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Review Article

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Newly Emerging Human Coronaviruses: Animal Models and Vaccine Research for SARS, MERS, and COVID-19

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Conflict of Interest
The authors declare no potential conflicts of interest.

Abbreviations
3Clpro, picornavirus 3C-like protease; AAV, adeno-associated virus; ACE, angiotensin converting enzyme; Ad, adenovirus; ADE, Ab-dependent enhancement; ARDS,

ABSTRACT

The recent emergence of the novel coronavirus (CoV) or severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2) poses a global threat to human health and economy. As of June 26, 2020, over 9.4 million cases of infection, including 482,730 deaths, had been confirmed across 216 countries. To combat a devastating virus pandemic, numerous studies on vaccine development are urgently being accelerated. In this review article, we take a brief look at the characteristics of SARS-CoV-2 in comparison to SARS and Middle East respiratory syndrome (MERS)-CoVs and discuss recent approaches to coronavirus disease-2019 (COVID-19) vaccine development.

Keywords: Coronavirus; Vaccines; SARS; MERS; COVID-19; SARS-CoV-2

INTRODUCTION

Coronaviruses are positive-sense RNA viruses belonging to the family *Coronaviridae*. They are divided into 4 genera: alpha (α), beta (β), gamma (γ), and delta (δ) coronaviruses, based on their phylogenetic relationships and genomic structures. α- and β-coronaviruses infect only mammals whereas the γ- and δ-coronaviruses mainly infect birds (1). Typically, coronaviruses are known to cause only mild illnesses, like the common cold in humans, but the outbreak of severe acute respiratory syndrome (SARS) in 2002 (2) demonstrated that coronaviruses originating from other animal species may cross the species barrier and could become life-threatening pathogens in humans. A decade after the SARS outbreak, Middle East respiratory syndrome coronavirus (MERS-CoV), another pathogenic coronavirus, emerged in Saudi Arabia (3). Most recently, another β-coronavirus—severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2)—has been newly identified from a cluster of patients with severe pneumonia (4,5). To date, 2 α-coronaviruses (human coronavirus [HCoV]-229E and HCoV-NL63) and 5 β-coronaviruses (HCoV-OC43, HKU1, SARS-CoV, MERS-CoV, and SARS-CoV-2) have been identified that infect humans (1).

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acute respiratory distress syndrome; BALF, bronchoalveolar lavage fluid; COVID-19, coronavirus disease-2019; CRISPR/Cas9, clustered regularly interspaced short palindromic repeats-associated protein-9 nuclease; DAD, diffuse alveolar damage; DPP4, dipeptidyl peptidase-4; E, envelope; ERGIC, endoplasmic reticulum-Golgi intermediate compartment; ExoN, exoribonuclease; FDA, Food and Drug Administration; hACE2, human ACE2; HCoV, human coronavirus; HE, hemagglutinin esterase; ICU, intensive care unit; IP, IFN-γ-induced protein; ISG, interferon-stimulated gene; M, membrane; M1, matrix 1; MERS, Middle East respiratory syndrome; MV, measles virus; MVA, modified vaccinia virus Ankara; MyD88, myeloid differentiation primary response 88; N, nucleocapsid; NHP, non-human primate; NIH, National Institutes of Health; nsp, nonstructural protein; NTD, N-terminal domain; ORF, open reading frame; PLpro or PLP, papain-like proteases; RBD, receptor-binding domain; RdRp, RNA-dependent RNA polymerase; RNP, ribonucleoprotein; S, spike; SARS, severe acute respiratory syndrome; TIM-3, T-cell immunoglobulin mucin-3; VAERD, vaccine-associated enhanced respiratory disease; VLP, virus-like particle; VSV, vesicular stomatitis virus; WHO, World Health Organization.

Author Contributions

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GENOME, VIRION, AND LIFE CYCLE

Coronaviruses are enveloped positive single-stranded RNA viruses. They have 26–32 kb genomic RNA, which is the largest among the genomes of RNA viruses (6,7). The genomic structures of all coronaviruses are similarly arranged: replicase genes encoded within two-thirds of the 5′ end and genes encoding structural proteins in the other one-third of the 3′ end. Replicase genes, which occupy 20–22 kb of the entire coronavirus genome, is composed of open reading frame (ORF) 1a and ORF1b. ORF1a includes papain-like proteases (PLpro or PLP) and picornavirus 3C-like protease (3Clpro) genes, and ORF1b features viral RNA-dependent RNA polymerase (RdRp), helicase, and exoribonuclease (ExoN) genes (8). Upon viral infection, translation first begins from the 5′ end of ORF1 to create a huge complex of ORF1a polyprotein. At a lesser frequency, the −1 ribosomal frameshift occurs at the pseudoknot immediately before the termination codon of ORF1a, and translation resumes to constitute the ORF1ab polyprotein. Subsequently, the polyprotein is cleaved by the viral proteases into 16 mature nonstructural proteins (nsp1–16). As the frequency of frameshift is roughly 25%–30%, proteins encoded by ORF1b are produced in relatively smaller amounts compared to those by ORF1a. Nevertheless, ORF1b is the most conserved gene within the coronavirus genome, suggesting that ORF1b plays a crucial role in viral replication.

The latter one-third of the 3′ end of genomic RNA encodes the four major coronavirus structural proteins, including spike (S), envelope (E), membrane (M), and nucleocapsid (N) proteins, along with accessory proteins that are virus species-specific with functions that are not fully understood (**Fig. 1**) (6,7). S, E, and M proteins are displayed on the coronavirus virion surface. Certain coronaviruses have hemagglutinin esterase (HE) on their surface, but SARS- and MERS-CoVs do not. The S protein is presented on the viral surface as trimers and protrude, forming corona-like structure on the envelope. S consists of two functional subunits—S1 is responsible for the binding to host receptors and S2 for the fusion of viral and cellular membranes. The E protein, formerly called sM, plays a major role in viral assembly and release by interacting with M protein via its cytoplasmic tails. In addition, E is closely associated with viral pathogenesis by interfering with the formation and maintenance of tight junctions in the lung mucosal epithelium, leading to acute alveolar damage (9). M protein is the most abundant protein in the virus envelope. It consists of a short N-terminal glycosylated domain and three transmembrane domains followed by a long C-terminal tail. Moreover, M is localized within the intracellular membrane in the endoplasmic reticulum-Golgi intermediate compartment (ERGIC), where virion assembly and budding out occur, thereby controlling viral assembly by interacting with S and N proteins. N protein is a basic RNA-binding protein composed of three highly conserved domains: N-terminal, C-terminal, and RNA-binding domains. N binds to viral genomic RNA to form a helical capsid structure called the ribonucleoprotein (RNP) complex. The interaction of N, E, and M proteins drive the incorporation of the RNP into the assembling virions. In addition to RNP formation, N proteins play a critical role in enhancing the replication and synthesis of genomic RNA by interacting with nsp3.

NEWLY EMERGING HUMAN CORONAVIRUSES

SARS-CoV

SARS was first reported in Guangdong province, China in November 2002 and promptly spread worldwide, resulting in 8,096 cases, including 774 deaths in 29 countries (10). The

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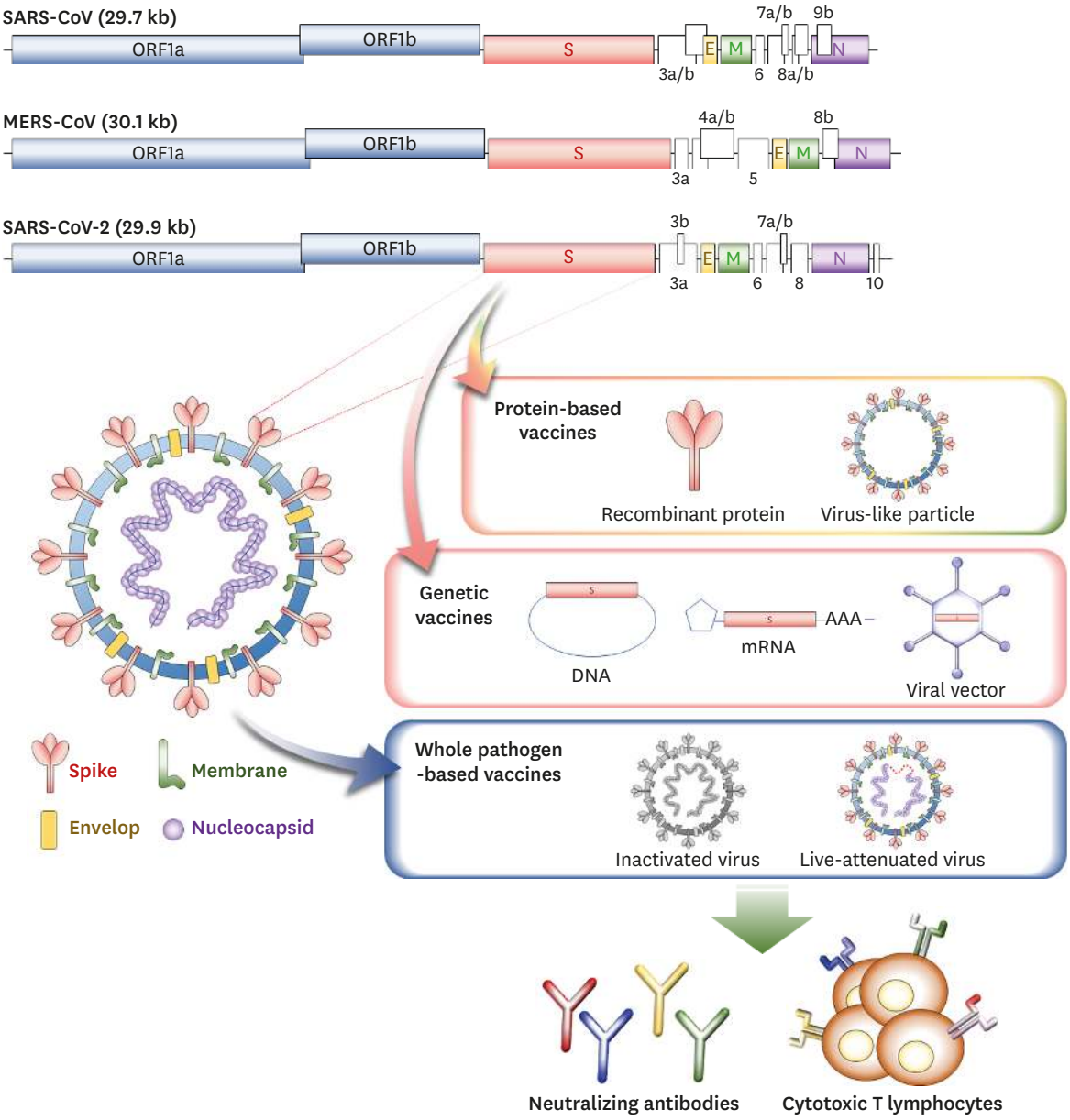

Figure 1. Genome structure of human coronaviruses (SARS-CoV, MERS-CoV, and SARS-CoV-2) and an overview of coronavirus vaccine platforms.

clinical symptoms of SARS are similar to those of other respiratory infections, like influenza. During the initial phase of infection, patients with SARS exhibited fever, cough, sore throat, and other mild symptoms, and some subsequently progressed to severe pneumonia (11). High levels of pro-inflammatory cytokines and chemokines were detected in the sera of SARS patients with severe disease (12). They also displayed low levels of anti-inflammatory cytokines, such as IL-10, compared to those of patients with mild symptoms.

The receptor binding domain (RBD) of S protein binds to host angiotensin-converting enzyme 2 (ACE2) for their entry into cells (13). ACE2 is expressed on a wide variety of body tissues and cells, including small intestine epithelium, arterial and venous endothelium, arterial smooth muscle, respiratory tract epithelium, alveolar monocytes, and alveolar

macrophages (14). Owing to the widespread expression of ACE2 throughout the body, SARS-CoV infects various tissues and causes lesions. Specifically, SARS-CoV primarily infects airway epithelial cells, resulting in the induction of large amounts of chemokines, such as CCL2, CCL3, CCL5, and CXCL10. The virus also infects hematopoietic cells, like dendritic cells (DCs) and macrophages. In such cases, DCs and macrophages exhibit the upregulated expression of pro-inflammatory cytokines and chemokines, such as TNF-α, IL-6, and CXCL10, but downregulated or delayed type I IFN (IFN-α/β) response (15). Consequently, the excess concentrations of pro-inflammatory molecules recruit various inflammatory immune cells into the lungs, leading to consolidation, hemorrhages, edema, and diffuse alveolar damage (DAD) (16).

MERS-CoV

Ten years after the SARS outbreak, MERS emerged in the Kingdom of Saudi Arabia in 2012. To date, 2,519 MERS cases with 866 deaths have been confirmed in 27 countries across the Middle East, Asia, and Europe (17). In 2015, 186 cases of infection, including 38 deaths, were reported in South Korea (18), which was the most unique and largest MERS outbreak outside the Arabian Peninsula.

Major clinical symptoms of MERS are fever, non-productive cough, dyspnea, myalgia, and sore throat (19). Unlike patients with influenza and SARS, certain patients with MERS distinctively presented gastrointestinal symptoms, including diarrhea and vomiting (20). The majority of MERS patients progressed to severe pneumonia; particularly, immunocompromised individuals and patients with comorbidities exhibited high incidences of acute respiratory distress syndrome (ARDS) and renal dysfunction (21). Similar to SARS, in MERS patients, a dysregulated immune response is thought to be the cause of pathological changes, such as extensive infiltration of immune cells into the lungs (22).

In contrast to the case of SARS, MERS-CoV particles or MERS-CoV-specific Abs were detected in a large number of dromedary camels in the Middle East and North Africa (23). This strongly indicates that the virus, which is thought to originate from bats (24,25), has been circulating for more than several decades in dromedary camels in those areas, which may be a reason for continuing zoonotic transmission of MERS-CoV. Human-to-human transmission of MERS-CoV mainly occurs through the nosocomial route, especially between hospitalized patients (26), probably because virus shedding takes place efficiently after the onset of disease symptoms.

MERS-CoV infects host cells by interacting with dipeptidyl peptidase 4 (DPP4) or CD26 (27). DPP4 is expressed in the respiratory tract epithelium, kidney, small intestine, liver, prostate, and also on activated leukocytes (28). The virus primarily infects airway epithelial cells, resulting in delayed IFN responses and upregulated pro-inflammatory cytokines, such as IL-6, IL-1β, and IL-8 (29). MERS-CoV-infected macrophages and DCs also produce high levels of pro-inflammatory cytokines and chemokines, such as CCL2, CCL3, CCL5, and IL-2 (30,31), and the concentration of these soluble factors closely correlates with disease severity (32). Increased numbers of neutrophils and monocytes were observed in the lungs of these types of patients (22), indicating that these cells are responsible for lung immunopathology.

SARS-CoV-2

In December 2019, severe cases of pneumonia with unknown etiology were reported in Wuhan, China (4). As the causative agent of the disease was identified as a coronavirus, the

disease and virus were named as coronavirus disease-2019 (COVID-19) and SARS-CoV-2, respectively (5,33). As the virus drastically disseminates on a global scale, the World Health Organization (WHO) declared a global pandemic on 11th March 2020. To date, over 9.4 million cases of SARS-CoV-2 infection, including 482,730 deaths, have been reported, and the infection curve is still rising at a steep angle (34).

SARS-CoV-2 was determined to be a lineage B β-coronavirus, sharing 79% of genome sequence identity with SARS-CoV (35). It is thought to originate from bats, like other human β-coronaviruses, and to be transmitted to humans through probable, but unproven, intermediate hosts, such as snakes or pangolins (36,37). However, despite ongoing studies, the hosts have not been specified to date, suggesting transmission took place incidentally, like in the case of SARS-CoV.

Similar to SARS-CoV, the S protein of SARS-CoV-2 binds to ACE2 as its receptor (38,39), and subsequently trigger fusion of viral and cellular membranes, thereafter entering host cells. As such, amino acid sequence and distribution of ACE2 is the major determinant of host and cell tropism (40). In the human body, ACE2 is expressed at a high level in the small intestine, testis, and kidneys, and at a relatively low level in the lungs and heart in healthy individuals. However, in the case of smokers and patients with heart conditions, ACE2 levels are elevated compared to that in the healthy (41,42), partially accounting for the high pathogenicity in those populations.

Most patients with COVID-19 exhibit mild to moderate clinical symptoms, such as fever and dry cough, and sometimes muscle and/or joint pain (43). However, the elderly or individuals with underlying diseases, such as asthma, heart conditions, and diabetes, are more vulnerable to SARS-CoV-2, leading to severe pneumonia or ARDS, the main cause of COVID-19-related death (43). Other less common symptoms have also been reported, such as gastrointestinal symptoms (44) and loss of taste or smell (45).

Upon infection, SARS-CoV-2 activates the innate immune system and induces pro-inflammatory cytokines and chemokines in the lungs along with recruitment of monocytes and T cells (43). In most healthy individuals, this local immune response contributes to the clearance of viral infection, but in patients with preconditions, dysregulated immune response results in massive infiltration of immune cells, respiratory failure, or systemic inflammation. In particular, unlike other respiratory viruses causing mild symptoms, SARS-CoV-2 is unique, as it drives low type I and III interferon levels and a moderate IFN-stimulated gene (ISG) response (46,47). Consistent with these observations, SARS-CoV-2 ORF3b, a 22-amino acid-long nonstructural protein, exhibited a significantly stronger antagonistic activity against type I IFN induction than that displayed by SARS-CoV ORF3b ortholog (48). Contrary to this weak antiviral response, inflammatory response represented by the production of proinflammatory cytokines and chemokines, such as IL-6, CCL8, and CXCL9, was markedly elevated under SARS-CoV-2 infection both *in vitro* and *in vivo* (46). Patients with severe COVID-19 also exhibited abundant distribution of proinflammatory monocyte-derived macrophages in the bronchoalveolar lavage fluid (BALF) (49) and high serum levels of proinflammatory cytokines and chemokines, such as IL-1β, IL-2, IL-6, IL-8, IL-17, IFN-γ-induced protein (IP)-10, MCP-1, TNF-α, G-CSF, and GM-CSF (43,50,51). This imbalanced—low antiviral but high inflammatory—host response is believed to be a major factor affecting disease outcome.

In addition to uncontrolled innate immune responses, impaired adaptive immune responses can affect disease severity. The number of lymphocytes including T, B, or NK cells was significantly reduced in patients with severe COVID-19 requiring intensive care unit (ICU) care (50-52). Decreased T cell number has also been observed in patients with SARS (53), and MERS-CoV has been reported to infect human T lymphocytes and activate apoptotic pathways in the infected cells (54). Further investigation is required to elucidate the reason underlying the decrease in the number of lymphocytes in patients with severe COVID-19. It is intriguing that T cells from patients with COVID-19 highly express PD-1 and T-cell immunoglobulin mucin-3 (TIM-3), which are exhaustion markers (52). Additionally, the frequency of T and NK cells producing CD107a, IFN-γ, IL-2, and granzyme B was significantly reduced in patients with severe infection, compared to those from healthy controls (55). However, despite the increased exhaustion marker levels and decreased cellular function, it should be carefully defined whether the T cells are really "exhausted" by continuous antigenic stimulation through Ag-specific TCRs.

ANIMAL MODELS

To elucidate the mechanisms of viral pathogenesis and develop optimal prophylactic and therapeutic strategies for newly emerging human coronaviruses, several animal models have been developed and evaluated (**Table 1**).

SARS-CoV and SARS-CoV-2

As mentioned earlier, SARS-CoV and SARS-CoV-2 enter target cells by binding to ACE2 as their receptor (38-40). In mice, SARS-CoV is able to interact with murine ACE2 and replicate in mouse tissues, including the lungs and small intestine (57), but disease symptoms are limited to mild respiratory disease and minimal bodyweight loss that is less than 5%. Aged mice present a relatively larger number of severe symptoms than young mice (59-61). To improve the availability of a murine SARS model, mouse-adapted SARS-CoV strains (149,150) and transgenic mice expressing human ACE2 (hACE2) were developed (62,63). Myeloid differentiation primary response 88 (MyD88) as well as STAT1 knock-out mice presented severe respiratory diseases, like pneumonitis and bronchiolitis, along with reduced survival compared to wild-type mice (58,64,65), suggesting that innate immunity involved with these molecules plays an important role in the clearance of SARS-CoV.

In addition to mice, various other animal models are available for SARS-CoV studies. Golden Syrian and Chinese hamsters (66) and ferrets (67,68) are susceptible to SARS-CoV infection and display moderate to severe respiratory symptoms. SARS-CoV infects non-human primates (NHPs), including rhesus macaques, cynomolgus macaques, African green monkeys, common marmosets, squirrel monkeys, and mustached tamarins because these NHP species express a form of ACE2 closely related to that of humans (69-71). More importantly, the virus successfully replicates in these NHPs and causes severe symptoms, like fever, pneumonitis, diarrhea, and hepatitis (72).

As SARS-CoV-2 was revealed to also utilize ACE2 for viral entry (38-40), SARS animal models were promptly tested in SARS-CoV-2 studies. hACE2 transgenic mice exhibited moderate interstitial pneumonia (84), and Golden Syrian hamsters presented clinical symptoms and histopathological findings closely resembling what is observed in humans (85). In ferrets, SARS-CoV-2 viral RNA was detected in the nasal turbinate, soft palate, and

Table 1. Epidemiology, biological characteristics, and vaccine studies of SARS-CoV, MERS-CoV, and SARS-CoV-2

	SARS	MERS	COVID-19
Emergence	2002 Nov Guangdong, China	2012 Jun The Kingdom of Saudi Arabia	2019 Nov Wuhan, China
Areas affected [No. of countries]	China, Hong Kong, etc. [29 countries]	Middle East, Korea, etc. [27 countries]	Worldwide [216 countries]
Cases (Death, case fatality rate)	8,096 (774, 9.6%) (10)	2,519 (866, 34.3%) (17)	9.4 million (482,730, 5.1%) (34)
Common symptoms	Fever, dry cough, shortness of breath, myalgia (11)	Fever, cough, shortness of breath, diarrhea, nausea vomiting (19)	Fever, dry cough, shortness of breath or difficulty breathing, loss of smell or taste (43-45)
Etiologic agents	SARS-CoV	MERS-CoV	SARS-CoV-2
Reservoirs → Intermediate hosts	Bats → Palm civets (56)	Bats (24) → Dromedary camels (23)	Bats (35) → Snakes (?) (36), pangolins (?) (37)
Host receptor	ACE2 (13)	DPP4 (27)	ACE2 (38-40)
Animal models	Mice (C57BL/6, BALB/c, 129S) (57,58), aged mice (59-61), hACE2 transgenic mice (62,63), knock-out mice (MyD88, STAT1, Rag1, etc.) (57,58,64,65), golden Syrian hamsters (66), ferrets (67,68), NHPs (rhesus macaques, cynomolgus macaques, African green monkeys, common marmosets, squirrel monkeys, and mustached tamarins) (69-72)	rAd-hDPP4-transduced mice (73), hDPP4 transgenic (74-76) or knock-in mice (77,78), camelid (76,79,80), NHPs (rhesus macaques, common marmosets) (76,81-83)	hACE2 transgenic mice (84), golden Syrian hamsters (85), ferrets (86,87), NHPs (rhesus macaques, cynomolgus macaques) (88,89), rAd-hACE2-transduced mice (90)
Vaccines			
Live-attenuated virus vaccine	rSARS-CoV-ΔE (91,92), nsp16 mutant SARS-CoV (D130A) (93)	rMERS-CoV-ΔE (94), nsp16 mutant MERS-CoV (D130A) (95), MERS-CoV-Δ3, MERS-CoV-Δ4ab, MERS-CoV-Δ5 (94)	-
Inactivated whole-virus vaccine	Inactivated with UV light (96,97), formaldehyde (97), or β-propiolactone (98)	Inactivated by gamma (γ) irradiation (99) or formaldehyde (100)	Inactivated with β-propiolactone (PiCoVacc) (101) Inactivated SARS-CoV-2* - Phase 1/2: ChiCTR2000031809 - Phase 1/2: ChiCTR2000032459 - Phase 1: NCT04412538 Inactivated SARS-CoV-2 with alum* - Phase 1/2: NCT04352608 - Phase 1/2: NCT04383574
Recombinant protein	Recombinant S, S1, RBD, trimeric form of S and RBD proteins (102-106)	Recombinant S, RBD, trimeric RBD, NTD proteins (107-115), S protein nanoparticles (116-118)	S protein nanoparticles with Matrix-M* - Phase 1/2: NCT04368988
Virus-like particle	VLP exhibiting S, M, and E proteins (119-121) Chimeric VLP consisting of S and influenza M1 (122)	VLP exhibiting S, M, and E proteins (123)	-
DNA vaccine	S, S1, RBD, N antigens (98,124-128)	S, S1 antigens (114,129,130)	S Ag (131,132) INO-4800* - Phase 1: NCT04336410 GX-19† - Phase 1/2a: NCT04445389
RNA vaccine	No investigated vaccine	No investigated vaccine	Lipid nanoparticle (LNP) encapsulated mRNA-1273* - Phase 1: NCT04283461 - Phase 2: NCT04405076 3LNP mRNA-BNT162* - Phase 1/2: 2020-001038-36 - Phase 1/2: NCT04368728 LNP-nCoVsaRNA* - Phase 1: ISRCTN17072692
Viral vector-based vaccine	rAd/S or N, rMVA/S (98,133-135)	Human rAdV/S, chimpanzee rAdV (ChAdOx1)/S, rMVA/S or N (136-147)	rAd5/S* - Phase 1: ChiCTR2000030906 - Phase 2: ChiCTR2000031781 ChAdOx1-S* - Phase 1/2: 2020-001072-15 - Phase2b/3: 2020-001228-32 - Phase 3: ISRCTN89951424 Gam-COVID-Vac and Gam-COVID-Vac Lyo* - Phase 1: NCT04436471 - Phase 1: NCT04437875

The list of clinical trials for COVID-19 vaccine has been adopted from *the database of the World Health Organization (148) and †the U.S National Library of Medicine (www.ClinicalTrials.gov).

tonsil, but the viral infection induced only mild clinical symptoms (86). In NHP models, the virus was excreted from the respiratory tract and detected in multiple organs in virus-infected cynomolgus macaques, but they did not develop any clinical signs (88). Aged rhesus macaques exhibited more severe and diffuse pneumonia along with serious inflammatory responses versus young monkeys (89), suggesting the age is a decisive factor in both NHP models and humans. SARS-CoV-2 susceptibility was also investigated in domesticated animals—SARS-CoV-2 can efficiently replicate in cats but does so poorly in dogs, pigs, chickens, and ducks (87). Most recently, a new mouse model using recombinant adenovirus 5 expressing hACE2 (Ad5-hACE2) was reported (90), which is similar to the model developed for a MERS study by the same group (73). Upon intranasal transduction with Ad5-hACE2, mice transiently expressed hACE2 in their respiratory tract and exhibited significant viral replication and lung inflammation upon subsequent SARS-CoV-2 infection.

MERS-CoV

MERS-CoV employs host cellular DPP4 as its receptor for entry (27). Whereas humans and NHPs are susceptible to MERS-CoV infection, hamsters, ferrets, and mice are not because of differences in major amino acid sequences of DPP4 (151-153). The first model for MERS-CoV study was based on mice transduced with recombinant adenovirus expressing human DPP4 (Ad5-hDPP4) (73). The Ad5-hDPP4-transduced mice developed clinical symptoms including pneumonitis and lung edema upon MERS-CoV infection. Global or tissue-specific hDPP4 transgenic mice were successfully infected with MERS-CoV and displayed respiratory symptoms and weight loss (74,75). hDPP4 knock-in mice have also been developed within which murine DPP4 is replaced by hDPP4 using CRISPR/Cas9 (77,78).

In addition to these mouse models, dromedary camels, an intermediate animal in MERS-CoV transmission, and alpacas were tested for a MERS study. Although they were susceptible to the virus, they were asymptomatic or exhibited, if any, only mild respiratory symptoms (79,80). In NHPs, MERS-CoV effectively infected the host cells and replicated within their lungs (81-83), but disease severity was higher in the common marmosets than that in rhesus macaques. Furthermore, symptoms observed in severe patients, such as lung consolidation and liver or kidney failure, were reproduced only in common marmosets (76), indicating that they are a more reliable animal model for MERS study.

VACCINE RESEARCH

Although there are still no approved vaccines for SARS and MERS, studies on the two previous coronaviruses provided important information about a strategy and considerations for COVID-19 vaccine development. First, as S protein, especially RBD, was known to be responsible for binding to host receptors, it has been extensively evaluated as a primary target Ag for vaccine development. Second, the majority of vaccine studies have utilized various recombinant vaccine platforms, including recombinant proteins, nucleic acids, and virus-vectored vaccines, rather than conventional live-attenuated or inactivated virus platforms (**Fig. 1** and **Table 1**).

Live-attenuated virus vaccines

As the live-attenuated virus vaccine is composed of almost all proteins of the virus, immune responses induced by attenuated virus vaccination are most similar to those by real viral infection (154). In a conventional method, live-attenuated virus has been made by serial culture of the virus, which leads to a spontaneous deletion of or mutation within a pathogenic gene.

However, in recent studies, recombination technology-based modification of the target gene has been more widely studied in coronavirus vaccines. Among the coronavirus proteins, E and nsp16 have been thought of as the most potential targets because of their potential association with the virulence *in vivo* (9). Immunization of engineered mutant SARS-CoV lacking the E protein (rSARS-CoV-ΔE) provided protective immunity in hACE2 transgenic mice and golden Syrian hamsters against viral challenge (91,92). E gene-deleted MERS-CoV (rMERS-CoV-ΔE) was able to replicate only by providing E protein in trans, but unable to propagate *in vivo* (94). Recombinant MERS-CoV lacking the accessory genes 3, 4a, and 5, was also replication-competent in vitro but propagation-defective in vivo, indicating that recombinant MERS-CoV could be a safe and promising vaccine candidate (94). SARS- and MERS-CoVs harboring a D130A mutation in each nsp16 gene (SARS-CoV [D130A] and MERS-CoV [D130A]) induced a neutralizing Ab response and protected against lethal virus challenge in young animals without any pathologic symptoms (93,95). Currently, 2 live-attenuated COVID-19 vaccines based on codon-deoptimization are under preclinical development (148).

Inactivated whole-virus vaccines

Inactivated whole-virus vaccines are prepared by inactivation of the cultured virus by heat, ultraviolet (UV), or chemicals, such as formalin. When compared to a live-attenuated virus vaccine, they can be produced relatively quickly and easily upon the emergence of a new or variant virus. In coronavirus vaccine studies, inactivated virus vaccine induced high-titer neutralizing Abs and cell-mediated immune responses. Immunization with SARS-CoV inactivated with UV light (96,97), formaldehyde (97), and β-propiolactone (98) induced potent neutralizing Abs and $CD4^+$ and $CD8^+$ T cell responses in mice and rabbits. In addition to SARS-CoV, MERS-CoV treated with γ-irradiation or formaldehyde also offered protection against MERS-CoV infection (99,100). Some studies have shown that inactivated SARS- and MERS-CoV vaccines were efficacious in protection from challenge in mice and NHPs (155-158). The efficacy of an inactivated virus COVID-19 vaccine has been tested in mice, rats, and rhesus macaques (101). Currently, at least nine research groups are developing COVID-19 vaccines using an inactivated virus platform. Among them, four institutions in China (Sinovac, Wuhan Institute of Biological Products, Beijing Institute of Biological Products, and Chinese Academy of Medical Sciences) have started clinical studies (148).

Recombinant protein vaccines

Recombinant protein vaccines have long been studied and assessed in terms of efficacy and safety. Many researchers have also evaluated human coronavirus vaccines, mainly focusing on S and RBD proteins of SARS- and MERS-CoVs. Immunization with the full-length, extracellular domain of S proteins or Fc-fused RBD proteins of SARS-CoV induced potent neutralizing Abs in mice and/or rabbits (102-105). Trimeric S or RBD proteins also induced humoral and cellular immune responses and provided protection against SARS-CoV infection in hamsters (106). Similar to the results from SARS vaccine studies, recombinant S, RBD, Fc-fused RBD, and trimeric RBD proteins of MERS-CoV also elicited neutralizing Abs in various animal models, including mice and monkeys, and exhibited protective effects upon viral challenge (107-114,116). Immunization of the N-terminal domain (NTD) of the MERS-CoV S protein also led to protection against MERS-CoV challenge in a transient hDPP4-expressing mice model (Ad5-hDPP4 mice) (115). In most of these experiments, recombinant protein Ags were used with an adjuvant, such as alum and MF59, to increase Ab or cell-mediated immune responses. Intriguingly, MERS-CoV S proteins alone self-assemble into nanoparticles of a size of approximately 25 nm. With this, the S nanoparticles effectively induce neutralizing Abs and Th1-type cellular immune responses in mice and NHPs (116,123), providing

protection against MERS-CoV infection in mice (117). In addition, heterologous prime-boost vaccination with adenoviral vector-expressing S and S nanoparticles led to balanced Th1/Th2 responses and safeguarded mice from MERS-CoV challenge (118).

In terms of SARS-CoV-2, Novavax (Gaithersburg, MD, USA) is developing a spike nanoparticle vaccine at phase 1/2 clinical stage, and another 50 institutions are working on recombinant protein vaccines focusing on S or RBD proteins at a pre-clinical stage (148). One of the most outstanding advances in the recent study of recombinant protein vaccines is the design of a prefusion form of viral surface Ags based on structural biology. Target Ags expressed as a stable prefusion form induced more potent and high-affinity neutralizing Ab responses than wild-type proteins in various infectious disease models (159-162). Several institutions, including Queensland University (Brisbane, Australia) and Clover Biopharmaceuticals (Chengdu, China), are applying this technology to COVID-19 vaccine development.

Meanwhile, for the optimal efficacy and dose sparing of recombinant protein Ags, it is essential to develop a vaccine in combination with an appropriate adjuvant. In previous SARS and MERS vaccine studies, the effects of diverse adjuvants such as alum (106,108,109,115,116,118,123), MF59® (109,110,112), Matrix™ M (116,117), Montanide ISA™ 51 (109,111,113), and monophosphoryl-lipid A plus trehalose dicorynomycolate (MPL® + TDM) (102,104,114), have been widely tested. During the development of a recombinant protein-based COVID-19 vaccine, the choice of an adjuvant would be a considerable factor affecting the quality of the immune response, the efficacy and safety of the vaccine, and the economic feasibility of the developer.

Virus-like particle (VLP) vaccines

VLPs are nano-sized particles composed of viral proteins with self-assembly properties. They mimic the morphology of a real virus particle but do not replicate owing to the lack of genomic material. As VLPs maintain the ideal conformation of native Ags, they can elicit an appropriate and strong immune response (163).

VLPs of SARS- and MERS-CoVs were produced by coexpressing the S, E, and M proteins from insect or mammalian cells (119,123), and these VLPs induced potent neutralizing Abs and Th1-biased cellular immune responses in mice and NHPs (120,121,123). Interestingly, SARS-CoV S and influenza virus matrix 1 (M1) coexpression efficiently formed chimeric VLP and induced protective immunity against SARS-CoV (122). At present, ten COVID-19 vaccine candidates are under pre-clinical investigation on the basis of VLP technology (148).

DNA and RNA vaccines

Since it was first reported that immunization of naked plasmid DNA encoding foreign protein induces an Ag-specific immune response in mammals in the early 1990s (164), DNA vaccines have been widely tested in various pathogen models. Although DNA vaccines have not been approved for humans to date, their immunogenicity and therapeutic effects have long been tested across various clinical trials for infectious diseases as well as human papillomavirus-mediated cervical intraepithelial neoplasia (165,166). DNA vaccines expressing full-length or truncated forms of SARS-CoV S protein induced humoral and cellular immunity, supplying protection against SARS-CoV infection in murine models (98,124,125). Further, heterologous prime-boost immunization with DNA vaccines and inactivated SARS-CoV induced strong $CD4^+$ T cell and Ab responses (126). Meanwhile, DNA vaccines encoding SARS-CoV N proteins induced Abs and T cell responses in mice, but their protective efficacy has not been fully investigated (127,128). In MERS vaccine studies, immunization of full-length or

S1 subunit-expressing plasmids also induced neutralizing Abs and T cell responses in mice, camels, and NHPs (114,129,130), while also alleviating clinical symptoms upon MERS-CoV infection in a rhesus macaque model (129).

mRNA vaccines are the most recent vaccine technology characterized by rapid development and production along with high potency. Recently, encapsulation and delivery methods have been substantially improved—the use of carrier molecules, such as liposomes, cationic polymers, and polysaccharide particles, significantly increases delivery efficacy, allowing for rapid uptake and high expression of target Ags. Owing to the safety, potent efficacy, as well as mass and prompt producibility, mRNA vaccines are being extensively evaluated in various infectious diseases and cancers (167). Yet, no striking results have been reported in SARS and MERS vaccine studies.

Currently, 12 DNA-based and 19 RNA-based COVID-19 vaccine candidates are under investigation and development (102). Moderna (Cambridge, MA, USA), together with the Vaccine Research Center at National Institutes of Health (NIH) of the USA, has promptly begun a phase 1 clinical trial, the first clinical study in COVID-19 vaccines, using mRNA vaccine, mRNA-1273. The immunogenicity and/or protective efficacy of COVID-19 DNA vaccine candidates has been evaluated in mice and rhesus macaques (131,132), and INOVIO Pharmaceuticals (Plymouth Meeting, PA, USA) and Genexine (Seongnam, South Korea) are undergoing phase 1 to 2a clinical trials (ClinicalTrials.gov: NCT04445389) (148).

Viral vector-based vaccines

As vesicular stomatitis virus (VSV)-based Ebola vaccine (ERVEBO®) has been approved for human use by the Food and Drug Administration (FDA) of the USA in 2019 (168), the use of viral vectors for vaccines against infectious diseases appears to be more flexible than how it was before. Viral vector-based vaccines are able to induce strong and rapid Ab and cell-mediated immune responses, and several viral vectors have been developed to date, including VSV, modified vaccinia Ankara (MVA), adenovirus (Ad), and adenovirus-associated virus (AAV) (169). In coronavirus vaccine studies, Ad and MVA are the most frequently employed. Replication-defective adenoviral vector expressing S and N proteins of SARS-CoV elicited humoral and cellular immune responses in mice (98,170). MVA expressing SARS-CoV S protein induced neutralizing Ab responses in mice, ferrets, and NHPs (133-135), and reduced lung viral titer in SARS-CoV-challenged mice (135). Immunization with adenoviral vector encoding S protein of MERS-CoV induced systemic neutralizing Abs and T cell responses (136,137). To avoid pre-existing immunity against human adenovirus, chimpanzee adenovirus (ChAdOx1) was utilized in recent vaccine development (138). The ChAdOx1 vector encoding MERS-CoV S induced neutralizing Abs and T cell responses in hDPP4 transgenic mice (139,140), and reduced virus shedding and nasal discharge in dromedary camels upon MERS-CoV infection (141). MVA encoding the S protein of MERS-CoV also induced neutralizing Abs and T cell responses, protecting Ad-hDPP4-transduced mice and camels from challenge with MERS-CoV (139,142,143). MVA expressing N protein of MERS-CoV elicited $CD8^+$ T cell responses in mice, but its protective efficacy was not determined (144). In addition to adenovirus and MVA, several viruses, such as Newcastle disease virus, live-attenuated measles virus (MV), rabies virus, Venezuelan equine encephalitis virus, and VSV have also been investigated in the context of MERS vaccine studies ((73,145-147,169).

A total of 37 vaccine institutions are developing viral vector-based COVID-19 vaccines, and among them, University of Oxford (Oxford, UK) and CanSino Biological (Tianjin, China) are

performing phase 1 to 2b/3 clinical trials using a replication-deficient chimpanzee adenovirus (ChAdOx1) and adenovirus type 5 (Ad5), respectively (148).

Lessons and remaining questions in SARS and MERS studies

Based on the reports introduced above, the Ab response inhibiting the interaction between S or RBD and the corresponding receptors is sufficient for the prevention of SARS- and MERS-CoV infection. Passive transfer of human monoclonal Abs also provided considerable protection against subsequent viral challenge in mice (171-173). Taken together, these results suggest that S is a promising target Ag for coronavirus vaccines. Meanwhile, the contribution of T cell immunity for the prevention and clearance of the virus has been widely advocated. $CD8^+$ T cells play a crucial role in viral clearance by secreting effector molecules directly to infected cells (73,174,175). Airway $CD4^+$ T cells also mediate protective immunity against SARS- and MERS-CoV infection through rapid production of IFN-γ (176). However, the long-term efficacy and safety of SARS or MERS vaccines in humans has not been tested to date. Moreover, in some animal studies, vaccine-induced or monoclonal S-specific neutralizing Abs markedly enhanced the infectivity of SARS- and MERS-CoVs (177,178), necessitating further dedicated investigation.

FACTORS TO BE CONSIDERED

As discussed previously, vaccine technology has significantly advanced over the last several decades. We also have gained useful information and materials for the study of vaccines for the novel coronavirus—how the virus enters the host cells, which Ag we should target, and what kind of animal models we can use. From the aspect of a pre-clinical study, some scientists appear to already have several successful vaccine candidates. However, in terms of a COVID-19 vaccine that is applicable to humans, several factors remain to be considered and intensively investigated.

First, safety issues must be initially evaluated. Certain vaccine formulations have induced sub-optimal Abs and inappropriate Th2-mediated immune responses, leading to Ab dependent enhancement (ADE) and/or vaccine-associated enhanced respiratory disease (VAERD) (99,179-183). Additionally, each candidate should be also tested for toxicity in rats or rabbits. Although nucleic acid vaccines are regarded as safe in the aspect of nonclinical toxicology, long-term safety of an RNA vaccine in humans should be carefully investigated.

The next factor to consider during COVID-19 vaccine development is efficacy, particularly in the elderly and immunocompromised. The mortality of the disease manifests a close correlation with age: less than 1.0% under the age 50, but significantly increasing up to 1.25, 3.99, and 8.61% in the 50s, 60s, and 70s, respectively, and surpassing 13% over 80 (184). This strongly suggests that the primary target population for COVID-19 vaccine should be the elderly. In the case of conventional vaccines, high-dose or adjuvanted vaccines are recommended to enhance weak immune responses in those populations (185-187). Taking this into account, the efficacy of COVID-19 vaccines in the elderly or immunocompromised must be carefully assessed. Nevertheless, even if a COVID-19 vaccine is unsatisfactory in those populations, it might still be beneficial because of indirect protection by establishing "herd immunity".

Other important questions to be addressed are how each immune response contributes to the protection against or clearance of the virus and for how long this effect can last

following vaccination or natural infection and recovery. In most pre-clinical vaccine studies, immunization of an S Ag or a part of it efficiently induced a potent neutralizing Ab response. Transfer of a SARS-CoV-2-specific monoclonal Ab and convalescent plasma also provided significant protection against the disease in an animal model and alleviated disease severity in humans (188,189). These reports suggest that neutralizing Abs play a key role in the protection or clearance, although partial, of the virus. However, the precise mechanism and extent of contribution of virus-specific T cells to the quality, quantity, and duration of the Ab response have not been addressed, and whether T cells *per se* provide sufficient protection or exert a therapeutic effect remains unknown (190). This knowledge is particularly important because it can provide critical information for designing a COVID-19 vaccine and developing a quarantine policy. Currently, the therapeutic effect of adoptively-transferred SARS-CoV-2-specific T cells is being tested in a clinical trial (ClinicalTrials.gov: NCT04351659).

CONCLUSIONS

This review has presented a brief introduction to three human coronaviruses, SARS-CoV, MERS-CoV, and SARS-CoV-2, and summarized previous and current coronavirus vaccine studies. In addition to the scientific issues discussed herein, there also remain several problems to be resolved in the effort to produce an "available" COVID-19 vaccine—the arrangement of existing infrastructure or build-up of new facilities for mass production, distribution of final goods, and vaccination of large proportions of the population, and so on. Facing a novel coronavirus pandemic, we are engaging in desperate efforts for the development of a safe and effective vaccine. Ultimately, the information in this review will be beneficial and valuable for a better understanding of human coronaviruses and COVID-19 vaccine development.

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