Microbes and Infection xxx (xxxx) xxx

Contents lists available at ScienceDirect

Microbes and Infection

journal homepage: www.elsevier.com/locate/micinf

Measures for diagnosing and treating infections by a novel coronavirus responsible for a pneumonia outbreak originating in Wuhan, China

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ARTICLE INFO

Article history:
Received 24 January 2020
Accepted 24 January 2020
Available online xxx

Keywords:
2019-nCoV
Diagnosis
Preventive
Therapeutic

ABSTRACT

On 10 January 2020, a new coronavirus causing a pneumonia outbreak in Wuhan City in central China was denoted as 2019-nCoV by the World Health Organization (WHO). As of 24 January 2020, there were 887 confirmed cases of 2019-nCoV infection, including 26 deaths, reported in China and other countries. Therefore, combating this new virus and stopping the epidemic is a matter of urgency. Here, we focus on advances in research and development of fast diagnosis methods, as well as potential prophylactics and therapeutics to prevent or treat 2019-nCoV infection.

Coronaviruses (CoVs), which are enveloped, positive-sense, single-stranded RNA viruses of zoonotic origin and belong to the family Coronaviridae in the order Nidovirales, are divided into four genera: alpha, beta, delta and gamma coronavirus. The emerging CoVs, including severe acute respiratory syndrome coronavirus (SARS-CoV) and Middle East respiratory syndrome coronavirus (MERS-CoV), both belonging to beta coronavirus, have caused recent pandemics of respiratory infectious diseases with high mortality.

At the end of December 2019, the Wuhan Municipal Health Commission reported the outbreak of viral pneumonia caused by an unknown pathogen in Wuhan, China [1]. Subsequently, the unknown pathogen was identified as a novel coronavirus denoted as 2019-nCoV by the World Health Organization (WHO) on 10 January 2020 [1]. On 12 and 13 January 2020, the full genomic sequence of 2019-nCoV, denoted WIV04 strain (GISAID accession no. EPI_ISL_402124), was released, with about 82% homology to that of SARS-CoV Tor2 (GenBank accession no. AY274119) and bat SARS-like coronavirus WIV1 (bat SL-CoV-WIV1, GenBank accession no. KF367457.1).

By 24 January, this new emerging virus had caused 887 confirmed cases, including 26 deaths, in the original epidemic area, Wuhan, and other cities in China and in foreign countries. More seriously, 15 healthcare workers were infected with 2019-nCoV after close contact with one infected patient, suggesting human-to-human transmission of 2019-nCoV.

Improved molecular technologies made it possible to rapidly identify this novel coronavirus. In this review, we summarize advances made in technologies for rapid diagnosis and identification of respiratory infections caused by coronavirus, as well as strategies for research and development of vaccines, prophylactics and therapeutics to combat 2019-nCoV and other emerging coronaviruses now or in the future.

1. Rapid identification of an emerging coronavirus

Identification of pathogens mainly includes virus isolation and viral nucleic acid detection. According to the traditional Koch's postulates, virus isolation is the "gold standard" for virus diagnosis

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https://doi.org/10.1016/j.micinf.2020.01.003
1286-4579/

in the laboratory. First, viral culture is a prerequisite for diagnosing viral infections. A variety of specimens (such as swabs, nasal swabs, nasopharynx or trachea extracts, sputum or lung tissue, blood and feces) should be retained for testing in a timely manner, which gives a higher rate of positive detection of lower respiratory tract specimens. Then, immunological methods – including immunofluorescence assay, protein microarray, direct fluorescent antibody assay, MAb-based rapid NP (nucleocapsid protein) detection, semiconductor quantum dots, and the microneutralization test – which measure binding between the antigen from the whole virus or protein of the coronavirus and corresponding antibody, are easy to operate rapidly but have a lower sensitivity and specificity [3,4]. In addition, other immunological methods, including microneutralization ppNT assay (pseudo-particle neutralization) are highly sensitive and specific by using the gene coding for the coronavirus spike protein [5,6]. In the case of 2019-nCoV, viral research institutions can conduct preliminary identification of the virus through the classical Koch's Postulates or observing its morphology through an electron microscopy [7]. Serology could also be used to identify the virus when 2019-nCoV-associated antigens and monoclonal antibodies are developed in the future [7–9]. All the examples above are traditional virus detection methods.

Viral nucleic acids can also be used for early diagnosis. The following are some of the new coronavirus detection methods. Polymerase chain reaction (PCR) is a molecular biological diagnosis technology based on the sequence of nucleic acids. The full gene sequence of 2019-nCoV has now been obtained [10], so patients who are suspected of being infected with 2019-nCoV [8] can be diagnosed by pan-coronavirus PCR for virus identification [11]. Reverse transcription polymerase chain reaction (RT-PCR) is a technology combining RNA reverse transcription (RT) with polymerase chain amplification (PCR) of cDNA. A duplex RT-PCR assay can be used to detect SARS-CoV and MERS-CoV using pUC57SARS-pS2 and pGEM-MERSS2 as templates, respectively [12]. Also, samples collected from the upper respiratory tract (oropharyngeal and nasopharyngeal) and lower respiratory tract (endotracheal aspirate, expectorated sputum, or bronchoalveolar lavage) of suspected 2019-nCoV patients can be diagnosed by RT-PCR [8]. Reverse transcription-insulated isothermal polymerase chain reaction (RT-iiPCR), quantitative real-time reverse transcription polymerase chain reaction (qRT-PCR), Real-time RT-PCR (rtRT-PCR), and one-step rtRT-PCR were all further optimized [13–16]. These optimized RT-PCR methods were used to detect the MERS-CoV envelope gene (upE) and the open reading frame 1a (ORF1a) or open reading frame 1b (ORF1b) genes separately. However, rtRT-PCR was used to identify 2019-nCoV through preliminary and validation detection of its E gene, RNA-dependent RNA polymerase (RdRp) gene, and N gene [17].

There are other molecular-based detection techniques in addition to RT-PCR and similar optimized detection techniques. For example, reverse transcription loop-mediated isothermal amplification (RT-LAMP) is an RNA amplification technique that detects the N gene of MERS-CoV and the ORF1a gene [18]. One-pot reverse transcription loop-mediated isothermal amplification (one-pot RT-LAMP) is the optimized RT-LAMP [19], while RT-LAMP-VF is the deformation of RT-LAMP [20], which is the combination of reverse transcription loop-mediated isothermal amplification and vertical flow visualization strips. Both are used to detect the N gene of MERS-CoV, making detection easier, faster, more efficient and highly specific. Besides these three methods, reverse transcription recombinase polymerase amplification assay (RT-PRA) is also used to identify MERS-CoV [21].

Finally, the following multiplex tests can detect both coronaviruses and other viruses. MCoV-MS (multiplexed CoV mass spectrometry) uses an array matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) system to accurately identify known human coronaviruses (hCoVs) and to provide phylogenetic evidence for emerging unknown hCoVs [22]. Another new test method, arch-shaped multiple-target sensor, is used to amplify the target for rapid identification of pathogens in clinical samples [23]. The method can detect hCoVs, and Zika and Ebola viruses. The last one, the paper-based colorimetric assay, uses Pyrrolidinyl Peptide Nucleic Acid-induced silver nanoparticles (AgNPs) aggregation of pathogen DNA testing [24]. The color change of AgNPs can distinguish between MERS-CoV, *Mycobacterium tuberculosis* (MTB), and human papillomavirus (HPV).

2. Research and development of vaccines

The cellular receptors of SARS-CoV and MERS-CoV have been identified [25,26], and the virion spike (S) glycoprotein, was also well studied. S glycoprotein includes two subunits [27], S1 and S2, resulting from cleavage of the one precursor into two parts. S1 determines the virus host range and cellular tropism with the key functional domain - receptor binding domain (RBD), while S2 contains two tandem domains, heptad repeats 1 (HR1) and heptad repeats 2 (HR2), to mediate virus-cell membrane fusion. It is believed that the fusion process is similar to that of HIV-1 [28]; for example, when S1 binds to the receptor on the cell membrane, the fusion peptide at the N terminus of S2 inserts into the cell membrane, then three HR1s attach to each other in parallel as a trimer, followed by binding of three HR2s separately onto the outside of the trimer to form a 6-helix bundle, thus bringing virus and cell membranes close to each other to trigger fusion.

As the major vaccine target, the S protein has been evaluated in different types of vaccines against infection by CoVs [29]. Apart from the inactive whole virus particle [30], live attenuated virus with gene deletion [31] , four more vaccines which mainly contain S protein were studied. These include a virus-like particle which incorporated S protein into hepatitis virus or influenza virus protein [32,33]; virus vectors, such as modified vaccinia virus Ankara (MVA) or Adenovirus carrying S protein [34,35]; S protein subunit vaccine, like RBD-based protein [29,36]; and DNA vaccine which encodes the full length or part of the S protein gene [37,38]. Most of them have been tested in mouse models and showed the ability to elicit neutralizing antibodies. The first SARS-CoV DNA vaccine was tested in humans only 19 months after the virus sequence was published [38], while the DNA vaccine GLS-5300, the first MERS-CoV vaccine, went to clinical trials in 2016 [39]. In addition to these conventional vaccines, Liu et al. analyzed the T cell epitopes of SARS-CoV and MERS-CoV, revealed the potential cross-reactivity of the coronaviruses, and assessed the possibility of developing universal vaccines against coronavirus infections [40].

Most CoVs share a similar viral structure, similar infection pathway, and a similar structure of the S proteins [41], suggesting that similar research strategies should also be applicable for the 2019-nCoV. For example, the study of MERS-CoV vaccines was accelerated by virtue of strategies that had been established for SARS-CoV [42]. It has been reported that the 2019-nCoV is also genetically close to SARS-CoV [43,44]. Therefore, to predict whether vaccines developed for SARS-CoV will also be effective against 2019-nCoV infection, the full length S protein sequences from the 2019-nCoV, a SARS-CoV, and two genetically similar bat CoV strains were selected for alignment (Fig. 1). The results indicated more than 50% homology of the viruses. However, the most variable residues are located in S1, a critical vaccine target, implying that neutralizing antibodies that were so effective against SARS-CoV infection may fail to recognize the 2019-nCoV, and that multiple amino acid differences at the receptor binding motif may modify virus tropism, a possible reason for cross-species transmission.

Please cite this article as: F. Yu et al., Measures for diagnosing and treating infections by a novel coronavirus responsible for a pneumonia outbreak originating in Wuhan, China, Microbes and Infection, https://doi.org/10.1016/j.micinf.2020.01.003

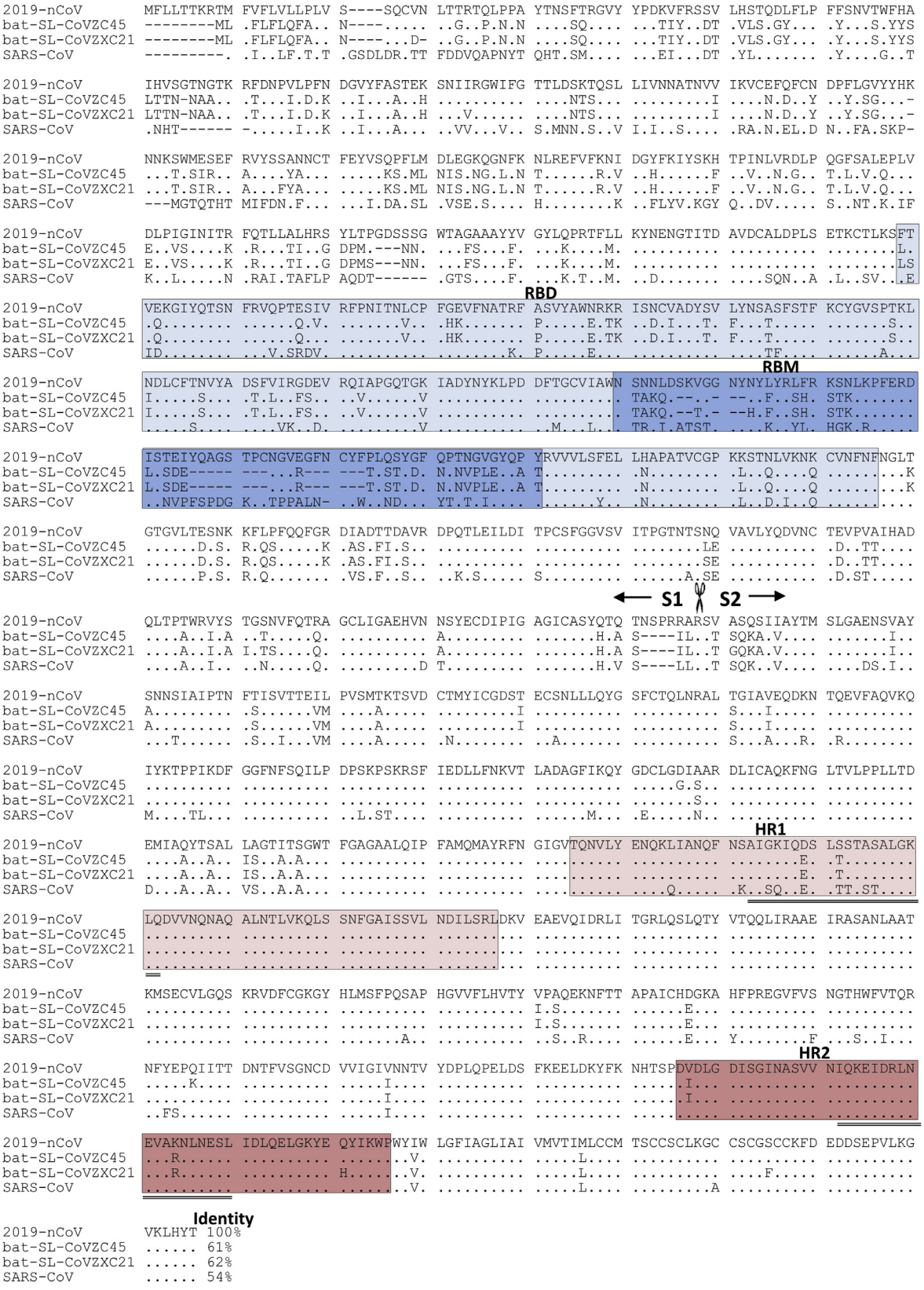

Fig. 1. Comparison of S protein sequences of coronaviruses. Multiple alignment of full amino acid sequences of S protein from 2019-nCoV (GISAID accession no. EPI_ISL_402124), SARS-CoV (GenBank accession no. AY278489), bat-SL-CoVZC45 (GenBank accession no. MG772933.1), and bat-SL-CoVZXC21 (GenBank accession no. MG772934.1) was performed and displayed with clustalx1.83 and MEGA4 respectively. "-" represents the unconfirmed amino acid residues, "." represents the identical amino acid residues. The functional domains were labeled based on the research on SARS-CoV [41]; light blue box was for RBD region; dark blue box for receptor binding motif (RBM); light purple box for HR1; and dark purple box for HR2, respectively. Double underlined regions in HR1 and HR2 are fusion cores, which are critical regions responsible for the formation of stable six-helical bundles between HR1 and HR2.

However, several bottlenecks typically delay the approval of vaccines to prevent CoVs infection. First, a lack of proper animal models for evaluating vaccine efficacy. Second, there are limitations from the S protein itself, such as mutations in the neutralization antibody epitopes in S protein that can cause virus escape [45], or non-neutralization antibody epitopes in vaccines that may elicit antibody-mediated disease enhancement (ADE) [46]. Third, DNA vaccines may recombine with other viruses. Fourth, pre-existing immunity may eliminate the vaccine by removing the general human virus vectors [47]. Finally, there is the problem of return on investment which may be slow and, hence, inhibit investments and slow down the clinical study.

Jiang and colleagues have demonstrated that RBD in the SARS-CoV S protein is the major target of neutralizing antibodies in SARS patients and is able to induce highly potent neutralizing antibody responses and long-term protective immunity in animal models. It contains 6 different conformational neutralizing epitopes, to which a series of mouse monoclonal antibodies (mAbs) with different neutralizing activity were generated. Interestingly, these mAbs exhibited cross-neutralizing activities against divergent SARS-CoV strains isolated from SARS patients at different stages of SARS epidemics in 2002−2004 and those from palm civets [48−52]. This group has also shown that these SARS-CoV-RBD-specific neutralizing mAbs can cross-neutralize bat SL-CoVs, such as bat SL-CoV-W1V1 [53], indicating that these antibodies may also cross-neutralize 2019-nCoV. Most importantly, RBD-based vaccine could induce neutralizing antibody responses and protection against SARS-CoV infection in the immunized animals, while it did not elicit ADE or other harmful immune responses, unlike the virus-inactivated vaccines or full-length S protein-based vaccines as discussed above. Therefore, this RBD-based SARS vaccine is expected to be safer and more effective than the vaccines targeting other sites in S protein. Jiang and Du's groups have collaborated with Hotez's group at Baylor College of Medicine in Houston and Tseng's group at the University of Texas Medical Branch at Galveston, Texas, USA in development of an effective and safe vaccine at the late stage of preclinical study [54]. The antibodies induced by this vaccine candidate are expected to cross-neutralize 2019-nCoV infection. If it is confirmed, this vaccine candidate has the great potential to be further developed promptly in clinical trials in both China and the United State through the continuous collaborations among the four groups of Drs. Hotez, Tseng, Du, and Jiang [55].

3. Research and development of therapeutics and prophylactics

At the present, no specific antiviral therapy has been approved for treatment of infection by human CoVs. As development of vaccines and compounds for prevention and treatment of infection have been brought to priority status by WHO and governments [56], numerous drug studies have been done or are moving forward. Some of them focus on the CoV fusion/entry process either by inhibition of S1 mediated virus attachment or by blocking of S2 mediated virus-cell membrane fusion, and some of them interfere with viral replication [57].

3.1. CoV fusion/entry inhibitors

Based on the previous experience in developing the HIV-1 fusion inhibitor SJ-2176 [58], Jiang et al. discovered the first anti-SARS-CoV peptide (SC-1) from the HR2 domain of SARS-CoV S protein S2 subunit. SC-1 could bind onto the HR1 domain to form a six-helical bundle (6-HB), blocking S protein-mediated membrane fusion and inhibiting SARS-CoV infection [59]. When MERS-CoV was circulating in human populations in 2012, following similar mechanistic design, Jiang's research group developed another peptide, designated HR2P, which was derived from the virus HR2 region as well and effectively inhibited MERS-CoV infection [60]. The further modified version of HR2P, HR2P-M2, presented even better anti-MERS-CoV activity and pharmaceutical properties.

Development of broad-spectrum pan-CoV fusion inhibitors would be an ideal way to cope with epidemics or pandemics caused by emerging HCoVs. The conservative amino acid sequence of the HR1 region across different CoVs has the potential to be a target domain for development of an inhibitor. Continuing to work on the HR1 and HR2 domains, Jiang's group discovered that the peptide OC43-HR2P, derived from the HR2 domain of HCoV-OC43, broadly inhibited fusion by multiple HCoVs. By optimization of this peptide, a pan-CoV fusion inhibitor, EK1, was generated. It could form a stable six-helix bundle (6-HB) structure with HR1s and showed significantly improved fusion-inhibitory activity and pharmaceutical properties [61]. The alignment of S protein in Fig. 1 exhibited 100% identity at the HR2 domains between the 2019-nCoV and SARS-CoV; however, they found 7 amino acid changes in the fusion core of the HR1, located in the EK1 binding motif. Fortunately, the substitutions were conservative replacements which would not dramatically disrupt the interactions between EK1 and HR1, meaning that EK1 would still have the potential to be an effective inhibitor for 2019-nCoV infection.

3.2. CoV S-RBD-specific neutralizing antibodies

So far, most neutralizing antibodies recognize the RBD in the S protein S2 of CoVs. Compared with the high mutation rate in the S1 protein, S2 is much more conservative, thereby decreasing the off-target risk caused by amino acid replacement [62], and also bypassing the special epitopes that may cause ADE [63]. This means that the cocktail of monoclonal antibodies binding to different epitopes of RBD would be more desirable for therapeutic purposes [64]. For treatment, the monoclonal antibodies are from a human source or are humanized antibodies, isolated or generated with various approaches. For example, wild-type mice were immunized with soluble recombinant RBD containing the S protein. Then mouse antibodies were humanized and isolated, or transgenic mice were directly immunized, to express human versions of the antibodies [50,65,66]. However, direct cloning of single B cells from human survivors, used in combination with the phage-display antibody library, could provide authentic human antibodies. Until now, it should be noted that many neutralizing antibodies have been successfully discovered for treatment of SARS-CoV [67] and MERS-CoV infection [45,68,69]. These antibodies have all been described favorably in the literature [29,70,71]. A similar approach is known as single chain fragment variable (scFv) library screening, whereby the use of RBD as a bait protein allows some neutralizing antibodies to be screened out from non-immune humans [72,73].

Antibodies effective at inhibiting SARS-CoV infection should also have the potential for treatment of 2019-nCoV as well, as long as the binding motif in RBD shares the same sequences. The new neutralizing monoclonal antibodies would also be isolated from the patients using the established techniques.

3.3. CoV replication inhibitors

Similar to developing vaccines, drugs effective against other RNA viruses were also repurposed for CoVs. Two major types of drugs being nucleoside analogues and immunomodulators. So far, the most common therapies tried in patients with CoVs are ribavirin, lopinavir/ritonavir, IFN, or their combinations [74]. Despite the antiviral activity observed with *in vitro* studies, the clinical effect was not consistent [75], in that ribavirin does not prolong the

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survival of SARS-CoV patients [74,76], while lopinavir/ritonavir plus ribavirin seemed to improve clinical outcomes for SARS patients [77], but the improvement was not confirmed in MERS-CoV patients. IFNs showed effective at inducing antiviral activity against both SARS-CoV and MRES-CoV, but without significant improvement in the outcomes for the patients [78,79]. In addition to the drug regimens used in patients, numerous drugs developed for the treatment of infection with CoVs were thoroughly discussed in the literature [57].

However, replication of an RNA virus usually generates progeny viruses with a highly diverse genome. Recombination also easily takes place between viral genomes [80], and these gene level changes may result in drug resistance if the mutations affect the drug target domain. Development of drugs is also hampered by various evaluation methods and animal models used for testing drug activity among different labs worldwide, which could postpone selection of the best drug for clinical trials.

4. Conclusion and prospects

Taken together, 2019-nCoV is a new coronavirus, and like SARS-CoV and MERS-CoV, it belongs to *Betacoronavirus*. Both SARS-CoV and MERS-CoV were able to spread around the globe and posed a major challenge to clinical management and a great threat to public health. Similarly to SARS-CoV and MERS-CoV, based on the monitoring and scientific forecast, 2019-nCoV may cause a worldwide threat to public health. Over the years, research on CoVs has resulted in multiple strategies for diagnosis, prevention and treatment of CoV infection. This brief review has demonstrated that such an achievement could very well apply to 2019-nCoV, or indeed, any newly emergent CoV in the future. At present, many companies engaged in the development of biologicals have marketed nucleic acid detection kits for 2019-nCoV, such as the new coronavirus nucleic acid detection kit (double fluorescence PCR method) from Shuoshi Biotechnology. Currently, however, no diagnostic test kit is available for the detection of antibodies to 2019-nCoV.

Declaration of Competing Interest

The authors declare that they have no conflicts of interest.

Acknowledgements

We thank Dr. Zengli Shi for providing us permission for analysis of the spike protein sequence of 2019-nCoV (GISAID accession no. EPI_ISL_402124) in this study.

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Please cite this article as: F. Yu et al., Measures for diagnosing and treating infections by a novel coronavirus responsible for a pneumonia outbreak originating in Wuhan, China, Microbes and Infection, https://doi.org/10.1016/j.micinf.2020.01.003