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REVIEW

Progress of Middle East respiratory syndrome coronavirus vaccines: a patent review

Jiwon Choi[a]*, Mi-Gyeong Kim[b]*, Yu-Kyoung Oh[b] and Young Bong Kim[a]

[a]College of Animal Bioscience & Technology, Konkuk University, Seoul, Republic of Korea; [b]College of Pharmacy, Seoul National University, Seoul, Republic of Korea

ABSTRACT

Introduction: Middle East respiratory syndrome coronavirus (MERS-CoV) has emerged as a new pathogen, causing severe complications and a high case fatality rate. No direct treatments are available as yet, highlighting the importance of prevention through suitable vaccination regimes. The viral spike (S) protein has been characterized as a key target antigen for vaccines. In particular, S protein domains have been utilized to produce high titers of neutralizing antibodies.

Areas covered: Since the first report of MERS-CoV infection, a limited number of MERS-CoV-specific patents have been filed. Patents related to MERS-CoV are categorized into three areas: treatments, antibodies, and vaccines (receptor-related). This review mainly focuses on the types and efficacies of vaccines, briefly covering treatments and antibodies against the virus. MERS-CoV vaccine forms and delivery systems, together with comparable development strategies against SARS-CoV are additionally addressed.

Expert opinion: Vaccines must be combined with delivery systems, administration routes, and adjuvants to maximize T-cell responses as well as neutralizing antibody production. High immune responses require further study in animal models, such as human receptor-expressing mice, non-human primates, and camels. Such a consideration of integrated actions should contribute to the rapid development of vaccines against MERS-CoV and related coronaviruses.

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1. Introduction

The recent outbreak of Middle East respiratory syndrome (MERS) has emerged as a major challenge to social safety and public health in Asian countries. MERS infection was initially reported in a male patient with pneumonia in Saudi Arabia in 2012 [1]. The patient died of progressive respiratory and renal failure caused by MERS coronavirus (MERS-CoV) 11 days after hospitalization [1].

Polymerase chain reaction (PCR) was used to diagnose infection with coronavirus after a series of negative assays failed to detect various virus candidates [1]. Sequences of the PCR fragment were identified as open reading frame 1b of the coronavirus, and the causative agent was reported as a novel virus—human coronavirus; Erasmus Medical Center later renamed the virus as MERS-CoV [2]. A comparison of the full genome sequence of MERS-CoV with those of other CoVs revealed a higher sequence homology and closer phylogenetic relationship with CoVs from a variety of bats than with other animal-derived CoVs [3]. Thus, early during the investigation period, MERS-CoV was suggested to originate from bats; however, to date, the virus has not been directly isolated from bats [4].

In addition to this seeming association of human MERS-CoV infection with bats, high antibody titers have been detected in camels, which have been suggested to play a major role in the spread of MERS-CoV to humans [5–7]. However, the transmission patterns, routes, and mechanisms of MERS-CoV infection in humans remain to be established. Recent surveys of serum samples from patients in Saudi Arabia have revealed that the seroprevalence of MERS-CoV antibodies is considerably higher in individuals with jobs involving high exposure to camels, who may, in turn, be a source of MERS-CoV spread to others in the absence of exposure to these animals [8].

Since the identification of the first MERS case in 2012, the virus has been detected not only in *Saudi Arabia* but also in several countries over a wide geographical range, including the United States, Italy, Germany, the Philippines, and Korea. Notably, the recent MERS-CoV outbreak in Korea in 2015 was initiated by a single infected Korean patient. The virus was transmitted to individuals in contact with secondarily infected individuals, resulting in infection of about 180 Korean citizens [9,10]. This pattern of outbreaks supports not only camel-to-human but also human-to-human transmission as major routes of MERS-CoV spread worldwide.

Despite the high case fatality rate (35%) of MERS-CoV infection relative to severe acute respiratory syndrome coronavirus (SARS-CoV) infection (10%) [11], no direct therapeutics or preventive treatments specific for MERS-CoV have yet been approved. Because MERS-CoV is a newly emerged human-infecting virus, few treatments using monoclonal antibodies have been patented to date [12–14]. Patent WO 2015/179535 A1 describes human antibodies against the MERS-CoV spike (S) protein, some of which target the receptor-binding domain (RBD) of S glycoprotein

CONTACT Young Bong Kim kimea@konkuk.ac.kr; Yu-Kyoung Oh ohyk@snu.ac.kr
*Both authors contributed equally to this work.

Article highlights

- The main objective is to develop Middle East respiratory syndrome coronavirus (MERS-CoV) vaccines, proteins or fragments that efficiently elicit high levels of neutralizing antibody over a short period of time based on knowledge and understanding of its closely related virus, severe acute respiratory syndrome coronavirus (SARS-CoV).
- For protection against infection by a broad range of MERS-CoV variants, DNA vaccines based on consensus DNA sequences of full or partial spike protein (S) have been designed. These DNA sequences have been further modified by including a leader peptide sequence and incorporating codon optimization to ensure stable, high expression of proteins.
- Similar to SARS-CoV, a MERS-CoV receptor-binding domain (RBD) in conjunction with Fc is an efficient vaccine that elicits sufficient neutralizing activity, blocking binding of S protein to its receptor, human dipeptidyl peptidase 4 (hDPP4), in the case of MERS-CoV.
- S proteins have been formulated in nanoparticles as trimers, which reflect their native conformation on the envelope of MERS-CoV. Nanoparticles mixed with optimal adjuvant are considered potent vaccine candidates. The importance of adjuvants has additionally been addressed.
- Viral vectors are attractive delivery tools for vaccines. Owing to their suitable characteristics, such as broad infectivity, including infection of mucosal tissues, adenovirus vaccines in particular have been applied to induce mucosal immunity in addition to T- and B-cell responses.
- Modified vaccinia virus Ankara (MVA), a strong immunostimulator, has been employed to express the S protein of MERS-CoV. The resulting vaccine, shown to be capable of inducing significant immune responses, was first administered to camels.
- hDPP4-expressing mouse models, which display susceptibility to MERS-CoV infection, have been developed by adenoviral-mediated delivery of hDPP4 or generation of hDPP4 transgenic mice.
- While a RBD-based vaccine is suggested as a promising candidate, it is important to consider the combination of efficient immunogens with various delivery systems, adjuvants, and administration routes. Moreover, in the case of DNA and subunit combination vaccines, vaccination sequence is another avenue that should be addressed.

This box summarizes key points contained in the article.

and thus inhibit binding of MERS-CoV to its receptor, human dipeptidyl peptidase 4 (hDPP4). One promising antibody candidate, m336, was shown to target an epitope that largely coincides with the receptor-binding site of S protein [15,16]. Filed therapeutics for MERS-CoV infections are additionally limited. Ranpirnase, a drug that promotes tRNA degradation in mammalian cells, has been shown to render cells resistant to some viral infections, including MERS-CoV [17].

In addition to limited treatment options and receptor-blocking antibodies, no protective vaccines are currently available for human use. Because MERS-CoV can cause zoonosis, veterinary control of the virus is additionally critical for managing human epidemics. However, no commercial animal vaccines are available at present. In a recent study, dromedary camels were administered modified vaccinia virus Ankara (MVA) expressing MERS-CoV S protein. Neutralizing antibodies (NAbs) were detected in association with reduced levels of infectious virus in the vaccinated camels [18]. However, the efficacy of experimental MERS vaccines for human use requires extensive evaluation before application in the clinic.

For vaccines to reach the human trial stage, potential antigens and delivery systems that elicit effective immune responses are initially tested in animals. The individual vaccines for MERS-CoV and associated delivery systems for which patents have been filed are discussed below.

Research progress in MERS-CoV vaccine development has been reported in recent reviews [19,20], which document the substantial progress on antigen types and efficacies of MERS-CoV vaccines. In the present review, we focus on the current status of MERS-CoV vaccine patents that may have higher potential for commercialization. MERS-CoV vaccine-related patents for the period 2012–2016 were searched and summarized with respect to vaccine design, delivery form, and administration route.

2. Important viral structures and receptors for MERS-CoV vaccines

MERS-CoV, initially isolated in humans, is classified as a member of the Coronaviridae family, lineage C of the genus betacoronavirus, as disclosed in patent US20150275183 [21]. Apart from MERS-CoV, only a few other coronaviruses are known to infect humans, including HCoV-229E, HCoV-NL63, HKU1, OC43, and SARS-CoV [21–23].

Similar to other viruses belonging to the Coronaviridae family, MERS-CoV is an enveloped virus containing a positive-sense RNA genome. Many coronaviruses, including MERS-CoV, have an unusually large RNA genome of ~30 kb containing at least 10 predicted open reading frames [24]. The genome starts with a leader sequence, followed by viral genes encoding (in order) replicase, spike protein (S), envelope protein (E), membrane protein (M), and nucleocapsid (N), which are flanked by 5'- and 3'-untranslated regions [25]. The virion is spherical in shape with a relatively large diameter of 120 nm [26,27]. The nucleocapsid encapsulating the RNA genome is surrounded by the envelope in which M and E are embedded; S proteins are attached to the envelope as homotrimers [28,29]. Glycosylated S proteins are cleaved into S1 and S2 proteins, such that S2 forms the stalk for a club-like protrusion of the S protein. N-terminus S1 proteins are important for viral entry and infection in the virus life cycle. The ectodomain of a mouse coronavirus S trimer was recently observed by single-particle cryo-electron microscopy [30], which revealed that the structure was maintained in a prefusion conformation, but reorganized to initiate viral entry and infection. In addition, this study showed that the sequence of S glycoprotein was conserved among various coronaviruses, including mouse hepatitis virus, SARS-CoV, and MERS-CoV. The accessible and highly conserved fusion peptide in coronavirus would thus be useful for the design of S-epitope-based vaccines capable of inducing NAbs against a broad spectrum of coronaviruses that share the peptide.

The receptors for S proteins of each coronavirus are distinct. SARS-CoV and MERS-CoV receptors are human angiotensin-converting enzyme 2 (ACE2) and dipeptidyl peptidase 4 (DPP4), respectively [31,32]. DPP4, also known as Cluster of Differentiation 26 (CD26), was identified using mass spectrometry and confirmed as the receptor of MERS-CoV based on infection of a nonpermissive cell line expressing DPP4 [32]. An hDPP4-expressing mouse model that is susceptible to MERS-CoV infection has been developed for use in developing

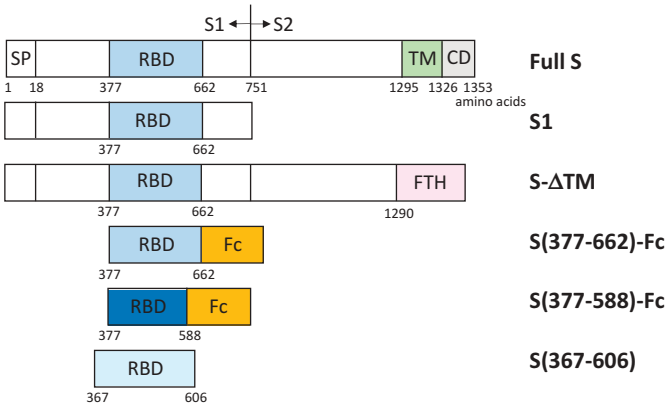

Figure 1. Schematic diagram of antigens used in MERS-CoV vaccine candidates. Full-length S protein is composed of S1 and S2 fragments. S1 contains the signal peptide (SP) and RBD, whereas S2 contains the transmembrane domain (TM) and cytoplasmic domain (CD). S-ΔTM contains the full-length S protein, except TM and CD, which were replaced with the fold-on trimerization domain (FTH). The peptide fused with human IgG Fc domain is designated S-Fc, differentiated by amino acid numbers at the start and endpoint in brackets.

strategies to prevent viral infection and to identify therapeutic antagonists against the receptor in humans [33]. An immunohistochemical analysis using an anti-human cluster of differentiation 26 antibody in this mouse model confirmed hDPP4 expression in tissues from organs including the intestine, kidney, heart, liver, lung, and brain [34]. This transgenic mouse also represents a useful model for determining the in vivo therapeutic efficacy of small molecules and protein antagonists that interact with the receptor in nonhuman animals for application in human trials.

The RBD of the MERS-CoV S protein is the ligand for hDPP4. Earlier crystallographic studies revealed that the extracellular domain of hDPP4 that forms the binding site for RBD is encoded by a sequence-spanning positions 367–606 [35]. The nucleic acid sequence of MERS-CoV RBD in humans is filed in patent US20150275183 [21]. Additional domains of the S protein have been illustrated based on their specific functions, as shown in Figure 1 and filed in patent WO2014134439 [36].

3. DNA vaccines targeting S protein

DNA vaccines offer several advantages over other types of vaccines from the perspective of safety, expression of necessary protein, and low production cost [37]. The gene encoding S protein has been a primary focus for the design of MERS DNA vaccines, based on the finding that S-protein-based vaccines are effective against SARS-CoV, a member of the same genus, betacoronavirus, as MERS-CoV. The S protein of SARS-CoV is highly immunogenic and induces NAbs, eliciting protective immune responses against a viral attack in challenged animals [38–40]. Among the MERS-CoV structural proteins, the outermost-located S protein responsible for receptor binding is one of the most prominent candidates for the development of an effective vaccine.

Despite the advantages of DNA vaccines, maintaining steady, high-level protein expression using them remains a challenge. To overcome these current limitations, researchers have engineered codon-optimized DNA encoding S protein that incorporates an added immunoglobulin E (IgE) leader sequence. The resulting DNA was cloned into the pVax1 expression vector [41–43]. DNA and amino acid sequences of full-length S protein and S protein with a deletion of the cytoplasmic domain (DCD) are illustrated in patent WO2015081155 [44].

DNA encoding the S protein of MERS-CoV was designed using a consensus sequence of clades A and B to protect against various strains and variants of the virus [44]. The synthetic DNA vaccine was intramuscularly injected three times at 3-week intervals in rhesus macaques, leading to cell-mediated and humoral immune responses. Rhesus macaques treated with the DNA vaccine showed increased secretion of the cytokines interferon (IFN)-γ, interleukin (IL)-2, and tumor necrosis factor (TNF)-α and exhibited increased NAb activity. The DNA vaccine was further administered to camels and rhesus monkeys three times at 3–4-week intervals, and its efficacy was determined by measuring NAb titers and IFN-γ levels representative of B- and T-cell responses, respectively.

NAb titers following the administration of DNA vaccines encoding shorter versions of S protein (S1 and the deleted transmembrane domain (S-ΔTM)) were lower than that achieved with a DNA vaccine encoding full-length S protein [45]. In addition, a DNA vaccine encoding MERS-CoV S protein was shown to induce cross-protection against eight MERS-CoV variants, including England1, Munich, Erasmus Medical Center, Buraidah1, Bisha1, Batin1, Hasa14b, and JordanN3, but not against SARS-CoV pseudo-type [45]. Multiple immunizations of rhesus macaques with full-length S DNA and S1 subunit protein induced NAb activity and provided protection against MERS-CoV-induced radiographic pneumonia. In this application, a plasmid encoding full-length S DNA was intramuscularly delivered via electroporation, and the S1 subunit protein was administered with alum as an adjuvant.

An antibody from MERS-CoV-immunized rhesus monkeys was shown to exert cross-clade neutralizing activity against MERS-CoV [41,46,47]. Rhesus monkeys exhibited less or no symptoms of MERS and a lower viral RNA load, as assessed using sensitive quantitative reverse transcription-PCR [41].

In addition to its utility in vaccine development, the S protein of MERS has been used to develop neutralization assays. To achieve this, two research groups created a pseudovirus bearing the S protein and human immunodeficiency virus (HIV) backbone in place of the entire MERS-CoV [47,48], generated by cotransfection of an HIV-1 luciferase reporter plasmid and a plasmid encoding the MERS-CoV S gene. These authors showed that MERS/HIV S protein pseudoparticles were useful for neutralization assays and antiviral drug screening [47,48].

4. S-protein-based vaccines

Several studies have confirmed that S protein is responsible for receptor binding and demonstrated that membrane fusion is critical in eliciting NAb responses, which block further viral invasion [35,49,50]. The S protein vaccine of SARS-CoV generates high NAb titers, providing protection against viral infection [51,52]. However, owing to vaccine-induced tissue

damage [53,54], other types of protein vaccines related to the S protein are under active investigation.

4.1. RBD as a MERS-CoV vaccine

The key region in the S protein for receptor binding is the RBD. The application of a soluble S glycoprotein fragment as a SARS-CoV vaccine together with its sequence has been filed in patent WO2005010034 [55]. The RBD of the MERS-CoV S protein was identified by two independent groups through a comparison of its sequence with that of the SARS-CoV RBD [56,57]. The RBD was mapped to a region within the MERS-CoV S-protein-spanning residues 377–662 by Du and colleagues [56] and to the 358–588 region by Bosch and coworkers [57].

Proteins expressed from both gene fragments contained the RBD of MERS-CoV, as evidenced by their coimmunoprecipitation from lysates of Human hemochromatotic (Huh)-7 cells expressing high levels of hDPP4, but not from ACE2/293 T cells. Furthermore, RBD was confirmed as an essential region for receptor interactions based on competition experiments using soluble hDPP4 in Huh-7 cells and hDPP4-expressing 293 T cells [49,57].

The RBD has been fused with other functional moieties to promote uptake by antigen-presenting cells and to enhance immunogenicity. For instance, the utility of RBD fused with the Fc region has been investigated. Fc-fused RBD has several advantages, including enhanced recognition by antigen-presenting cells, convenience of purification, and increased stability [58,59].

The 377–588 fragment fused with Fc was determined to be extremely immunogenic in terms of inducing a NAb response. Accordingly, RBD-Fc was described as a potent vaccine warranting further analysis in patent WO2014134439 [36]. The smaller region encompassing amino acids 484–567 in the RBD was identified as a receptor-binding motif based on crystallographic studies. The extended 377–588 fragment is proposed to be the optimal length for maintaining a stable conformation that results in a neutralizing epitope [49]. The RBD-Fc fusion protein was shown to efficiently elicit an antibody response not only to S1 but also to full-length MERS-CoV S protein, producing high titers of NAbs in mice. The elicited antibodies included IgG1 (Th2) and IgG2a (Th1) [49].

In addition to optimization of vaccination efficacy, other aspects of RBD-Fc-based vaccines, including vaccination route, effective adjuvant, and minimal antigen dose, have also been investigated. Intranasal and subcutaneous routes were found to induce comparable systemic RBD-specific humoral responses with high NAb titers [60]. However, local mucosal immunity, represented by RBD-specific IgA antibody responses, was significantly higher with the intranasal than subcutaneous route [55].

Combination with adjuvant is another strategy used to increase the efficiency of RBD-based vaccines [61,62]. The use of MF59 as an adjuvant was shown to increase the immunogenicity of intranasal RBD-Fc vaccines relative to other adjuvants, such as Freund's adjuvant, aluminum, monophosphoryl lipid A, and montanide ISA51 [62]. Because of the low productivity of subunit vaccines, it is important to establish minimal doses of antigens capable of inducing NAb responses. In an investigation of optimal doses of RBD-Fc vaccines, Zhou and colleagues reported that a 1 μg dose of recombinant antigen protein induced a NAb response comparable to that of 5 and 20 μg doses of recombinant antigen in the presence of MF59 adjuvant, protecting mice from MERS-CoV challenge [63,64]. Establishing efficient antigens, adjuvants, vaccination routes, and optimal antigen amounts will provide practical guidance and ensure effective and safe application of MERS-CoV vaccines in clinical trials in the near future.

4.2. S protein nanoparticles

The unavailability of approved vaccines using live attenuated or recombinant virus for human coronavirus has prompted the investigation of virus-like particles (VLPs). VLPs of nonenveloped viruses are composed of (nucleo)capsid proteins, whereas those of enveloped viruses comprise S proteins along with other structural proteins. A SARS-CoV VLP assembled from SARS-CoV S, E, M, and N structural proteins expressed in a baculovirus system [65] or live cells has been described, as filed in patents EP218406756 and WO2005035556 [66,67].

SARS-CoV VLPs with two different compositions have been reported: one consists of the structural proteins (M, E, N) from hepatitis virus and the S protein from SARS-CoV, and the other consists of a SARS S protein containing the influenza hemagglutinin transmembrane domain and influenza M1 protein [68–70]. The constituent proteins were expressed using a heterologous protein expression system and assembled into VLPs with a ~ 100-nm particulate structure. Because structural proteins are able to stably and reproducibly maintain the native conformation of their epitopes in VLPs and VLPs are highly immunogenic, these particles have been extensively researched for vaccine applications. Despite its potential advantages, vaccination with SARS-CoV VLPs produces relatively low yields of VLPs and elicits only partial protection against the virus; thus, additional improvement is required.

In contrast to SARS-CoV VLPs, MERS-CoV nanoparticles contain at least one trimer of S protein [71]. The S protein protruding from the viral envelope maintains a native trimeric conformation, which is critical for receptor binding and subsequent viral entry. A previous study reported that trimeric S protein is significantly more potent in inducing NAbs than monomeric S1 protein [72]. These nanoparticles are ~25 nm in diameter and are relatively homogeneous. The component S proteins were extracted from cellular membranes using a nonionic detergent, and S protein oligomers were purified from soluble materials using anion exchange, affinity, and size-exclusion chromatography [71]. Intramuscular coadministration of nanoparticles of MERS-CoV S proteins with adjuvant was shown to induce higher levels of NAbs than nanoparticles alone [71]. In particular, the Matrix M1 adjuvant induced significantly higher NAb titers than nanoparticles alone or mixed with alum adjuvant in mice [71]. Because the amount of nanoparticles and duration of vaccination was not found to significantly affect the induction of NAbs, formulation of nanoparticles with an optimal adjuvant may be a key factor in determining the efficacy of nanoparticles as vaccines [73].

Cross-protection against MERS-CoV and SARS-CoV using a single nanoparticle type would be ideal. At present, however, MERS-CoV nanoparticles only provide effective protection against infection by the same virus.

5. Virus-based vaccines expressing S protein

Viral-vector-based vaccines are efficient in delivering antigen-encoding genes into cells and expressing immunogens. In contrast to plasmid-DNA-based vaccines, viral vectors invade cells and have the potential to activate the immune system, similar to adjuvants. In the case of protein vaccines, multiple injections are usually required to induce and maintain systemic immune responses [60,64]. In contrast to protein subunit vaccines, which suffer from transient and low-level immune responses, viral-vector-based vaccines are very promising because of their potential induction of strong immune responses and efficient delivery to target cells based on their natural tropism [74,75].

5.1. Adenovirus expressing MERS-CoV S protein

Adenoviral vectors are considered leading candidates for the delivery of antigen-encoding genes. For the SARS-CoV vaccine, introduction of a SARS-CoV S-protein-encoding gene into adenovirus type 5 (Ad5) vector has been filed in patent US2008267992 [76]. Ad5-based vectors have been actively investigated as potential vaccine delivery systems owing to their broad host range, high infectivity, high protein expression, and safety, the latter of which reflects their replication deficiency. Additionally, because adenoviruses effectively induce mucosal immune responses, considerable research attention has focused on their use as delivery vectors for vaccines against airway pathogens, such as MERS-CoV, which infects respiratory tract mucosal tissues constituting the first line of defense.

Although the prevalence of prior infection by adenovirus is a major limitation for clinical application in humans, adenoviruses remain attractive candidates for use as a veterinary vaccine. Camels have been investigated for application of MERS-CoV vaccine because they present a putative animal reservoir of MERS-CoV, and their vaccination is an effective method for controlling infection—research that could ultimately impact development of a human vaccine.

Intramuscularly injected recombinant Ad5-expressing-codon-optimized MERS-CoV full-length S protein (Ad5.MERS-S) or S1 protein (Ad5.MERS-S1) was shown to elicit generation of S- and S1-specific antibodies, respectively. Moreover, following vaccination in mice, Ad5.MERS-S1 induced elevated IgG2a responses (Th1) compared with Ad5.MERS-S and Ad5 vector alone [77]. Vaccination with Ad5.MERS-S1, but not with the adenovirus itself, also led to a robust level of NAb activity in camels [77]. The use of adenovirus vaccination is particularly encouraging in dromedary camels. Compared with full-length S protein, Ad.MERS-S1 as a vaccine exerts higher efficacy and provides an added benefit of lower risk of recombination with wild-type virus. Adenovirally expressed N-terminal subunit S1 protein was not found to be more immunogenic than the full-length S protein in DNA-vaccine-immunized animals. Indeed, DNA vaccine expressing full-length S protein induced a higher NAb titer compared with DNA corresponding to the S1 protein [45].

The optimal vaccination route and adenovirus subtype were further investigated for MERS-CoV. Ad41, which targets the gastrointestinal tract and is resistant to hostile factors in the environment, was found to be more suitable for oral vaccination compared with the airway pathogen, Ad5 [78]. Because of the natural tropism of Ad41, oral administration was considered to enhance mucosal immunity and decrease MERS-CoV infection via the mucosa. A single dose of either Ad5.MERS-S- or Ad41-expressing S protein resulted in the production of S-specific Th1 cytokines as well as humoral immune responses.

Interestingly, although Ad41 exhibits tropism toward the gastrointestinal tract, both Ad41 and Ad5 were found to induce significantly higher immune responses upon intramuscular compared with intragastric administration [79,80]. Despite the suitable characteristics of Ad41, such as preference for the gastrointestinal tract and resistance to inactivating conditions, the limited strength of immune responses following mucosal administration must be overcome to afford protection against infection by mucosal pathogens. Despite insufficient immune responses, mucosal-tropic Ad41-based vaccines remain tempting from a development standpoint because of the convenience of their administration method. Intramuscular injection of Ad41 as a possible challenge vaccine also provides an alternative vaccine strategy in subjects who produce antibodies against Ad5.

5.2. MVA expressing MERS-CoV S protein

MVA has been utilized as a gene-delivery tool for various viral vaccines, including influenza virus, which is replication-deficient and can be handled in biosafety level I [81]. Unlike wild-type vaccinia virus, MVA readily loses many immune-evasion factors. Moreover, MVA infection triggers the production of IFNs, inflammatory cytokines, and chemokines and stimulates immigration of lymphocytes/monocytes. Importantly, the intrinsic immunostimulatory activities and protective capacities of MVA are advantageous for vaccine application [82,83]. In view of these characteristics, MVA is the preferred candidate for a MERS-CoV vaccine in dromedary camels.

An MVA vaccine expressing the gene encoding full-length S protein (MVA-MERS-S) under the control of the vaccinia virus early/late promoter, modified H5 promoter, was developed and confirmed to express glycosylated S protein as a co- and posttranslational modification [84]. Vaccination of mice with MVA-MERS-S elicited production of NAbs, even after a single immunization, and NAb titer was increased by a booster dose. Additionally, INF-γ-secreting memory $CD8^+$ T cells were significantly activated in an antigen-specific manner, and a histopathological analysis of lungs and bronchi revealed that MERS-CoV replication was efficiently inhibited upon virus challenge [85].

Based on the promising results obtained from mice immunized with MVA-MERS-S and MVA expressing SARS-CoV S protein [86,87], the efficacy of the vaccine against MERS-CoV was assessed in dromedary camels. Camels immunized by intranasal or intramuscular routes developed NAbs specific for S protein and showed dramatically reduced levels of MERS-CoV, as confirmed by histological analysis as well as virus and viral RNA measurements [18]. In addition, the vaccinated camels produced neutralizing activity against camelpox virus, indicating that immunization with MVA-MERS-S has the further advantage of offering dual protection. This vaccine is currently under consideration for further evaluation in clinical trials on humans working in close contact with camels [17].

6. Candidates vaccine molecules other than S protein and corresponding DNA

The majority of recent strategies for MERS-CoV vaccine development have focused on DNA and RBD of full or partial S protein for generation of immune responses. Other candidates for NAb production include an attenuated live vaccine of MERS-CoV and the structural protein, N.

6.1. Live attenuated MERS-CoV

Live attenuated viruses are expected to elicit mucosal immunity more efficiently than nonreplicating antigens, which elicit a low level of secretory immune responses [88–90]. Live attenuated viruses can be generated by deletion of the genes responsible for virulence in a cDNA clone of the viral genome in a bacterial artificial chromosome (BAC). An attenuated MERS-CoV vaccine was constructed by deletion of the gene encoding E protein from an infectious cDNA clone of the MERS-CoV genome (pBAC-MERS-DE) [91], resulting in a rMERS-CoV-DE virus expressing structural proteins, N and S, in primary infected cells and production of syncytia [91]. The replication-competent, propagation-deficient rMERS-CoV-DE virus expressed structural proteins and elicited a strong immune response [92–94].

Patent WO2006136448 focused on SARS-CoV lacking a functional E gene (SARS-CoV-DE) as a strong vaccine candidate [95]. Hamsters and mice immunized with SARS-CoV-DE produced NAbs and SARS-CoV-specific CD4 and CD8 T-cell responses [89,96]. In addition to immune reactions, the attenuated vaccine likely prevents emergence of mutated viruses during viral adaptation. Other viral proteins, including structural proteins, are produced upon replication of rMERS-CoV-DE. Thus, immune responses against viral proteins are different and advantageous compared with those generated by S-protein-related vaccines. Viruses containing a large RNA genome have a higher probability of genetic mutation owing to protein variations resulting from the inherent infidelity of viral RNA polymerase. Antibodies against various viral proteins, including structural proteins, produced by rMERS-CoV-DE may effectively combat infection by viruses with genetic variations.

6.2. N protein as a potential protective immunogen

Balanced induction of both NAb and cellular immunity would be crucial for an effective MERS-CoV vaccine. Although S proteins and derivatives have been the most studied potential targets for a MERS-CoV vaccine because of their strong induction of NAbs against MERS-CoV infection, mutation of S protein may result in the escape of NAbs against MERS-CoV.

An immunoinformatics-driven genome-wide screen of effective immunogens against MERS-CoV predicted that N protein, rather than S protein, could be a suitable immunogen candidate with the potential to elicit both humoral and cell-mediated immune responses [97]. An in silico study based on genome sequence data determined that the N protein of MERS-CoV possessed epitope candidates for B cells, helper T cells, and cytotoxic T lymphocytes (CTLs). Optimal B-cell epitopes were selected by calculating surface accessibility and fragment flexibility, protrusion index, and allergenicity. Putative epitopes with maximal binding affinity for human leukocyte antigen (HLA) alleles were selected for helper T-cell response, and CTL epitopes were identified based on predictions of MHC-I binding using an immune epitope database and bound MHC-I-specific HLA alleles. This study provided additional insight, showing that various epitopes in the N protein can be further developed as vaccine targets. However, the ability of these candidates to induce humoral and cellular immunity has not yet been studied. Thus, the potential of N protein to serve as preventive and therapeutic vaccines will require further investigation in MERS-CoV challenge and infectious models, respectively.

N protein of SARS-CoV was reported to stimulate IgG production and filed as a vaccine in patent WO2006024543 [98]. N protein has been shown to elicit $CD8^+$ CTL responses and increase secretion of the cytokines IFN-γ and IL-2 [99,100]. Promising findings with SARS-CoV, together with in silico prediction of epitopes from MERS-CoV N protein, support the importance of conducting further in vitro and in vivo studies to validate the utility of N protein as a novel epitope-based vaccine candidate.

7. Expert opinions

MERS-CoV and SARS-CoV belong to the same genus, betacoronavirus, and share several common characteristics, including virus structure, genome organization, and symptoms. Strategies for designing a competent vaccine against MERS-CoV have been adopted from those established for SARS-CoV.

A key finding in MERS vaccine studies would be demonstrating the potential of S and S-derived proteins of MERS-CoV as major vaccine candidates. S and S-related proteins have been intensively exploited as MERS vaccine candidates capable of inducing S/S-related specific antibody responses and neutralizing MERS-CoV infection [101]. Notably, NAb production by RBD was shown to be sufficient to protect against SARS-CoV infection [102].

Although significant progress has been made in the field of MERS-CoV vaccines through evaluation of in vivo efficacy in animal models, several issues remain to be resolved for

effective future translation to the clinic. Currently unresolved issues include unambiguous assessment of vaccine efficacy and safety, limitations of target antigens, and establishment of relevant animal models.

First, S-protein-related vaccines require further assessment in terms of efficacy. To date, three types of S and S-related proteins—full-length S, S1, and RBD—have been shown to elicit NAb activity. However, except for adenoviral expression of full-length S and S1 reported by Kim et al. [77] and protein vaccines of S-ΔTM and S1 reported by Wang et al. [45], a fair comparison of the efficacies of these three vaccines using the same delivery system has not been performed. Although immune responses to S, S-ΔTM, and S1 have been analyzed, the delivery system for each antigen —adenovirus-, DNA-, and protein-based—was different. Owing to these differences in delivery system, it is difficult to compare the immunogenicity of the antigens themselves. Moreover, the immunogenicity of RBD alone has not been compared with that of other protein vaccine candidates, and thus requires further investigation.

Second, the safety of the vaccines needs to be considered, especially for the full-length S protein of SARS-CoV. A previous study reported harmful immune responses after vaccination with full-length S protein of SARS-CoV in ferrets [53]. Moreover, the gene for full-length S protein delivered as a DNA vaccine may have increased opportunity to recombine with other virus genomes. Therefore, the safety of vaccines must be further considered, which may compromise their efficacy.

Third, another weakness in the MERS-CoV field is the lack of diversity in vaccine candidate proteins. Although most MERS-CoV vaccine studies have focused on S protein vaccines, recent studies have reported that mutation of the S protein results in escape of NAbs against SARS-CoV infection [39,40]. Thus, N protein and other new antigen candidates that elicit more diverse NAb repertoires need to be studied.

The challenges in this field would be the limitation in animal models. The majority of MERS-CoV vaccine studies to date have used mice as animal models. For example, a lethal transgenic mouse model for pathogenesis assessment and preclinical evaluation of vaccine candidates against MERS-CoV infection and disease was developed by estimating both a 50% infectious dose and a lethal dose of MERS-CoV; weight loss and mortality were also monitored [103]. Following intranasal administration of MERS-CoV, 100% of mice died at virus doses 10^2–10^6-fold greater than the 50% tissue culture infective dose, and mice lost more than 20% of their body weight at virus doses of 10^3 and higher. Because wild-type BALB/c mice fail to exhibit symptoms following challenge with MERS-CoV [73], a transgenic BALB/c mouse expressing adenovirally delivered human CD26/DPP4 was also developed [104]. However, the validity of such animal models is likely limited by the transduction efficiency of viral vectors. Moreover, the experimental conditions in mice are distinct from physiological conditions or natural disease progression. Nonhuman primates, such as rhesus macaques or marmosets, exhibit immunological similarity to humans and have been used as animal models of MERS-CoV infection. Rhesus macaques, which can be infected by MERS-CoV, have been tested for immune responses to recombinant RBD [105]. In a separate study, rhesus macaques were intratracheally inoculated with a MERS-CoV isolate and then clinical symptoms, pathology, and NAb production were assessed [106]. In addition, Feldmann and colleagues investigated the outcome of combined treatment with IFN-α2b and ribavirin following intratracheal, intranasal, oral, or ocular challenge of rhesus macaques with MERS-CoV, reporting histological effects in the lung [107]. However, since these studies lacked a control group (inoculated and untreated), it is not certain whether the observed clinical signs in these animal models resulted from MERS-CoV. Moreover, symptoms caused by viral infection in macaques do not reflect the pathogenesis in humans [108]. Accordingly, a marmoset model has been suggested as an effective alternative in view of the observation that typical symptoms of MERS-CoV in this model closely resemble disease progression and immune responses in humans [109]. In any case, vaccine candidates tested in mice need to be confirmed in nonhuman primate animal models to ensure their efficacy and safety prior to clinical trials in humans. It should be noted that translation of MERS-CoV vaccine studies from preclinical testing to clinical trials is hampered by financial concerns and the availability of research facilities with appropriate safety ratings.

The induction of NAbs in animal models suggests the feasibility of developing S-protein–based preventive vaccines [110]. Ultimately, however, both preventive and therapeutic vaccines should be developed to minimize outbreaks and reduce the high case fatality rate. Given the issue of animal reservoirs of MERS-CoV, potent MERS-CoV vaccines for both humans and reservoir animals should be developed.

Combined induction of humoral and cellular immune responses requires the design of vaccines that can stimulate the intracellular processing of antigens in both MHC class I and II pathways. To date, most studies have focused on the induction of NAbs using single-protein-type vaccines. One way to stimulate the cytoplasmic MHC class I pathway would be through intracellular expression of antigen protein by externally administered DNA. However, few studies have adequately assessed delivery strategies for MERS vaccine antigens. Nanoparticles, which exist as polymeric structures, represent a vaccine delivery form for protein vaccines. In contrast to SARS-CoV VLPs, the MERS-CoV nanoparticle vaccine described in WO2015042373 consists of homogeneous S proteins [71], and no studies on heterogeneous VLPs of MERS-CoV have been reported as yet.

Owing to the relatively brief history of MERS-CoV, the vaccine types developed to date are not as extensive as those for SARS-CoV. For SARS-CoV, DNA vaccine priming followed by boosting with S peptide or viral vector was shown to elicit T-cell as well as humoral responses [111,112]; it also may be more protective against a broad range of viral variants. Similar to SARS-CoV vaccines, a DNA-primed protein vaccine for MERS-CoV was shown to elicit a higher immune response compared with a protein vaccine alone [45]. This finding implies that a more efficient vaccine strategy can be achieved by testing combinations of various antigens with different delivery systems. The types of antigens, delivery forms,

Table 1. Antigens and administration routes of MERS-CoV vaccine candidates.

Antigens	Forms of delivery	Administration routes	Adjuvants	Ref.
Full S	Naked DNA	Intramuscular/ electroporation	–	45
	DNA in Ad5 adenoviral vector	IM, IG	–	79
		IM, IN	–	77
	DNA in Ad41 adenoviral vector	IM, IG	–	79
	DNA in MVA viral vector	IM	–	84
	Proteins loaded in S nanoparticles	IM	Aluminum hydroxide, Matrix M1	73
S-ΔCD	Naked DNA	IM-E	–	45
S-ΔTM + FTH	Protein	IM-E	Ribi	45
S1	Naked DNA	IM-E	–	45
	Protein	IM-E	Ribi	45
	DNA in Ad5 adenoviral vector	IM, IN	–	77
RBD	Peptide S(377–662)-Fc	N/A	Montanide ISA 51	56
		IN	Poly(I:C),	60
	Peptide S(377–588)-Fc	N/A	Montanide ISA 51	49
		SC	MF59	64
	Peptide S(367–606)	IM, SC		61

S-ΔCD, S gene in which the cytoplasmic domain (CD) was removed; S-ΔTM + FTH, S gene in which the transmembrane domain (TM) was replaced with a fold-on trimerization domain (FTH); IM-E, intramuscular injection, followed by electroporation; IN, intranasal; SC, subcutaneous; IG, intragastric; N/A, information not available; –, not added.

administration routes, and adjuvants of MERS-CoV vaccines are summarized in Table 1.

In the near term, studies will focus more on improving existing S protein vaccines using new delivery technologies and optimization strategies. Because the optimal dose depends on the administration route and delivery system, each delivery module should be optimized separately. Another avenue would be to study combinations of the S protein and relevant adjuvants.

Together with suitable antigen delivery systems, the development of optimal adjuvants and immunization routes are important factors that affect the efficiency of MERS-CoV vaccines. However, there are few document instances where diverse adjuvants and vaccination routes have been tested, highlighting the importance of further studies on adjuvants and delivery routes in animal models in optimizing vaccine formulations for clinical trials.

One issue that is not uniquely related to the MERS-CoV vaccine field is the question of combining relevant adjuvants and nanotechnology-based formulations. To date, only a few studies have investigated nanoparticle formulations of S protein. The use of nanotechnology to improve uptake by antigen-presenting cells is another new approach for enhancing vaccine efficacy that warrants attention. Technologies capable of codelivering both adjuvants and antigens to the immune system would further advance the field.

Since MERS-CoV is a relatively new disease, only a limited number of patents directly related to it have been filed. Using the amino acid and nucleotide sequences of MERS-CoV, existing patents have applied methodologies used for SARS-CoV vaccines to attempt to develop a safe and effective MERS-CoV vaccine. Further studies to establish optimal combinations of antigens, delivery systems, adjuvants, and administration routes in animal models should contribute to improving the efficacy of MERS-CoV vaccines, with the aim of achieving approval for human clinical trials in the near future.

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Declaration of interest

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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