles or organelles of different morphologies induced by CoVs act as a platform for the formation of replication–transcription complexes (RTCs) and sequester newly formed RNAs away from host immune sensors [88,89]. Both viral and hijacked host proteins are used in this process, taking advantage of cellular pathways and lipid modifying enzymes to the benefit of the virus [90,91]. This usurping comes about through the

commandeering of normal secretory pathways used by noninfected cells to transport and deliver protein cargos; rather than encode proteins to build DMVs anew, CoVs redirect and reorganize the cellular processes already in place [92].

Two principle mechanisms have been described for moving and delivering cargo proteins through the secretory pathway; cisternal maturation and the formation of megavesicles [93]. In both cases the detail remains incomplete [94]. During CoV infection, such as for MHV, virions have been observed in large vesicle depots resembling megavesicles derived from Golgi/ERGIC membranes, indicating that remodeling of the Golgi complex may be crucial for virion trafficking [14]. As noted, nsp6 may initiate cellular autophagy and a general ER stress response also occurs during the formation of DMVs [95,96]. Atg5 is necessary for the formation the crescent membranes and if is knocked out the yield of MHV is reduced although this is not a universal finding [97]. Although the precise mechanisms are ill defined, biological bilayers of proteins and lipids [98] are key to the separation and control of biological processes and their occurrence and composition is dynamic [99]. Bending, that is positive or negative membrane curvature, is driven by the acquisition and loss of peripheral membrane proteins, integral membrane proteins and by lipid composition [100,101]. Membrane wrapping may occur around intrinsically curved proteins in which positively charged amino acids interact with negatively charged lipid head groups, for example, in the dynamin and BAR domain interactions, also known as scaffolding [102,103]. Alternatively, crowding mechanisms may effect membrane curvature as a result of the asymmetric distribution of proteins either side of a cellular membrane [99,104], and the insertion of an amphipathic helix which can act as a wedge to expand one side of the membrane more than the other can also cause curvature as revealed by studies on influenza virus M2 protein, Epsins and Sar 1p [102,105,106].

Virus egress

During assembly, all enveloped viruses face the challenge of combining capsids proteins and genome produced in the cytosol with glycoproteins that predominantly occur in another cellular compartment, the luminal side of the ER. A cell membrane separates these components and must be breached or used in the assembly of the complete virion and this is achieved in three stages (Figure 2). First, the virus proteins coalesce on the membrane, capsid proteins grouping together underneath the patch of membrane where viral glycoproteins are embedded. Second, the membrane bulges outward to form a bud decorated by the viral transmembrane proteins and enclosing the capsid proteins and genome. Third and finally, the bud splits from the rest of the membrane by scission, a pinching-off at the base which releases the virion either into an intracellular vesicle as in the case for CoVs or directly out of the cell [1]. For many enveloped viruses these processes are actioned by viral protein interaction with host proteins of the endosomal sorting complexes required for transport (ESCRT) machinery [107]. Surprisingly however, perhaps because of incompatibility with the extensive membrane rearrangements induced in infected cells, CoVs appear not to use ESCRT proteins for egress, rather the S protein has a signal for ERGIC retention in its cytoplasmic tail [108] while the M protein locates to the ERGIC and cis-Golgi via its first TM domain where it also oligomerises [109] to drive the budding process. M–N interactions ensure that the viral RNPs also occur at these budding sites allowing the budding virus to incorporate a copy of the new genome [46,110]. The E protein, as a viroporin, has been implicated in membrane scission as E is present in virus particles at only a very low level and most is left associated with the ERGIC and *cis* Golgi consistent with a predominant role as a mediator of virus assembly and release at this location [111]. The lipid content at these locations may also enhance virus budding [112,113].

CoV membranes as antiviral targets

As CoVs cause such extensive membrane perturbation and as there is an acknowledged lack of available antiviral compounds to combat CoV-induced disease, it is not surprising that membrane rearrangement has been considered as a target for the development of inhibitors that could act as antivirals, along with the more classical targets of the polymerase and proteases [114]. Peptide therapeutics are promising antagonists in this regard as they compete directly for membrane binding or inhibit the conformational mechanisms involved and several peptides have been demonstrated to target various steps in the CoV replication cycle. A HR2 competitive peptide blocked the fusion mechanism of MERS-CoV and prevented virus entry when measured using a pseudotype assay [115] and a more complex 5 helix bundle, designed as a mimic of the final S fusion intermediate, was also active when measured similarly [116]. SARS-CoV has been inhibited similarly [117]. As membrane microdomains are implicated in CoV membrane interaction, drugs that alter microdomain composition, particularly the level of cholesterol present, have been shown to have an effect on some CoVs [118,119]. More general still is the use of drugs which alter intracellular vesicle pH and so inhibit the entry or exit of enveloped viruses, including CoVs [114,120]. Vaccines

and passive immunotherapy options have also targeted crucial CoV–membrane interactions. The predominant antibody response to S is to the S1 domain which has been shown to be a successful vaccine candidate [121,122] but the binding of antibodies directed here is subject to antigenic drift and may not be effective for all serotypes. The S2 domain by contrast is generally immunologically silent. Rare antibodies that do target S2 in the stem of S and inhibiting the fusion mechanism, are broadly reactive and so relatively impervious to serotype change [123]. The use of such broadly reactive monoclonal antibodies as therapies may be particularly suitable for the treatment of serious but sporadic CoV infections where general vaccination of the target population is not warranted or is impractical.

Future perspective

With their large, adaptable genomes and their extensive distribution in the biosphere, CoVs will certainly feature in future zoonotic outbreaks; SARS and MERS will not be the last. While vaccination remains the cornerstone of control for viral diseases, it is not quick, a new vaccine may take 15 years to develop and it is very virus specific, a MERS vaccine will not protect against SARS and vice versa. Similarly, antiviral drugs targeting the main enzyme functions of the virus risk being ineffective as a result of sequence variation in the target genes. Targeting the common physiological features of CoV replication; however, offers the possibility of developing panCoV treatments that focus on what is common to this family of viruses rather that what is distinct. There are obvious problems, viral stages that are so closely associated with host biology that toxicity would be expected, but there is also sufficient novelty, nsp-based membrane remodeling, for example, that clear targets for intervention exist. Such a strategy could offer the possibility for the development of panCoV agents of the future. More immediately, as membrane remodeling by CoVs is fundamental to immune evasion, targeting the proteins responsible for the remodeling could reveal the infection to the host immune system much sooner than would otherwise be the case and lead to the curtailment of the infection at a much earlier time, before extensive collateral damage is done. Together, a further understanding of the role of virus proteins in membrane interaction and remodeling, directly and via interaction with host factors, is likely to increase the underpinning data that lead to an increase in the therapeutic options for the control of CoV infections in the future.

Executive summary

- Coronaviruses (CoVs) are diverse, complex, adaptable viruses that have a significant impact on human health and animal productivity. Despite their diversity, common features exist, including the formation of membrane organelles which are driven by virus-encoded membrane-binding proteins.
- Both structural and nonstructural proteins of the virus contribute to membrane reorganization and viral protein interaction with membranes occurs at several stages of the virus replication cycle.
- The precise role of each protein and of individual domains within each protein in contacting the membrane and initiating its deformation remains work in progress and may vary across the family.
- Certainty has improved considerably for the structural proteins as a number of protein structures now exist, including structures for large molecules and multimeric assemblies such as the Spike protein trimer, obtained by cryoelectron microscopy. Models for the mechanism of protein function based on such structures allows them to be tested.
- Some certainty also applies to the nonstructural proteins in that certain combinations of proteins, notably nonstructural protein (nsp) 3, 4 and 6, can produce membrane deformation and structures that resemble those formed during virus infection. However, the precise contribution of each protein and the role of host proteins in the overall process remains to be determined. Ironically, it is the hydrophobic nature of the proteins concerned that makes them difficult targets for structural biology.
- Regardless of the precise mechanisms of membrane curvature the central role of membrane perturbation in the CoV replication cycle suggests itself as a target for designed intervention. A lack of membrane structures would clearly prevent virus replication but more reasonably even a partial inhibition might result in revelation of the replicative intermediates to the immune system and accelerate virus clearance.
- Study of the membrane reorganization associated with CoV infection is likely to contribute to a greater understanding of membrane biogenesis in general and to offer opportunities for rational design.
- As a universal feature of CoV replication, inhibition of membrane reorganization would likely apply to future zoonotic outbreak strains, as well as, to established and characterized viruses.

Financial & competing interests disclosure

The authors have no relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript. This includes employment, consultancies, honoraria, stock ownership or options, expert testimony, grants or patents received or pending, or royalties.

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Review

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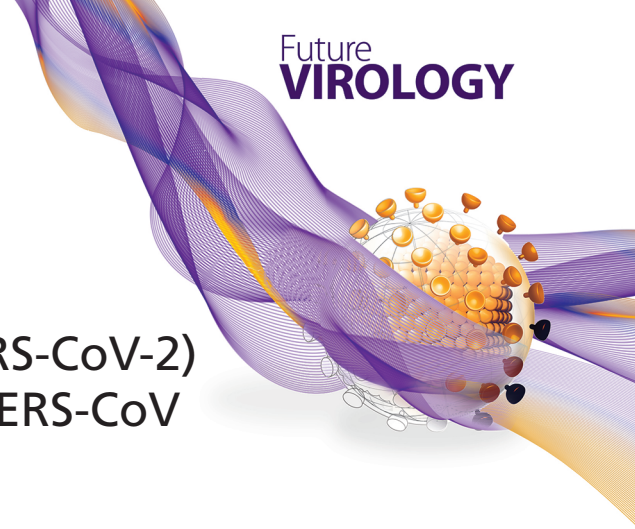

Comparison of the COVID-2019 (SARS-CoV-2) pathogenesis with SARS-CoV and MERS-CoV infections

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The severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), was first identified in several patients who traveled to Wuhan or went to a seafood wholesale market in Wuhan. The phylogenetic tree showed that SARS-CoV-2 was 96.2% identical to bat β-coronaviruses from lineage B. Also, several studies reported that SARS-CoV-2 uses the SARS-CoV receptor, angiotensin-converting enzyme 2, for entry to target cells. Lung alveolar and small intestine are potential targets for SARS-CoV-2 due to the high expression of the angiotensin-converting enzyme 2 receptor. In this review, we focused on the zoonotic β-coronaviruses and given there is no specific drug or vaccine for coronavirus disease 2019, we reviewed the literature on the therapy options for SARS and Middle East respiratory syndrome coronavirus infection, in order to discover their possible use in the treatment of SARS-CoV-2 infections.

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Keywords: ACE2 • coronavirus • COVID-19 • SARS-CoV-2 • zoonotic diseases

Although several coronaviruses are known as the major causes of morbidity and mortality in animals, their importance was highlighted after the emergence of SARS (severe acute respiratory syndrome). Furthermore, some studies indicated a coronavirus interspecies transmission from animals to human that is significant for global health institutions. Generally, coronavirus has a wide host range including birds, felines, pigs, cows, turkeys and dogs; causes respiratory, enteric, hepatic and neurologic infections. Coronaviruses cause mild-to-severe disease in humans and they have newly emerged from a zoonotic source. On the other hand, nowadays it is believed that approximately 75% of infectious diseases are zoonotic. Documented evidence indicates the mutation of existing strains, leading to the emergence of novel strains and new illnesses in animals [1].

Coronavirus belongs to the family *Coronaviridae* and the subfamily *Coronavirinae* and based on genetic properties, this subfamily has been divided into four genera: α-coronavirus, β-coronavirus, γ-coronavirus and δ-coronavirus [2].

In the past two decades, β-coronavirus has been a major subject of research due to it emerging and re-emerging. Human coronavirus (HCoV) infects the upper and lower respiratory tract in children, aged people and patients with underlying heart and respiratory diseases [3].

HCoV is a positive-sense RNA virus and has the largest genome known among RNA viruses. Also, 229E, OC43, NL63, HKU1, SARS, MERS (Middle East respiratory syndrome) and coronavirus disease 2019 (COVID-19; SARS-CoV-2) species cause respiratory tract infection. Among them, 229E, OC43, NL63 and HKU1 strains result in common cold symptoms in individuals. The two other species, SARS-CoV and MERS-CoV which belong to β-coronavirus genus sometimes are associated with fatal disease. Recently, the SARS-CoV-2 strain was reported by the Chinese Center for Disease Control and Prevention (China CDC) in Wuhan city on 31 December 2019 [4].

Structural proteins are essential for the assembly and infection of coronavirus: spike glycoprotein (S) on the surface of the particle consists of S1 and S2 subunits. The S1 subunit contains the receptor binding domain (RBD) and binds to the cellular receptor and the S2 subunit facilitates the fusion and entrance process. Membrane (M) protein by increasing the membrane curvature, promotes the viral assembly. Envelope (E) protein is essential to

release the virus. Nucleocapsid (N) protein is interferon (IFN) antagonistic and supports viral replication. The nonstructural proteins of coronaviruses can block the host immune system for viral replication [4].

RNA-dependent RNA polymerase (RdRp) enzyme in coronaviruses has proofreading-activity, so the mutation rate in this family is lower than other RNA viruses, while homologous recombination frequently occurs in this family [5].

In this review, we compared the pathogenesis of SARS-CoV-2 infection with SARS-CoV and MERS-CoV infections and briefly mentioned the symptoms and transmission pathway of COVID-19. We also introduced the potential targets for therapeutic options to treat COVID-19.

Etiology of severe acute respiratory syndrome coronavirus infection

SARS-CoV was a pandemic agent of the SARS from 2002 to 2003 in 33 countries with 8096 cases and 774 deaths [6]. In 2003, Holmes reported that the sudden emergence of SARS-CoV did not correlate to mutation or recombination between previous HCoV. On the other hand, genome sequencing and epidemiologic reports demonstrated that SARS-CoV was a new virus which was not similar to known HCoV [7]. However, the genome sequences of human SARS-CoV were similar to animal isolates and in addition, several serological studies confirmed that animal traders had specific antibody (IgG) against the SARS-CoV infection. These results displayed that SARS-CoV was a zoonotic virus and originated from animal and bird species before outbreaks in humans [1]. Moreover, in 2006, Li *et al.* reported that significant genetic changes occurred in the spike glycoprotein (S glycoprotein) of bat SARS-CoV to infect humans. Finally, the sequence data of SARS-CoV exhibited 87–92% identity with bat SARS-CoV and it was concluded bats were the potential natural reservoir for the outbreak of SARS in 2003 [8]. In fact, exotic animals have transmitted SARS-CoV to humans through intermediate hosts (civet cats and raccoon dogs) and subsequently, person-to-person transmission resulted in the outbreak of SARS-CoV in hotels and hospitals [9].

Several risk factors including age, diabetes and heart disease can increase the risk of death. SARS can infect the respiratory tract of individuals in all age groups, principally through droplet transmission. SARS-CoV infection is associated with several common signs such as fever, diarrhea, myalgia, malaise and chills [9].

The entry of SARS-CoV is facilitated by attachment of S glycoprotein to ACE2, subsequently, the conformational changes of S glycoprotein take place in the endosome microenvironment by cellular serine protease cathepsins B and L to facilitate the fusion process [9].

In 2005, Li *et al.* reported that residues 318–510 of the S1 domain encode the RBD, but two of amino acids are not conserved in SARS-CoV strains. Probably, the adaptation of S glycoprotein with ACE2 permits the efficient infection of human cells and also cause the unusual severity of SARS-CoV [10].

The ACE2 is expressed on epithelial cells of the lung, tongue, kidney, heart and liver. The attachment of S glycoprotein to ACE2 can cause the loss of cilia, squamous metaplasia and an increase in macrophages in the alveoli that cause diffuse alveolar damage to the lung [11].

Furthermore, SARS-CoV produces 3a and 7a proteins that cause apoptosis in the lungs, kidneys and liver cells. Also, activation of TH1 and increasing inflammatory cytokines and interleukins such as IFN-γ -IP-10, IFN-γ, IL-1B, IL-6, IL-8, IL-12 and MCP-1 happen in SARS-CoV infection [12].

Etiology of MERS-CoV infection

In 2012, a new human disease that was caused by MERS-CoV emerged in the Middle East with 2494 confirmed cases and 858 fatalities [13,14]. For a long time, the origin of MERS-CoV was controversial, previously it was thought that bat was the reservoir due to phylogenetic similarity of MERS-CoV with certain bat coronaviruses. But serological and phylogenetic studies demonstrated that dromedary camels suffer from human-MERS-CoV-like disease. After camel–human contact, human-to-human transmission occurred, especially in healthcare communities [13].

The average incubation period for MERS-CoV is 5–7 days but can be as long as 2–14 days. Also, MERS-CoV infects men more than women. The clinical symptoms of MERS may be asymptomatic, mild and can lead to severe disease with multi-organ failure. Also, MERS is associated with metabolic syndromes including diabetes mellitus, cardiovascular diseases and obesity. Subsequently, metabolic syndrome can interfere with innate and humoral immune and can render patients more susceptible to infectious diseases.

DPP4, CD26 is the receptor for attachment of MERS-CoV to the pneumocytes and epithelial cells of the respiratory tract [15]. Moreover, MERS-CoV has a specific RBD that is 231-amino-acid in S glycoprotein and binds to DDP4 on host target cells. DPP4 affects glucose metabolism, T cell activation, cytotoxic modulation,

cell adhesion and apoptosis. MERS-CoV RBD comprises the core structure and a RBM. Although the core structures of MERS CoV and SARS-CoV RBDs are highly similar, their RBMs are divergent and lead to different receptor specificities. Also, it was supposed that MERS-CoV transmission from bats-to-humans and then from human-to-human occurred through little or no adaptation in RBD [16].

Etiology of SARS-CoV-2 infection

The new coronavirus was named SARS-CoV-2, belongs to β-coronaviruses based on the genome sequence and infects the upper and lower respiratory tract. Symptoms of the novel coronavirus strain are milder than SARS and MERS, but it transmits from human-to-human faster than them. Besides, the mortality rate of SARS-CoV-2 is lower (3.4%) than that of SARS-CoV (9.6%) and MERS (35%) [5].

More recent studies, have confirmed that diabetes and hypertension may relate to the pathogenesis of SARS-CoV-2. By blocking the function of lymphocytes and macrophages, these disorders reduce IFN-γ and interleukin synthesis to downregulate the host innate immune response [2,12].

Despite SARS-CoV-2 mostly affecting the middle-aged and older people with underlying disease, it does not mean that children are less susceptible to novel coronaviruses. Maybe their relative resistance to SARS-CoV-2 infection may be due to the active immune system and the healthy respiratory tract compared with adults. Laboratory mice models showed that the ACE2 expression as the receptor of the SARS-CoV-2 decreases with age. Although this result contradicts with low susceptibility of children to SARS-CoV-2 infection, the lung is protected by ACE2 against SARS, influenza A H5N1 virus, respiratory syncytial virus infections. It can be explained that ACE2 in healthy people and children modulates the renin–angiotensin system via cleaving angiotensin (Ang)-II to Ang-1–7 to prevent severe acute lung failure. Indeed, the severe lung injury arising from a viral respiratory infection is associated with ACE2 deficiency and increasing of Ang II [17,18].

Zhou *et al.* demonstrated that Asian men are more susceptible to SARS-CoV-2 infection compared with women and other races due to more expression of the ACE2 receptor [19].

According to the latest studies, SARS-CoV-2 has the highest number of casualties in more than 80 countries and is now a pandemic.

The incubation period and the epidemiological, clinical, laboratory and radiological features of patients with confirmed COVID-19 were similar to SARS-infected people in 2003, but phylogenetic tree analysis showed that the SARS-CoV-2 is separate from SARS and MERS. On the other hand, the outbreak of SARS-CoV-2 has probably started from the wholesale market of Huanan seafood, where wildlife such as snakes, bats, birds, frogs, hedgehogs and rabbits are sold. Wei Ji *et al.* using sequence analysis of different species of coronavirus revealed that SARS-CoV-2 is a recombinant virus between the bat coronavirus and a source-unknown coronavirus, but the possible intermediate host of SARS-CoV-2 is the pangolin [11]. So, these results indicate the outbreak in Wuhan city was a zoonosis disease similar to bat SARS [2,12]. Moreover, the viral genome sequencing confirmed that SARS-CoV-2 is 96.2% identical to some bat coronaviruses and also more distantly correlated to SARS-CoV and MERS-CoV (about 79 and 50%, respectively). Also, the genome sequencing of SARS-CoV-2 S2 protein confirmed the similarity of 93% with bat coronaviruses.

Moreover, the SARS-CoV-2 and SARS-CoV are distinct from each other in the genome sequence of RNA-dependent RNA polymerase (RdRp). Therefore SARS-COV-2 was clustered within an independent subclade in the β-coronavirus genus [14].

The phylogenetic analysis of RBD showed that the SARS-CoV-2 was close to SARS-CoV that was located in lineage B, also, SARS-CoV-2 can infect BHK-21 cells. These results suggest that SARS-CoV-2 uses the ACE2 as a cell receptor and cellular proteases like TMPRSS2 protease for SARS-COV-2 S glycoprotein priming [16].

Lu *et al.* observed that several residues in RBD of SARS-CoV S glycoprotein were variable in SARS-CoV-2 [14]. Also, biophysical and cryo-EM structure evidence revealed the affinity of SARS-CoV-2 S protein to ACE2 is 10–20-times higher than the SARS-CoV one. These findings support the theory of the higher contagion of SARS-CoV-2 compared with SARS-CoV [11,20].

Indeed, the ACE2 is a physiologically related receptor during coronavirus infections and responsible for the localization of viruses in infected human and animals. Therefore, the infection efficiency correlates with the ability of the ACE2 of each species to support viral replication. It should be noted that several bat SARS-CoVs did not employ ACE2 for entry to the target cell. The analysis reports showed that the most amino acids in RBM of S glycoprotein of SARS-CoV-2 were similar to bat SARS-CoV in lineage B which uses ACE2 on the target cell surface [16].

Table 1. Detection methods and genes for diagnosis of severe acute respiratory syndrome coronavirus 2.

Sample	Method	Gene	Primers & probe (5′-3′)	Comments	Ref.
Throat swab sample	rRT-PCR	ORF1ab	(F)primer: CCCTGTGGGTTTTACACTTAA (R)primer: ACGATTGTGCATCAGCTGA Probe: VIC-CCGTCTGCGGTATGTGGAAAGGTTATGG-BHQ1	Respiratory tract specimens were used to diagnose NCIP through RT-PCR. The serum of patients was not obtained to evaluate the viremia. The viral load is a potentially useful marker associated with disease severity of coronavirus infection and this should be determined in NCIP.	[21]
		N gene	(F)primer: GGGGAACTTCTCCTGCTAGAAT (R)primer: CAGACATTTTGCTCTCAAGCTG Probe: FAM- TTGCTGCTGCTTGACAGATT-TAMRA		
Blood, sputum, feces, urine and nasal samples	rRT-PCR	ORF1ab	(F)primer: CCCTGTGGGTTTTACACTTAA (R)primer: ACGATTGTGCATCAGCTGA Probe: VIC-CCGTCTGCGGTATGTGGAAAGGTTATGG-BHQ1	1. Lower respiratory tract samples were positive. 2. Live virus was detected in feces	[22]
Oral swabs, anal swabs and blood samples throat swabs, sputum, urine and stool	qRT-PCR assay	S gene	(F)primer: CAATGGTTTAACAGGCACAGG (R)primer: CTCAAGTGTCTGTGGATCACG Probe: NM	The virus may be present in anal swabs or blood of patients when oral swabs detection negative. Patients infected with SARS-CoV-2 may harbor the virus in the intestine at the early or late stage of disease.	[19,23]
Throat swabs, sputum, urine and stool	qRT-PCR assay	N gene	Primers: NM Probe: NM	The peaks of viral loads in throat swab and sputum samples were 5–6 days after symptom onset, ranging from around 10^4 to 10^7 copies per ml during. Sputum samples generally showed higher viral loads than throat swab samples.	[24]
Nasopharyngeal and throat swabs and stool and urine samples	Real-time RT-PCR	S gene	(F)primer: CCTACTAAATTAAATGATCTCTGCTTTACT (R)primer: CAAGCTATAACGCAGCCTGTA		[2]
	Multiplex RT-PCR	RdRp	(F)primer: CAAGTGGGGTAAGGCTAGACTTT (R)primer: ACTTAGGATAATCCCAACCCAT		
Nasal and pharyngeal swabs, bronchoalveolar lavage fluid, sputum or bronchial aspirates	NGS & real-time RT-PCR	E	(F)primer: TCAGAATGCCAATCTCCCCAAC (R)primer: AAAGGTCCACCCGATACATTGA Probe: CY5-CTAGTTACACTAGCCATCCTTACTGC-BHQ1		[12]
Blood, stool and urine samples and nasopharyngeal swabs	PCR	RdRp	NM	Virus was detected by PCR in 50% stool, 8% in whole blood and virus was not detected in urine	[25]

NM: Not mentioned; NCIP: Novel coronavirus (2019-nCoV)-infected pneumonia; rRT-PCR: Real-time reverse transcriptase-PCR; SARS-CoV-2: Severe acute respiratory syndrome coronavirus 2.

Diagnosis of SARS-CoV-2 infection

Fever, cough and fatigue are common symptoms of COVID-19. Also, muscle ache, chest pain, dyspnea, sore throat, vomiting, diarrhea and confusion are observed in SARS-CoV-2 infection. Along with clinical symptoms, the C-reactive protein and cytokine level increases and the total white blood cell, lymphocyte, platelet and thromboplastin time decrease. Acute respiratory distress syndrome is a common complication in patients, followed by anemia, acute heart damage and secondary infections. Unilateral and bilateral pneumonia is found in the chest computed tomography (CT) images or chest x-ray in the patients with COVID-19.

Real-time reverse transcriptase-PCR of nasopharyngeal swab is routinely used to detect SARS-CoV-2 (Table 1).

Because chest CT is more sensitive than real-time reverse transcriptase-PCR, the combination of SARS-CoV-2 molecular tests and clinical features is used to diagnose COVID-19 [26].

Therapeutic options for SARS-CoV-2 infection

The SARS-CoV-2 infection appears to be out of control, so drugs and vaccines will be needed to prevent public health threats.

To date, no licensed vaccines or proven therapies exist against SARS-CoV-2, but the combination of IFNs and ribavirin is effective for coronaviruses infection. Ribavirin targets viral replication to block the viral RNA synthesis and mRNA capping [11].

Individuals with COVID-19 have high amounts of IL-1B, IFNγ, IP10 and MCP1 that can lead to activated Th1 cell responses. On the other hand, in contrast to SARS-CoV infection, secretion of IL-4 and IL-10 has been reported in COVID-19, that suppress inflammation, which could be one of the reasons for the lower severity of COVID-19 compared with SARS infection [12]. Vaccination can provide the best line of defense against disease compared with chemotherapeutic drugs. Generally, more research is required on Th1 and Th2 responses against SARS-CoV-2 to clarify the pathogenesis.

In a study, it was demonstrated that the serum from a patient with SARS-CoV S glycoprotein prevent the entry of SARS-CoV-2 [27]. More broadly, *in vitro* researches are needed to determine the inhibitory effect of SARS-CoV-infected serum on replication of SARS-CoV-2.

Lopinavir and ritonavir are anti-CoV drugs that target the nonstructural proteins of chymotrypsin-like protease (3CLpro) and polymerases, however, none of them are licensed for clinical trials yet [28].

S glycoprotein and ACE2 are critical in SARS-CoV-2 infection, thus, employing them can help to develop antiviral agents. Chloroquine is a potent drug against SARS-CoV-2 infection that increases endosomal pH and also blocks the cathepsin function, moreover, chloroquine can interfere with the virus cell binding [16]. Therefore, TMPRSS2 may be as a suitable therapeutic option, because TMPRSS2 in SARS-CoV-2, like SARS-CoV, help to spread SARS-CoV-2 via virus/cell to cell fusion and also by diminishing viral identification by neutralizing antibodies. Thus, using a protease inhibitor such as camostat mesylate could block the TMPRSS2 function.

Remdesivir and favipiravir target the RdRp enzyme and lead to premature termination during virus transcription, therefore, they can be used in the treatment of COVID-19. But further studies on the effect of chloroquine, remdesivir and favipiravir on extracellular proteases are required in an *in vivo* setting. Furthermore, several anti-HIV drugs including darunavir, cobicistat and ASC09F have been considered for the clinical trials against SARS-CoV-2 infection [11].

In 2003, Kumar *et al.* reported that ACE2 in the form of nucleic acid shuttles can treat acute respiratory and lung failure arising from SARS-CoV infections [27]. Also, in 2016, Gu *et al.* published a paper in which they demonstrated that a recombinant ACE2 reduces the lung injury and regulates the innate immune system [18]. Moreover, in 2018, Guangyu found that nanobodies (single-domain antibodies) can target the MERS-CoV RBD to inhibit the binding of S glycoprotein to DPP4 [29]. Thus, ACE2 receptor and RBD can be other therapeutic choices.

Conclusion

Bat seems the common natural origin of SARS-CoV and MERS-CoV and SARS-CoV-2. The clinical features of them are similar and unlike SARS-CoV and MERS-CoV, SARS-CoV-2 spreads rapidly. On the other hand, the adaptation of the S glycoprotein and its affinity for ACE2 can determine the severity of SARS-CoV-2 infection. Thus, a vaccine containing S glycoprotein and inactivated SARS-CoV-2 could have the potential to prevent COVID-19. What is now needed is research on recombination events and genetic diversity of SARS-CoV-2 to present an effective vaccine or drug.

Future perspective

There are concerns about the emergence of large numbers of people infected with SARS-CoV-2 in the short term, which could lead to an increase in the mortality rate. As the number of patients increase, the process of controlling the disease is further disrupted in the healthcare system, which is more detrimental to individuals with a history of immunodeficiency. To date, there are no effective vaccines and drugs against COVID-19. By reviewing the literature on the therapeutic options for people infected with SARS and MERS-CoV, we introduced therapeutic options for SARS-CoV-2 infection. However, further clinical studies would clarify the value of our findings.

Author contributions

M Fani searched and wrote the primary manuscript, A Teimoori scientifically checked the manuscript and S Ghafari edited and scientifically checked the manuscript.

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Ethical conduct of research

This is a review article written based on a search of scientific databases.

Summary points

- Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) infects the upper and lower respiratory tract.
- SARS-CoV-2 Spike (S) protein had higher affinity to human angiotensin-converting enzyme 2 receptor than that of SARS-CoV.
- COVID-19 is a zoonotic disease similar to bat SARS infection.
- Viral genome sequencing confirmed that SARS-CoV-2 is 96.2% identical to some bat coronaviruses.
- Fever, cough and fatigue are common symptoms of COVID-19.
- Real-time reverse transcriptase-PCR of nasopharyngeal swab is routinely used to detect the SARS-CoV-2.
- Chloroquine and remdesivir showed the most powerful antiviral activities against SARS-CoV-2 infection.

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