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MERS-CoV Standards, Assays and Animal Models for Vaccine Development Landscape Analysis
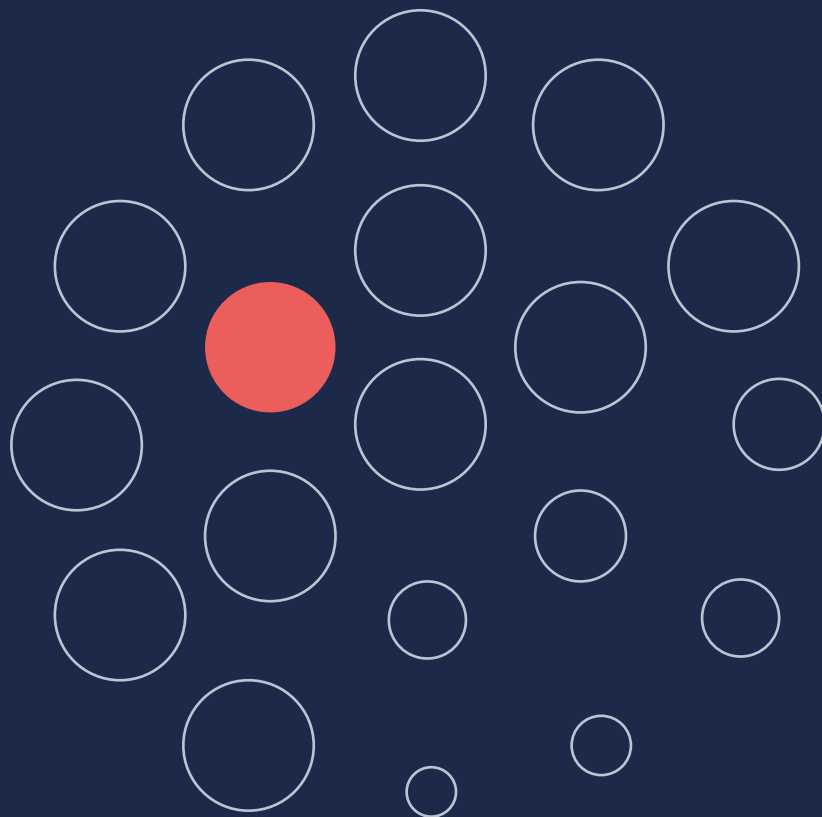
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Author: Vadim Tsvetnitsky, Ph.D.
IAVI

Secondary authors / reviewers:

Albert Price, IAVI

Lisa Gieber, IAVI

Eddy Sayeed, IAVI

Thomas Hassell, IAVI

Donata Sizemore, IAVI

Karie Youngdahl IAVI

Celine Gurry, CEPI

Johan Holst, CEPI

Raul Gomez Roman, CEPI

Carolyn Clark, CEPI

Paul Kristiansen, CEPI

Amy Shurtleff, CEPI

Valentina Bernasconi, CEPI

List of abbreviations

Ad	adenovirus
AGM	African green monkey
BSL	biosafety level
CEPI	Coalition for Epidemic Preparedness Innovations
CFR	case fatality rate
COPD	chronic obstructive pulmonary disease
CoV	coronavirus
CP	convalescent plasma
dpi	day post infection (post inoculation)
DPP4	dipeptidyl peptidase 4
EMC	Erasmus Medical Centre
FDA	Food & Drug Administration
GCV	geometric coefficient of variation
HPV	human papillomavirus
IFN	interferon
IgA	immunoglobulin A
IgG	immunoglobulin G
IIFT	indirect immunofluorescence
IM	intramuscular
IN	intranasal
IQR	interquartile range
IRP	International Reference Preparation
IS	International Standard
IT	intratracheal
KSA	Kingdom of Saudi Arabia
LRT	lower respiratory tract
MERS	Middle East Respiratory Syndrome
MVA	Modified Virus Ankara
NIBSC	National Institute for Biological Standards and Control (UK)
ORF	open reading frame
PFU	plaque-forming unit
ppNT	pseudoparticle neutralization test
PRNT	plaque-reduction neutralization test
R0	basic reproductive rate
R&D	Research and Development
RABV	rhabdovirus
RBD	receptor-binding domain
RNA	ribonucleic acid
RSV	respiratory syncytial virus
RT-PCR	reverse transcription polymerase chain reaction
SARS	Severe Acute Respiratory Syndrome
TPP	Target Product Profile
UAE	United Arab Emirates
URT	upper respiratory tract
WHO	World Health Organization

1. INTRODUCTION

Middle East Respiratory Syndrome coronavirus (MERS-CoV) infections have been a cause for global concern since the virus was identified in 2012 due to the high fatality rate of the resulting disease, and because of its similarity to Severe Acute Respiratory Syndrome coronavirus (SARS)-CoV that caused a high impact epidemic with serious consequences in 2003 [1].

MERS-CoV is a zoonotic virus that has been repeatedly introduced into the human population via direct or indirect contact with infected dromedary camels in the Arabian Peninsula [2], resulting in human-to-human transmission. Although MERS-CoV may have originated in insectivorous bats, humans do not commonly interact with these species, thus limiting the opportunities for viral transmission. Conversely, direct contact between humans and dromedary camels occurs on a regular basis in regions with high numbers of reported human MERS-CoV cases, and dromedary camels have been strongly implicated as a source for human MERS-CoV infections. Despite ongoing efforts to develop MERS-CoV countermeasures, best practices in infection control and supportive clinical management have remained the mainstay of MERS-CoV prevention and treatment.

MERS-CoV is one of the pathogens prioritized by the World Health Organization (WHO) in its R&D Blueprint [3, 4] and accelerated development of an effective and affordable MERS-CoV vaccine is one of the goals of the Coalition for Epidemic Preparedness Innovations (CEPI), which currently supports development of five vaccine candidates against MERS-CoV (Table 1, also see CEPI website).

Table 1. MERS-CoV vaccine projects supported by CEPI (as of July 2019)

Funded Consortium Lead Partner	Technology Platform
IDT Biologika	Viral vector: MVA
Themis Biosciences	Viral vector: Measles
Janssen Vaccines & University of Oxford	Viral vector: Chimpanzee Adenovirus
Inovio Pharmaceuticals	DNA
University of Queensland	Protein subunit

Development of new vaccines against any disease is most efficient when there is harmonization of key R&D tools, particularly analytical methods, reagents and animal models. This enables direct and confident comparison of experimental results obtained by different investigators and developers. The purpose of this landscape analysis document, prepared for CEPI, is to analyze current published reports describing the state of MERS-CoV standards, assays and animal models currently in use within the context of MERS-CoV biology, epidemiology and vaccine development to guide scientific and policy planning for standardization of these R&D tools.

This landscaping analysis document has been funded by the National Institute of Allergy and Infectious Diseases, National Institutes of Health, Department of Health and Human Services, with additional support provided by CEPI for attendance of Workshop on Standards and Assays in Oslo, June 2019.

2. BACKGROUND

MERS-CoV Epidemiology

MERS–CoV is one of six human coronaviruses (CoV) that can cause a disease. Four endemic respiratory coronaviruses 229E, OC43, NL63 and HKU1 — are known [5] and cause only mild disease. The first coronavirus to cause serious disease was Severe Acute Respiratory Syndrome (SARS) coronavirus, identified in 2003. It caused a serious epidemic and subsequently disappeared [6]. MERS–CoV was first discovered in a patient in Saudi Arabia in 2012 [7] and continues to infect people. Globally, as of July 2019 a total of 27 countries reported 2,449 laboratory–confirmed MERS–CoV cases. Over 80% of cases reported to date have occurred in countries of the Arabian Peninsula. There have been no community–acquired cases reported from outside the Near East region. The wide distribution of the animal reservoir (dromedary camels), and cultural and social norms related to human and camel interactions have been proposed as reasons for MERS–CoV spread. It is however unclear which factors play a role in disease prevalence being so focused in one geographical area.

As of July 2019, the WHO has been notified of 2,449 laboratory–confirmed human cases of infection with MERS–CoV with 845 deaths (case fatality rate [CFR] 34.5%). Although the majority of cases (2,058 with 760 related deaths, CFR 37.3%) have occurred in the Kingdom of Saudi Arabia (KSA), 26 other countries (Table 2) have reported cases of MERS–CoV infection.

Table 2. Number of laboratory-confirmed MERS cases reported by countries since 2012

Country	Number of laboratory-confirmed MERS-CoV cases reported as of 16 July 2019
Algeria	2
Austria	2
Bahrain	1
China	1
Egypt	1
France	2
Germany	3
Greece	1
Iran	6
Italy	1
Jordan	28
Kuwait	4
Lebanon	2
Malaysia	2
Netherlands	2
Oman	24
Philippines	2
Qatar	19
Republic of Korea	186
Saudi Arabia	2058
Thailand	3
Tunisia	3
Turkey	1
United Kingdom	5
United Arab Emirates	87
United States of America	2
Yemen	1
Total	**2449**

A large hospital-associated outbreak (16,752 suspected cases, 186 confirmed cases, 38 deaths) occurred in the Republic of Korea in 2015 [8] and other hospital-associated outbreaks were also reported in the KSA and United Arab Emirates (UAE) [9, 10]. Drivers of transmission and the exact modes of transmission in health care settings have not been articulated and are currently the focus of collaborative scientific research. The current body of evidence does not show any change in the epidemiology of the MERS-CoV virus or its transmission characteristics since the virus discovery in 2012 (Figure 1).

Figure 1. Epidemiological curve of confirmed global cases of MERS-CoV as of July 16, 2019
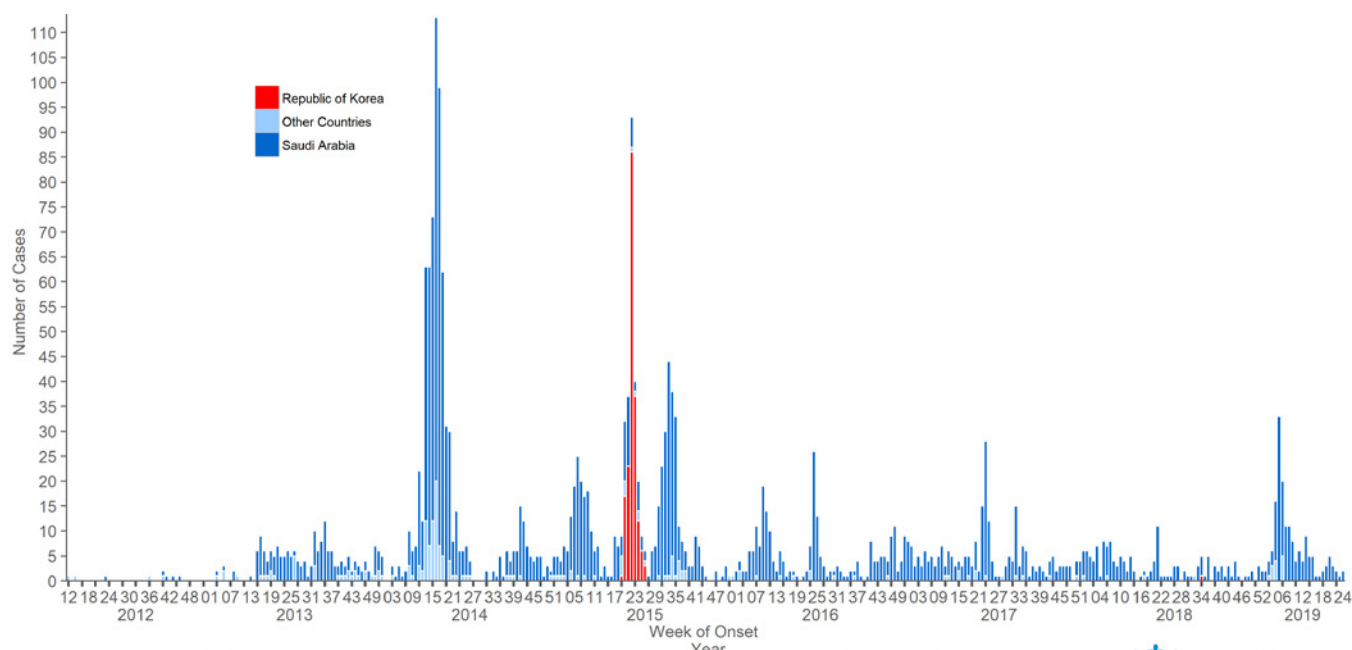
Other countries: Algeria, Austria, Bahrain, China, Egypt, France, Germany, Greece, Iran, Italy, Jordan, Kuwait, Lebanon, Malaysia, Netherlands, Oman, Philippines, Qatar, Thailand, Tunisia, Turkey, United Arab Emirates, United Kingdom, United States of America, Yemen
Please note that the underlying data is subject to change as the investigations around cases are ongoing. Onset date estimated if not available.

The transmission pattern seen so far represented repeated sporadic introduction of the virus mostly from dromedary camels to humans resulting in limited human-to-human transmission (Figure 2). Either direct or indirect contact with dromedaries continue to be the primary risk factors for human infections that are acquired in the community. However, certain primary cases cannot be traced to contact with camels; and with their source for infection undetermined and/or undocumented community or environmental transmission remains possible. Despite the bulk of information pointing to camels as the source of human infections, a possibility remains for another animal reservoir.

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Figure 2. Ecology and transmission of MERS-CoV (adapted from [11])

Environmental source and risk
Bats and dromedary camels, perhaps other animals, intermediary sources

Camel birthing

Camel racing

Camel milking

Camel owner contact

Direct or Indirect transmission to human beings

Nasal discharge or saliva
Perhaps excreta (faeces and urine)
Diary products, meat handling

Contact, inhalation, ingestion, or mucosal invasion?

MERS-CoV infection

Incubation period
MERS-CoV replication and viraemia → **Subclinical infection**
Asymptomatic

Family, friends, visitors and health-careworkers

Prodrome
Early symptoms and signs
Malaise, chills, fatigue, nausea, headache, myalgia, sneezing → **Recovery**

Transmission
Droplet
Contact (vomit, faeces, urine)
Airborne

Acute illness
Cough, chest pain, dyspnoea, vomiting, diarrhoea, other

Fulminant illness (intensive care unit)
Acute respiratory distress syndrome, renal failure, multiorgan failure

Death
Comorbid risk factors: diabetes, kidney or liver diseases, immunosuppression

Chronic sequelae?

Secondary transmission continues to occur among close contacts of laboratory-confirmed symptomatic cases, mostly in health care settings and households. Human-to-human spread requires close contact and is likely through large droplets, although aerosol or fomite transmission has not been ruled out. MERS-CoV may persist in the environment for up to 24 hours, which may also be a source for human infection.

MERS-CoV human-to-human transmission appears to be inefficient, with few documented cases beyond quaternary transmission. For example, one study found that the transmission rate of infected patients to household contacts was only 4% [12]. Many of these household contacts were asymptomatic or had mild disease. The basic reproductive rate (R0), which is the number of secondary cases one case generates on average over the course of its infectious period in an otherwise uninfected population, has been estimated to be less than 0.7 and likely closer to 0.5 for MERS-CoV. This is significantly lower than an R0 of 1, which describes epidemic potential [13]. The R0 can be higher in health care settings, as has been seen in several health care associated outbreaks in Saudi Arabia and the Republic of Korea [8]. Despite apparently inefficient human-to-human transmission, serologic and RT-PCR studies showed that as many as 45,000 people in Saudi Arabia were infected by MERS-CoV [14]. Although not clinically severely ill, these individuals may play a role in spreading MERS-CoV.

Males above the age of 60 are at a higher risk of severe disease, including death. The WHO Global Summary and Assessment of Risk in 2018 reported the median age of all laboratory confirmed cases of MERS-CoV infection was 52 (IQR 36-65; range >1-109 years old) with males comprising 67.2% of all laboratory-confirmed cases reported to that date (n=2228). At the time of that report, 21% of the 2,228 cases were reported to have no or mild symptoms, while 46% had severe disease or died. Overall, 18.6% of the cases reported to date are health care workers. Fatal outcomes are more common in infected individuals with underlying medical conditions such as diabetes, chronic renal disease, obesity, hypertension, smoking and lung disease such as asthma or chronic obstructive pulmonary disease (COPD).

MERS-CoV in Camels

Neutralizing antibodies against MERS-CoV, or a closely related virus, have been detected in dromedary camel serum samples collected from several Middle Eastern and African countries as far back as 1983 [15]. Moreover, MERS-CoV RNA has been identified in nasal and conjunctival swabs, milk, and, rarely, rectal swabs from dromedary camels. Although MERS-CoV seropositivity is more common in adult camels, MERS-CoV RNA is most often detected in nasal swabs from juvenile camels, suggesting that active infections typically occur in younger camels. MERS-CoV infections in camels appear to be acute and transient [16] and the virus causes mostly an upper respiratory tract (URT) disease [17]. Most infected camels are asymptomatic; however, some camels develop rhinorrhea and an increase in body temperature. Experimental inoculation of young adult dromedary camels with MERS-CoV has been shown to cause mild disease consisting of degeneration and necrosis of the respiratory epithelium in the upper and lower respiratory tract (LRT) in the absence of pneumonia [18]. Viral antigen and high levels of infectious virus were detected in affected tissues, and the nasal turbinate respiratory epithelium was identified as the main site of virus replication. High amounts of infectious virus were isolated from nasal discharge despite development of only mild clinical disease.

A few other livestock species, including llamas (alpacas) and pigs, are susceptible to infection with MERS-CoV [19, 20], however to date there has been no evidence of circulation of MERS-CoV in animal species other than camels.

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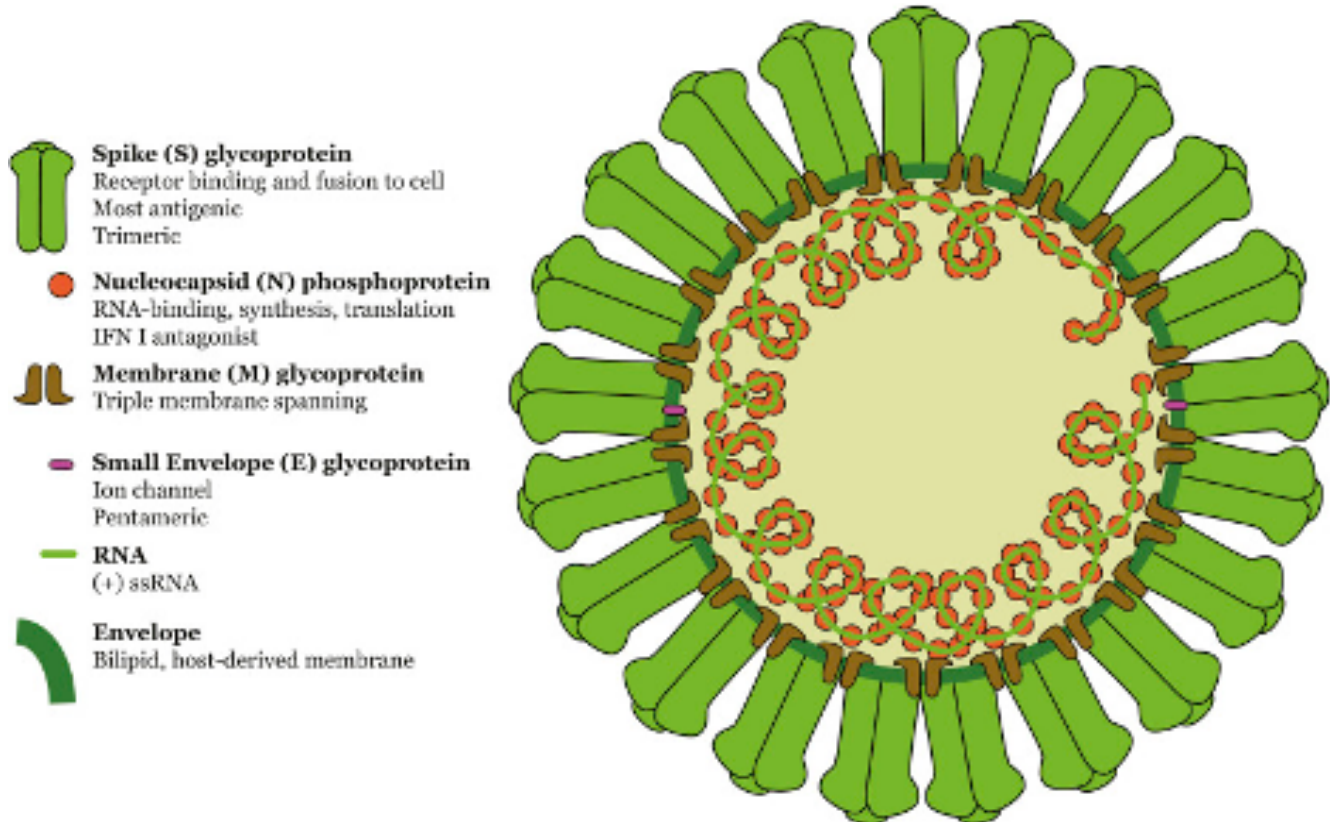
MERS-CoV Molecular Biology and Structure

Coronaviruses (CoVs), large positive-sense single-stranded RNA viruses of the order Nidovirales, were formerly considered to be minor human pathogens, causing cold-like symptoms and only occasionally associated with pneumonia and more severe diseases. However, the emergence of SARS-CoV in 2002 and MERS-CoV in 2012 marked a shift in our understanding of the pathogenic potential of CoVs. As these virulent viruses are genetically similar to those currently circulating in Pipistrellus or Tylonycteris bats (MERS-CoV) [6] and horseshoe bats (SARS) [21], CoVs may pose a threat for future zoonoses.

MERS-CoV has a genomic structure similar to SARS-CoV and that of other CoVs. It contains a 30-kb positive-strand RNA genome encoding 11 open reading frames (ORFs) that are ordered 5′ to 3′: ORF1a, ORF1b, spike (S), ORF3, ORF4a/b, ORF5, envelope (E), membrane (M), nucleocapsid (N) and ORF8b. MERS-CoV is a spherical enveloped coronavirus (Figure 3) and its genome encodes four major structural proteins: spike (S), membrane (M), envelope (E) and nucleocapsid (N), and two nonstructural replicase polyproteins (ORF1a and ORF1b). Proteins produced by other open reading frames play an important role in MERS-CoV infection and pathogenesis, including inhibiting interferon (IFN) pathway activation, attenuation and modulation of inflammation [22].

Figure 3. Molecular structure of MERS-CoV

Spike (S) glycoprotein
Receptor binding and fusion to cell
Most antigenic
Trimeric

Nucleocapsid (N) phosphoprotein
RNA-binding, synthesis, translation
IFN I antagonist

Membrane (M) glycoprotein
Triple membrane spanning

Small Envelope (E) glycoprotein
Ion channel
Pentameric

RNA
(+) ssRNA

Envelope
Bilipid, host-derived membrane

The most immunogenic of the viral proteins is the S protein (spike). It is a trimeric, envelope-anchored type I fusion glycoprotein that interfaces with its human host cognate receptor, dipeptidyl peptidase 4 (DPP4, also known as CD26) [23]. The S protein consists of two subunits as depicted in Figure 4: S1 contains the receptor binding domain (RBD) and determines cell tropism; S2 contains cell fusion machinery and comprises epitopes cross-reactive with homologous epitopes of other coronaviruses. Most vaccine candidates currently in development for MERS-CoV target the S protein (Tables 4-6).

Figure 4. Schematic diagram of functional domains of MERS-CoV spike protein (adapted from [24])
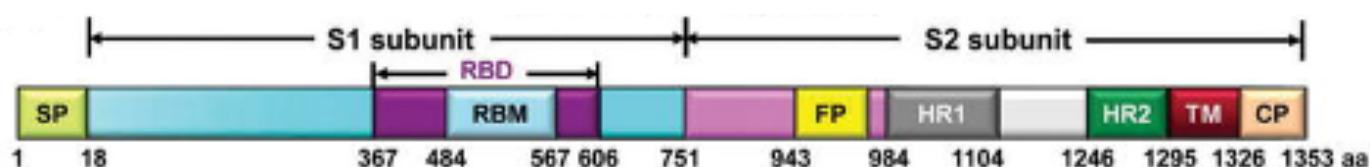
S protein comprises S1 and S2 subunits. SP, signal peptide; RBD, receptor-binding domain; RBM, receptor-binding motif; FP, fusion peptide; HR1 and HR2, heptad repeat region 1 and 2; TM, transmembrane; CP, cytoplasmic tail. Two N-linked glycans (N410 and N487) are located in the core and RBM of S1, respectively.

The DPP4 receptor is required for viral binding and entry into host cells [25]. In addition to binding to DPP4 receptor, MERS-CoV can bind to sialic acid via the S1 subunit of S protein, or utilize the membrane-associated 78 kDa glucose-regulated protein (GRP78) to attach to target cells, suggesting importance of these molecules for virion attachment [26, 27].

DPP4 is a type II transmembrane glycoprotein that is expressed on epithelial and endothelial cells throughout the body. Although DPP4 is evolutionarily conserved, differences in the amino acids present in its extracellular domain, which interacts with MERS-CoV S protein, have been noted among various animal species and humans [28]. Specifically, 14 amino acids in DPP4 appear to be critical in determining whether the MERS-CoV S can bind to DPP4. MERS-CoV S protein cannot bind to DPP4 in species that have significant differences in these 14 amino acids as compared with human DPP4 (hDPP4), such as ferrets, hamsters and mice; thus, these species are resistant to infection [29, 30]. Species with few or no differences in the 14 amino acids are infectible with MERS-CoV, including rhesus macaques, common marmosets and camels. Researchers have identified several other species that are susceptible to MERS-CoV infection, including rabbits [31], hDPP4-transduced and hDPP4-transgenic mice [32].

MERS-CoV Clinical Features and Pathogenesis in Humans

The clinical spectrum of MERS-CoV infection ranges from no symptoms (asymptomatic) or mild respiratory symptoms to severe acute respiratory disease and death [33]. The mean incubation period is around 5 days (range 2-13 days), and typical presentation of MERS-CoV disease is fever, cough, and shortness of breath. Pneumonia is a common finding, but not always present. Gastrointestinal symptoms, including diarrhea, have also been reported. Severe illness can cause respiratory failure that requires mechanical ventilation and support in an intensive care unit. The virus appears to cause more severe disease in older people, people with weakened immune systems, and those with chronic diseases such as renal disease, cancer, chronic lung disease and diabetes.

As of July 2019, the WHO has been notified of 2,449 laboratory-confirmed human cases of infection with MERS-CoV with 845 deaths (CFR 34.5%).

Because the clinical symptoms of MERS-CoV infection overlap with those of other respiratory illnesses and are not pathognomonic (i.e., specific characteristics) for MERS-CoV, prompt access to and use of sensitive and specific diagnostic tools [34] is critical for appropriate clinical management (https://www.who.int/csr/disease/coronavirus_infections/case-management-ipc/en/).

Asymptomatic or mild MERS-CoV infections mainly occur in previously healthy immunocompetent individuals. The recent apparent increase in reported numbers of asymptomatic cases in the KSA is attributed to more effective contact tracing and to a policy change in which all high-risk contacts are tested for MERS-CoV regardless of the development of symptoms.

Although DPP4, a cognate receptor for MERS-CoV spike protein, has a broad tissue distribution, most of the clinical manifestations of MERS-CoV can be attributed to infection of cells of the LRT [35]. Importantly, smoking and COPD upregulate DPP4 expression in the lungs. In healthy human lungs, DPP4 is almost exclusively expressed in type II pneumocytes, which are small cuboidal cells that can regenerate alveolar epithelium upon injury and cover roughly 2% of the alveolar surface area. Meanwhile, around 95% of the surface area of the alveolus is occupied by type I pneumocytes. In the lungs of smokers and COPD patients, unlike in healthy human lungs, DPP4 is prominently expressed in both type I and II pneumocytes significantly increasing the risk of diffuse alveolar damage stemming from damage to type I cells in case of MERS-CoV infection [35].

However, MERS-CoV can also cause significant dysfunction of the gastrointestinal, cardiovascular, renal and neurologic systems. MERS-CoV tends to cause greatest harm to older individuals with concurrent comorbidities of one or more of these organ systems. It is of interest that DPP4 has an uncanny dual nature, being an immunologic signaling glycoprotein component cluster of differentiation-26 (CD26) on T cells as well as an enzyme best known for its catalytic affinity on incretins from which DPP4-inhibitor therapy was developed as an additional sword in the arsenal against the diabetes "pandemic." As diabetes is a risk factor for

MERS-CoV infection, DPP4's role in both conditions deserve further elucidation.

Ancillary tests have detected abnormalities in complete blood counts, biochemistry panels and radiographs in MERS-CoV-infected patients. Lymphopenia, neutrophilia, thrombocytopenia and increased levels of C-reactive protein have all been reported [36]. Increases in creatine kinase, alanine aminotransferase, lactate dehydrogenase, creatinine and blood urea nitrogen have been identified in some individuals, suggesting the development of hepatic and renal disease [37]. Pulmonary interstitial infiltrates have been documented in radiographs from individuals with pneumonia [7]. MERS-CoV infections are commonly diagnosed by detecting viral RNA from several specimen types using real-time reverse transcription polymerase chain reaction (RT-PCR). Samples obtained from the LRT, such as bronchoalveolar lavage samples or sputum, are considered the most reliable for testing purposes since the highest viral loads have typically been found in these fluids. However, MERS-CoV has also occasionally been detected in nasal or oropharyngeal swabs, urine, feces, serum and blood.

Human infections with MERS-CoV are expected to continue to occur on the Arabian Peninsula because of the prevalence of MERS-CoV in dromedary camels and the cultural importance of these camels in the region, including for food, milk, transportation and racing purposes. Camels are also used for display in "beauty contests" and for social status purposes. Some of the ongoing research determined that the MERS-CoV isolates from Egypt and Nigeria (African strains, clade A) are genetically divergent from other MERS-CoV strains seen in the Arabian Peninsula (clade B) [38]. Although the sequencing shows divergence between strains, these variations do not appear to translate into a change in antigenic expression as demonstrated by viral neutralization assays [39]. The MERS-CoV sequences from

dromedary camels in Saudi Arabia and Qatar were closely related to sequences found in humans and did not show major genetic variability that would support long-term evolution of MERS-CoV in camels.

While still unexplained, no autochthonous (i.e., found in a locality in which it originates as an infectious disease) human cases of MERS-CoV have been reported from African countries in the Greater Horn of Africa region. The countries in this region host 80% of the world's dromedary camel population and exports up to 300,000 dromedaries to the Arabian Peninsula per year. While the seroprevalence in dromedary camels within this region was demonstrated [40], the lack of reported human cases in the Greater Horn of Africa countries might be explained by 1) different from Arabian Peninsula socioeconomic or age groups of people interacting with camels, 2) different cultural norms related to human-camel interactions, 3) diversity of MERS-related CoV in dromedaries in Africa that are less efficient in human transmissibility or 4) lack of reporting due to underdeveloped capacities of health systems in countries of this area to recognize the disease.

MERS-CoV Vaccine Development

While a range of therapeutics have been explored for MERS-CoV disease [41], a MERS-CoV vaccine would offer a powerful, practical and cost-effective prophylactic measure. Current therapeutics studies are limited to case reports and case series with no control arm. Very limited use of convalescent plasma (CP) in a South Korea MERS-CoV outbreak in 2015 has been reported. Three of thirteen MERS-CoV patients with respiratory failure received four CP infusions from convalesced MERS-CoV-infected patients. It should be noted that only two of four CPs showed neutralizing activity, this information apparently unavailable at the time of CP administration. Donor plasma with a plaque-reduction neutralization test (PRNT) titer of 1:80 demonstrated meaningful serological response after CP infusion (in one patient), while that with a PRNT titer of 1:40 did not (in one other patient) [42]. A third patient received CP with no neutralizing PRNT titer. Collecting CP from patients with confirmed or suspected MERS-CoV infection is predicted be challenging [43] due to the limited pool size of potential donors with sufficient antibody titers.

DPP4-targeting therapeutic agents, including antibodies specific to DPP4 and DPP4 antagonist, can block the binding or interaction between MERS-CoV receptor-binding domain (RBD) and DPP4, and thus inhibit MERS-CoV infection in animal models [24]. Therapeutics tested in humans include antivirals such as lopinavir, ritonavir, ribavirin (alone or in combination with interferon) and convalescent plasma, in addition to few other options [2]. While ribavirin appeared to be an effective anti-viral in vitro, it has been recognized that its concentrations required to inhibit MERS-CoV need to be far higher than what can be clinically acceptable in humans. Clinically tested antivirals are listed in Table 3. Data from other combination studies in humans are not available yet.

Table 3. Clinical experience with combination anti-viral therapy for MERS-CoV infection

Study type	Treatment	Treatment group n/N (% survival)	Ref
Case series	Ribavirin and IFN alpha 2b	0/5 (0)	[44]
Retrospective cohort study	Ribavirin and IFN alpha 2b	14/20 (70 at 14 days) 6/20 (30 at 28 days)	[45]
Case series	Ribavirin and IFN alpha 2b	11/11 (100)	[46]
Case series	Ribavirin and IFN alpha 2b	11/13 (85)	[47]
Case series	Ribavirin and IFN b2a	7/11 (64)	[47]
Case report	Lopinavir /ritonavir / ribavirin and interferon alpha	4	[48]
Case series	Ribavirin and Interferon alpha 2b	3/6 (50)	[49]

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Currently a vaccine for MERS-CoV is not available, although several candidates have been developed using a variety of approaches listed in Tables 4-6. Vaccine studies were initially hampered by a lack of small animal models of MERS-CoV disease. Rodents' DPP4 sequences vary in the critical 14 amino acid loop region which is responsible for binding to S protein of MERS-CoV. Thus, while rodents possess homologues for hDPP4, most of the traditionally used small animal models are refractory to MERS-CoV infection. However, several in vivo approaches have been developed to overcome these barriers and facilitate MERS-CoV vaccine testing in small animal models (see Section 5).

Table 4. Whole virus MERS-CoV human vaccine candidates in development

Vaccine platform	Composition (MERS-CoV strain) §	Immunization strategy‡			Animal model#	Efficacy	Ref
		Schedule	Route	Dosage			
MERS-CoV whole virus	Inactivated EMC/2012	2 doses (3 weeks interval)	i.m.	1×106 TCID50 Alum / MF59	hDPP4-Tg mice	nAb↑ Viral Load ↓	[50]
		3 doses (4 weeks interval)	i.m.	1 µg S (equivalent) Alum + CpG	Ad5-hDPP4 mice	nAb↑ (against RBD) Viral Load ↓ Pathology ↓	[51]
	Virus-like particle	4 doses (2 weeks interval)	i.m.	250 µg VLPs 250 µg Alum	NHPs	nAb↑ Cellular Immunity ↑	[52]

§ Composition indicates specific virus strain, truncation of DNA / protein, or adjuvants used in the vaccine design. Modified Vaccinia Ankara (MVA), Venezuelan Equine Encephalitis Virus (VEEV), Vesicular Stomatitis Virus without G protein (VSV-ΔG), Rabies virus (RABV), tissue plasminogen activator (tPA). S indicates full length Spike Glycoprotein with the transmembrane domain (TM).

‡ Abbreviations for vaccination route: intramuscular (i.m.), intranasal (i.n.), subcutaneous (s.c.), intragastric (i.g.), and intraperitoneal (i.p.).

Different units are applied to describe doses in each platform: plaque forming units (PFU), virus particle (vp), half-tissue-culture-infectious-dose (TCID50), and infectious units (IU).

Abbreviations for animal models: human DPP4 transgenic (hDPP4-Tg) mice with global/epithelial hDPP4 expression, human DPP4 knock-in (hDPP4-KI) mice with hDPP4 replacing mDPP4 in situ, mice transduced with human adenovirus 5 vector expressing hDPP4 (Ad5-hDPP4 mice), and non-human primates (NHPs).

* Efficacy in the specific animal model listed in the previous column. Neutralizing antibody (nAb). ↑ indicates more, while ↓ indicates less.

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Table 5. Viral vector based MERS-CoV human vaccines in development

Vaccine platform	Composition (MERS-CoV strain) §	Immunization strategy‡			Animal model#	Efficacy	Ref
		Schedule	Route	Dosage			
Viral vector based	MVA						
	S	2 doses (3 weeks interval)	i.m./s.c.	1×108 PFU	Ad5-hDPP4 mice	nAb↑ Cellular Immunity ↑ Viral Load ↓ Pathology ↓	[53, 54]
	S	2 doses (4 weeks interval)	i.n. i.m.	2×108 PFU (i.n.) + 1×108 PFU (i.m.)	Dromedary Camel	nAb↑ (against S1) Viral Load ↓ Pathology ↓	[55]
	Ad5-S/S1	2 doses (week 0 i.m.+ week 3 i.n.)	i.m. i.n.	1×1011 vp	BALB/c mice	nAb ↑ (against S)	[56]
	Ad5-S Ad41-S	1 dose	i.m./ i.g.	1×109 vp 5×109 vp	BALB/c mice	nAb ↑ (against RBD) Cellular Immunity ↑ (i.m.)	[57]
	Adenovirus ChAdOx1-S	1 dose	i.n./i.m.	1×108 IU	hDPP4-Tg mice	nAb↑ Cellular Immunity ↑ Viral Load ↓ Pathology ↓	[58, 59]
	Ad5-S1-CD40L	2 doses (4 weeks interval)	i.m.	1×109 PFU	hDPP4-Tg mice	nAb↑ Viral Load ↓ Pathology ↓	[60]
	AdC68-S	1 dose	i.n.	2×109 vp	hDPP4-KI mice	nAb↑ Cellular Immunity ↑ Viral Load ↓ Pathology ↓	[61]
	Measles Virus S	2 doses (4 weeks interval)	i.p.	1×105 TCID50	Ad5-hDPP4 mice	nAb↑ Cellular Immunity ↑ Viral Load ↓ Pathology ↓	62, 63
	VEEV Replicon Particle S	2 doses (4 weeks interval)	foot-pad	1×105 IU	Ad5-hDPP4 mice hDPP4-Tg mice	Viral Load ↓	[64, 65]
	VSV-ΔG S	1 dose	i.n./i.m.	2 ×107 FFU	NHPs (also in mice)	nAb↑ Cellular Immunity ↑	[66]
	RABV S1	3 doses (1-2 weeks interval)	i.m.	10 µg inactivated virus	Ad5-hDPP4 mice	nAb ↑ Viral Load ↓	[67]
Viral vector + nanoparticle	Ad5-S+Nanoparticle(S)	1×Ad5-S 2 x nanoparticle (2-3 weeks interval)	i.m.	1×109 IU 5 µg S+Alum	BALB/c mice	nAb↑ Cellular Immunity ↑ Pathology ↓	[68]

§ Composition indicates specific virus strain, truncation of DNA / protein, or adjuvants used in the vaccine design. Modified Vaccinia Ankara (MVA), Venezuelan Equine Encephalitis Virus (VEEV), Vesicular Stomatitis Virus without G protein (VSV-ΔG), Rabies virus (RABV), tissue plasminogen activator (tPA). S indicates full length Spike Glycoprotein with the transmembrane domain (TM).

‡ Abbreviations for vaccination route: intramuscular (i.m.), intranasal (i.n.), subcutaneous (s.c.), intragastric (i.g.), and intraperitoneal (i.p.).

Different units are applied to describe doses in each platform: plaque forming units (PFU), virus particle (vp), half-tissue-culture-infectious-dose (TCID50), and infectious units (IU).

Abbreviations for animal models: human DPP4 transgenic (hDPP4-Tg) mice with global/epithelial hDPP4 expression, human DPP4 knock-in (hDPP4-KI) mice with hDPP4 replacing mDPP4 in situ, mice transduced with human adenovirus 5 vector expressing hDPP4 (Ad5-hDPP4 mice), and non-human primates (NHPs).

* Efficacy in the specific animal model listed in the previous column. Neutralizing antibody (nAb). ↑ indicates more, while ↓ indicates less.

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Table 6. DNA, protein and nanoparticle based MERS-CoV human vaccines in development

Vaccine platform	Composition (MERS-CoV strain) §		Immunization strategy‡			Animal model#	Efficacy	Ref
			Schedule	Route	Dosage			
DNA	S (consensus sequence)		3 doses (3 weeks interval)	i.m.	0.5–2 mg	NHPs (also in mice and camels)	nAb↑ Cellular Immunity ↑ Viral Load ↓ Pathology ↓	[69]
	S1 (1–725)		3 doses (3 weeks interval)	i.m.	0.1 mg	Ad5-hDPP4 mice	nAb↑ Cellular Immunity ↑ Viral Load ↓	[70]
DNA + protein	S DNA + S1 Protein		2×DNA 1×Protein Boost (4 weeks interval)	i.m.	1 mg DNA 100 μg Protein	NHPs (also in mice)	nAb ↑ Pathology ↓	[71]
Protein subunit	RBD-Fc (377–588)	MF59	3 doses (3 weeks interval)	s.c.	1–10 μg	Ad5-hDPP4 mice	nAb↑ Cellular Immunity ↑ Viral Load ↓	[72–75]
		Alum	2 doses (4 weeks interval)	s.c.	5 μg	hDPP4-Tg mice	nAb ↑ Pathology ↓	[76]
	RBD trimer (377–588)	Alum	2 doses (4 weeks interval)	i.m.	5 μg	hDPP4-Tg mice	nAb ↑ Pathology ↓	[77]
	RBD-Fc (377–662)	Poly(I:C)	5 doses (Week 0, 3, 6, 12, 24)	i.n.	10 μg	BALB/c mice	nAb ↑ (against RBD)	[78]
	RBD (367–606)	Alum	3 doses (Week 0, 8, 25)	i.m.	200 + 2×100μg 50 + 2×25μg	NHPs (also in mice)	nAb↑ Cellular Immunity ↑ Viral Load ↓ Pathology ↓	[79, 80]
	NTD (18–353)	Alum + CpG	3 doses (4 weeks interval)	i.m.	10 μg	Ad5-hDPP4 mice	nAb↑ Cellular Immunity ↑ Pathology ↓	[81]
	S prefusion trimer	Sigma adjuvant	2 doses (3 weeks interval)	i.m.	10 μg	BALB/c mice	nAb↑	[82]
Nanoparticle	S		2 doses (3 weeks interval)	i.m.	1–10 μg Spike	Ad5-hDPP4 mice	nAb ↑ Viral Load ↓	[83, 84]

§ Composition indicates specific virus strain, truncation of DNA / protein, or adjuvants used in the vaccine design. Modified Vaccinia Ankara (MVA), Venezuelan Equine Encephalitis Virus (VEEV), Vesicular Stomatitis Virus without G protein (VSV-ΔG), Rabies virus (RABV), tissue plasminogen activator (tPA). S indicates full length Spike Glycoprotein with the transmembrane domain (TM).

‡ Abbreviations for vaccination route: intramuscular (i.m.), intranasal (i.n.), subcutaneous (s.c.), intragastric (i.g.), and intraperitoneal (i.p.).

Different units are applied to describe doses in each platform: plaque forming units (PFU), virus particle (vp), half-tissue-culture-infectious-dose (TCID50), and infectious units (IU).

Abbreviations for animal models: human DPP4 transgenic (hDPP4-Tg) mice with global/epithelial hDPP4 expression, human DPP4 knock-in (hDPP4-KI) mice with hDPP4 replacing mDPP4 in situ, mice transduced with human adenovirus 5 vector expressing hDPP4 (Ad5-hDPP4 mice), and non-human primates (NHPs).

* Efficacy in the specific animal model listed in the previous column. Neutralizing antibody (nAb). ↑ indicates more, while ↓ indicates less.

The One Health concept focuses on the relationship and interconnectedness between humans, animals and the environment, and recognizes that the health and well-being of humans is intimately connected to the health of animals and their environment (and vice versa). It is ideally suited to the MERS-CoV situation in which camels, humans and environmental factors are central to virus persistence and evolution. An animal vaccine strategy may thus be the best way to prevent human outbreaks and may have a faster development and licensing pathway.

Pursuing animal vaccine approach, one paper describes a rhabdovirus (RABV)-based vaccine with MERS-CoV S1 domain of the MERS-CoV S protein fused to the RABV G protein [67] which offered protection against MERS-CoV challenge in mouse model. Rabies virus-based vectors have been proven to be efficient dual vaccines against rabies and emergent infectious diseases such as Ebola virus disease. Inactivated rabies vaccine, from which MERS-CoV candidate vaccine was derived in this study, has been used in millions of humans, leading authors to suggest their MERS-CoV candidate vaccine for further development for both human and veterinary use.

Another paper described two major advantages of an orthopoxvirus-based MERS-CoV vaccine, which include its capacity to induce protective immunity in the presence of preexisting (e.g., maternal) antibodies and the observation that MVA-specific

antibodies cross-neutralize camelpox virus, revealing the potential dual use of this candidate MERS-CoV vaccine in dromedaries [55]. Infection with MERS-CoV in dromedary camels is mildly symptomatic and localized primarily in the URT. Thus, the main objective of a camel vaccine is to prevent transmission of MERS-CoV within dromedary herds and prevent transmission from camels to humans. This will require prevention of infection or reduction in viral shedding, which may require mucosal delivery of the vaccine for eliciting local immunoglobulin A (IgA) protection [55]. Young camels could be a priority group for vaccination because they appear to be at high risk for MERS-CoV infection [85]. However, the loss of maternal MERS-CoV antibodies at approximately 5–6 months after birth suggests a short time window for vaccination [16].

A major challenge to this approach is that dromedary camels can be re-infected with MERS-CoV [86]; it was noted in a study by Farag et al. [87] that no correlation was identified between MERS-CoV RNA levels and neutralizing antibodies in camels, suggesting that antibodies may not be protective against infection or that protection wanes over time. It is unknown if humans can be reinfected with MERS-CoV. Because of reinfection concerns, a camel vaccination strategy may require multiple dosing and booster vaccination to increase effectiveness over time. Assuming successful development of a camel vaccine, additional novel approaches, such as creating

appropriate incentives for camel owners, would be necessary for a camel vaccination strategy to succeed in the real world. It will also be important to determine the duration of protective window following the immunization to determine the frequency of possible boosting.

In 2017 the WHO developed a Target Product Profile (TPP) for MERS-CoV vaccine, including preferred as well as critical or minimal product characteristics for three vaccines:

1. Dromedary camel vaccine — for prevention of transmission of MERS-CoV among camels and from camels to humans.

2. Human vaccine — for long term protection of persons at high ongoing risk of MERS-CoV such as health care workers and those working with potentially infected animals.

3. Human vaccine — for reactive use in outbreak settings with rapid onset of immunity.

Key desirable vaccine performance attributes recommended in the TPP are summarized in Table 7.

Table 7. Key performance characteristics for future MERS-CoV vaccines

Vaccine platform	Camel vaccine		Human vaccine for long-term protection		Human vaccine for outbreak use	
Characteristic	**Preferred**	**Critical or minimal**	**Preferred**	**Critical or minimal**	**Preferred**	**Critical or minimal**
Efficacy	Multiple log reduction in virus shedding	90% reduction of viral shedding	At least 90% efficacy in preventing MERS; prevention of virus shedding.	At least 70% efficacy in preventing MERS	At least 90% efficacy in preventing MERS; prevention of virus shedding. Onset of immunity in 1 week or less	At least 70% efficacy in preventing MERS. Onset of immunity in less than 2 weeks
Dose regimen	Single dose	Up to 3 doses, with some protection after 1st; booster dose no more frequent than annually	Single dose	No more than 3 doses; booster no more frequent than 3 years	Single dose	No more than 2 doses
Durability of protection	At least 3 years	At least 1 year after last vaccination	At least 5 years, maintained by booster	At least 3 years, maintained by booster	At least 1 year	At least 6 months
Route of administration	Spray for mucosal delivery	Injectable (IM, SC)	Injectable (IM, ID or SC), while oral or non-parenteral desirable	Injectable (IM, ID or SC),	Injectable (IM, ID or SC), while oral or non-parenteral desirable	Injectable (IM, ID or SC),
Shelf life	5 years at 2–8°C	At least 12 months at –20°C and at least 6 months at 2–8°C	5 years at 2–8°C	At least 12 months at –20°C and at least 6 months at 2–8°C	5 years at 2–8°C	At least 12 months at –20°C and at least 1 month at 2–8°C

In all cases the target antigen has been the S protein (Tables 5–6). Due to safety issues associated with production (i.e., biosafety level three [BSL-3] containment) and administration of a live attenuated MERS-CoV vaccine, most attempts at MERS-CoV vaccine development have focused on recombinant viral vectors and adjuvanted protein subunit vaccines. An interesting approach, built on idea from attenuated influenza virus development, involved mutating the non-structural protein NSP16 in MERS-CoV to obtain attenuation [88]. A candidate vaccine strain capable of protection from heterologous virus challenge, with efficacy in aged mice and no evidence for reversion, was produced. It appears, however, that substantial work will be required to resolve safety concerns associated with live attenuated MERS-CoV prior to human testing.

The first result of human testing of MERS-CoV vaccine has recently been published, describing durable responses to a DNA vaccine GLS-5300 [89]. The study enrolled healthy adults aged 18-50 years; exclusion criteria included previous infection or treatment of MERS. Eligible participants were enrolled sequentially using a dose-escalation protocol to receive 0·67 mg, 2 mg or 6 mg GLS-5300 administered by trained clinical site staff via a single intramuscular 1 mL injection at each vaccination at baseline, week 4 and week 12 followed immediately by colocalized intramuscular electroporation. The primary outcome of the study was safety, assessed in all participants who received at least one study treatment and for whom post-dose study data were available, during the vaccination period with follow-up through to 48 weeks after dose 3. The secondary outcome was immunogenicity.

Seroconversion measured by S1-ELISA occurred in 59 (86%) of 69 participants and 61 (94%) of 65 participants after two and three vaccinations, respectively. Neutralizing antibodies were detected in 34 (50%) of 68 participants. T-cell responses were detected in 47 (71%) of 66 participants after two vaccinations and in 44 (76%) of 58 participants after three vaccinations. Interestingly, there were no differences in immune responses between dose groups after 6 weeks. At week 60, vaccine-induced humoral and cellular responses were detected in 51 (77%) of 66 participants and 42 (64%) of 66, respectively. Immune responses were durable through 1 year of follow-up. It was concluded that the GLS-5300 MERS-CoV vaccine was well tolerated with no vaccine-associated serious adverse events.

Potential safety concerns for MERS-CoV vaccines

For respiratory diseases there are some concerns of vaccine-induced pulmonary immunopathology as was observed previously with a candidate SARS-CoV vaccine in mice after challenge with SARS virus [90] or much earlier with respiratory syncytial virus (RSV) vaccine [91] or with measles vaccine [92, 93]. Importantly, those observations were made for inactivated vaccines. In RSV and atypical measles cases, vaccines failed to elicit long-lived protective antibody and to promote cytotoxic T lymphocyte responses. Post-vaccination exposure to respective wild type virus (RSV or measles) was associated with immune complex deposition in affected tissues, vigorous CD4 T lymphocyte proliferative response, and a Th2 bias of the immune response.

Few recent publications reported MERS-CoV immunopathology concerns after vaccination followed by exposure to the virus, and this fact should warrant special attention for any developed vaccine candidate. A recent publication reported hypersensitive type lung pathology in challenged transgenic hDPP4/CD26 mice vaccinated with inactivated MERS-CoV [50] suggesting a similar risk as seen with inactivated SARS vaccine. In another study rabbits asymptomatic after primary infection with MERS-CoV and in the absence of neutralizing antibodies exhibited enhanced pulmonary inflammation upon reinfection with the same virus [94]. Even passive transfer of serum from previously infected rabbits to naïve rabbits was associated with enhanced inflammation upon infection. This inflammation was accompanied by increased recruitment of complement proteins compared to primary infection. However, reinfection elicited neutralizing antibodies that protected rabbits from subsequent viral challenge.

It is unclear if non-neutralizing antibodies contributed to the observed phenomena, but the observations point out potential risk upon re-exposure to MERS-CoV patients who fail to develop a protective response after initial infection or vaccination. Since concerns over SARS-related pathology led to a U.S. Food and Drug Administration (FDA) clinical hold on vaccine studies, investigation of MERS-CoV vaccine candidates to induce virus-enhancing antibodies and harmful immune response in animal models could be informative before human clinical trials are initiated.

3. BIOLOGICAL STANDARDS AND ASSAY STANDARDIZATION

Assays and animal models to quantify or characterize immune responses elicited by vaccination are, by their nature, inherently variable.

Some reasons for this include the molecular complexity of the samples (serum or other biological samples); the need to produce reagents in complex biological systems such as cell culture or in vivo; and the need to test immune responses in vivo, which is itself a very complex biological process. Nevertheless, modern vaccine development requires vaccines and samples from vaccinated humans and animals be tested with the highest possible accuracy and precision. The task is further complicated by the collaborative and global nature of modern vaccine development. Multiple research laboratories, vaccine developers, non-governmental organizations and regulatory agencies are often involved in the development process and vaccine candidates utilizing different platform technologies are often evaluated for the same disease indication. Thus, harmonization and standardization of methods and reagents is important to facilitate development of new vaccines such as for MERS-CoV. The goal is to enable "like-versus-like" comparisons of data between different laboratories and products. Recognizing the value of standardization early in the vaccine development process, CEPI is promoting assay, reagents and animal model standardization to accelerate development of vaccines and to facilitate comparison between various vaccine formulations for MERS-CoV and other priority diseases in its portfolio.

Immune Serum Reference Standards

One of the most important tools for standardization of serological assays is the availability of (a common) immune reference serum. Even when similar assay formats are used for detection of antigen-binding antibodies or virus-neutralizing antibodies, the resulting data can be highly variable between laboratories due to differences in assay methods and reagents. For example, a 10-laboratory collaborative study assessing the precision of assays for detection of serum antibodies against Human Papillomavirus 16 (HPV 16) revealed inter-laboratory variations in anti-HPV titer of up to 25-fold for the same test sample [95]. A similar, 15-laboratory collaborative study evaluating assays for serum antibodies against H5N1 influenza showed inter-laboratory variations in titer of 10- to 35-fold, depending on the sample and type of assay [96]. The purpose of establishing immune reference standards is to provide a common, external control to improve the comparability of assay data between laboratories. With the standard in place, test results are reported relative to the activity of the reference standard [97]. In the studies cited above, use of a common reference standard significantly reduced intra-laboratory assay variability substantially. Reference standards have been developed for many vaccine indications, both in development and commercial manufacturing. Recent examples include HPV 16 [98], typhoid fever [99], RSV [100] and Zika virus.

Three key factors determine the fitness of material for use as a biological standard. First, the material must have similar composition and in vitro behavior to the human sera test articles. For this reason, monoclonal antibodies are dis-favored, and animal-derived sera are approached with caution. Second, the standard should be commutable, meaning it should work for a wide range of vaccine approaches being tested. Thus, convalescent sera with broad polyclonal specificity are generally favored over immune sera from vaccine recipients, which may have more restricted antibody specificities. Note, however, that these are not absolute requirements and the approach may be adjusted as necessary. For example, sera from vaccinated volunteers was used for the International Standard (IS) for anti-typhoid capsular Vi polysaccharide IgG [99]. Finally, a multi-laboratory collaborative study must demonstrate the utility of the standard for reducing intra-laboratory assay variability.

Serum reference standards for a new vaccine are often established in a staged manner as the development process progresses. For R&D and early clinical trials, a working standard or interim standard may be established by a collaborative study involving a relatively limited number of laboratories. The preferred source material for standards is high-titer immune serum from disease-convalescent humans. However, if this is not feasible due to poor availability of suitable sera, an interim standard may be established from humans vaccinated in clinical trials or from disease-convalescent animals. A single, large lot of reference standard is preferred to avoid

potential variability between multiple lots and the need for subsequent bridging studies. Once suitable reference standard sera candidates are available for evaluation, a collaborative study is performed to evaluate serological assays performed by a number of participating laboratories. A broad panel of test samples from different sources (i.e. sera from naturally infected humans, animals infected in the laboratory and vaccinated humans or animals) is assayed blindly along with the candidate reference standard, and the intra-laboratory variability in assay results is assessed. Finally, the test sample absolute values (for example, geometric mean titers) are expressed relative to the candidate reference standard (arbitrary units), and the ability of the reference standard to improve intra-laboratory comparability of test results is assessed. Once the reference standard has been chosen, a full storage stability program is conducted to ensure the quality of the material over time.

Establishment of an interim standard usually precedes establishment of an IS under the endorsement of the WHO Expert Committee on Biological Standardization; or rather called International Reference Preparation (IRP). This is a more formal process, taking up to 36 months and involving a larger and a more in-depth collaborative study than for a working or interim standard. Regulatory agencies generally expect an established IS to be used in pivotal clinical trials for vaccine approval, unless specific justification is provided.

A very recent collaborative study with examples of potential MERS-CoV reference standard underscored the importance of

using a standard reagent to allow better comparison of results from different laboratories or interpretation of results from different studies or clinical trials [101]. The study participants used their routine methods to analyze blinded MERS-CoV serum samples and while the individual results (raw titers) between the laboratories varied, sometimes dramatically, the use of common reagent tightened the values from the laboratories for all the samples, enhanced comparability and reduced the geometric coefficient of variation (GCV) percentage among all laboratories.

Given the sporadic nature of MERS-CoV outbreaks and relatively small number of cases (and available survivors), establishment of a serum reference standard using convalescent human sera is likely to be challenging to develop. Since most children by the age of seven are seropositive for coronaviruses, the baseline level of cross reactivity with MERS-CoV in any convalescent sera will have to be carefully assessed [102]. CEPI has one agreement in place with International Vaccine Institute (IVI) in Seoul, South Korea, to collect serum from four MERS-CoV survivors from the South Korean outbreak in 2015. This will amount to a total of 1L and will be developed into an International Reference interim standard as well as an international reference reparation under a partnership agreement with the National Institute for Biological Standards and Control (NIBSC) in the U.K. It is understood that obtaining convalescent sera from MERS-CoV survivors from countries in the Arabian Peninsula requires more negotiations (CEPI Workshop on Standards and Assays, Oslo, June 2019). One other possibility

that has been considered is to collect large volumes of bovine sera from transgenic animals carrying a human IgG repertoire and vaccinated with whole inactivated MERS-CoV [103], but cross-reactivity against bovine coronaviruses needs to be accounted for. In addition, vaccination is unlikely to give as broad a polyclonal response as a natural disease.

There is also a need for more coordinated efforts to sequence circulating viruses and to correlate those data with phenotypic outcomes, such as viral fitness, virulence and structure-function relationships of the surface spike protein and other MERS-CoV proteins.

Standardization of Other Biological Assay Reagents and Methods

Many aspects of biological assays for vaccine testing may be standardized to improve the comparability of inter- and intra-laboratory data. Common reagents (reference sera, antigens, virus stocks) may be produced and standardized assay methods established and validated. For example, potency testing for release of subunit seasonal influenza vaccines is performed under a high degree of standardization using common reagents (reference sera and antigens) provided by regulatory agencies and using a single, validated assay method to quickly test and release new seasonal vaccine formulations produced by several manufacturers. However, the need, feasibility and level of standardization is typically considered on a case-by-case basis according to the stage of vaccine development, the types of assays in use and the potential of standards to facilitate development and licensure. Standardized reference sera are relatively easy to implement since they should be commutable across many assay types and are broadly recognized for improving both intra- and inter-laboratory assay consistency. In contrast, standardized assay formats are more challenging to implement, especially for newer vaccines and those in development, since there is less consensus on the ideal format. CEPI is promoting harmonization by providing a standard antigen to all CEPI-funded vaccine developers. An agreement is in discussion with the University of Queensland (UQ) in Australia to produce common reagents including the spike (MERS-S) protein for ELISA assays. CEPI and UQ aim to make these reagents available to the MERS-CoV vaccine development community by the end of 2019.

4. SEROLOGICAL ASSAYS FOR MERS-COV

Robust serological assays for quantifying and characterizing humoral immune responses in humans and animals are critical for vaccine development. A number of methods listed in Table 8 have been developed for MERS-CoV serology [34], and the refinement and standardization of such methods will be essential for facilitating development of safe and effective human vaccines. This section describes the commonly used serological assays for measurements of immune responses against MERS-CoV.

Table 8. Commercial and regulated assays for MERS-CoV

Commercial ELISA and IFT/IFA								
Developer	System	Sample Type	Target	LOD	Sensitivity/ PPA	Specificity/ NPA	Specimens tested	Reference assay
Alpha Diagnostic International (US)	Camel Anti-MERS-NP IgG ELISA Kit	Serum, plasma or other biological fluids	Recombinant (sf9), purified (95%, ~46 kDa), full length MERS-NP protein MERS-CoV) ([Human betacoronavirus 2c EMC/2012]) is used as antigen.	<0.75 µg/mL IgG or IgM	no info	no info	no info	no info
Alpha Diagnostic International (US)	Human Anti-MERS-RBD IgG ELISA Kit	Serum, plasma or other biological fluids	Recombinant purified (95%, MERS-CoV-RBD, 383–502 aa ~14.5 kDa) protein (Human betacoronavirus 2c EMC/2012) is used as antigen.	<0.75 µg/mL IgG or IgM	no info	no info	no info	no info
Alpha Diagnostic International (US)	Human Anti-MERS-S2 IgG ELISA Kit	Serum, plasma or other biological fluids	Recombinant (sf9), MERS-S2 (95%, ~66 kDa, full length) MERS-S2 protein) (Human betacoronavirus 2c EMC/2012) is used as antigen.	<0.75 µg/mL IgG or IgM	no info	no info	no info	no info
EUROIMMUN (GER) (acquired by Perkin Elmer 2017)	Camel Anti-MERS-CoV-ELISA (IgG)	Camel serum or plasma	purified spike protein domain S1 antigen of MERS coronavirus (MERS-CoV S1)	The lower detection limit of the Anti-MERS-CoV ELISA (IgG) is ratio 0.04.	100% (151 specimens)	100% (33 specimens)	Sera from 184 camels (151 positive, 33 negative)	in-house assays (rIFA, vIFA) of the Institute of Virology, University of Bonn, Germany
EUROIMMUN (GER) (acquired by Perkin Elmer 2017)	Camel Anti-MERS-CoV IIFT (IgG, IgM)	Camel serum and plasma	MERS coronavirus-infected and non-infected cells (species EU 14)	Titer < 1: 100	99.4%	100%	Sera from 196 camels (163 positive, 33 negative)	in-house assays (rIFA, vIFA) of the Institute of Virology, University of Bonn, Germany
EUROIMMUN (GER) (acquired by Perkin Elmer 2017)	Human Anti-MERS-CoV-ELISA (IgG)	Human (serum)	purified spike protein domain S1 antigen of MERS coronavirus (MERS-CoV S1)	The lower detection limit of the Anti-MERS-CoV ELISA (IgG) is ratio 0.04.	100% (4 specimens)	99.8% (500 specimens)	Sera from four patients with MERS and 500 blood donors with other infections	in-house assays (rIFA, vIFA) of the Institute of Virology, University of Bonn, Germany
EUROIMMUN (GER) (acquired by Perkin Elmer 2017)	Human Anti-MERS-CoV IIFT (IgG, IgM)	Human serum and plasma	For the detection of antibodies against MERS coronavirus by indirect immunofluorescence, MERS coronavirus infected cells (species EU 14) are used.	MERS IgG: Positive reaction at 1:100 indicates former or acute infection. MERS IgM: Positive reaction at 1:10 indicates acute infection.	100% sens	100% spec	IgG: 29 clinically characterized MERS positive; 264 negative samples IgM: 5 clinically characterized MERS positive; 165 negative samples	in-house assays (rIFA, vIFA) of the Institute of Virology, University of Bonn, Germany

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Commercial Rapid Diagnostic Tests (RDTs)

Developer	System	Sample Type	Target	LOD	Sensitivity/ PPA	Specificity/ NPA	Specimens tested	Reference assay
BioNote Inc. (KOR) subsid of SD Biosensor	Camel Rapid MERS–CoV Ag Test (BioNote, RG1805SG)	Camel nasal swab	MERS–CoV antigen	3.125 ng/mL of recombinant nucleocapsid Ag of MERS CoV	93.9%	99.6% (OIE value) bovine/ canine/feline corona virus	66 pos, 523 neg	UpE and Orf1A rRT-PCR
SD Biosensor (KOR)	Human SD Q Line MERS–CoV Ag	Sputum and bronchoalveolar lavage, tracheal aspirate and pleural fluid	MERS–CoV antigen	no info	no info	no info	no info	no info

Coalition for Epidemic Preparedness Innovations

Quantitation of MERS-CoV Antigen-Specific Serum IgG

The quantitation of serum-neutralizing antibodies is essential for measuring vaccine potency and is also important for evaluating correlates of protection. Humoral immunity is currently considered to be the predictor of protective responses, and all vaccine candidates in development aim to elicit anti-spike (S protein) antibodies. However, no information is available about the protective levels of such antibodies that would correlate with clinical protection (in real-life situations). Reported reinfection of seropositive camels — the natural reservoir of MERS-CoV – appears to suggest that protective levels may wane over time [16, 85]. While cellular immunity is thought to play a role in clearing the MERS-CoV infection [104], it is currently difficult to predict if a certain level of polyfunctional T cell would prevent a reinfection. However, it is also tempting to speculate that presence of cross-reactive T cells (including to other circulating coronaviruses) in at-risk populations is at least partly responsible for many asymptomatic and mild cases of MERS-CoV infections.

The most reliable laboratory methods for detection of antibodies against MERS-CoV are indirect immunofluorescence (IIFT), ELISA [105] and neutralization tests. Similar to the ELISA, IIFT is used when it is difficult to evaluate specific antigens individually by enzyme immunoassays or there is a preference for broader analysis of an immobilized specimen. Companies such as Euroimmun have commercial tests offering camel and human IgG detection by IIFT and ELISA methods (https://www.vet.euroimmun.com/produkte/kamel/mers-coronavirus.html). The anti-MERS-CoV IIFT (for IgG, IgM) is based on MERS-CoV-infected eukaryotic cells and the anti-MERS-CoV ELISA (IgG) on purified S1 antigens of MERS-CoV. Owing to this, a high sensitivity and specificity is achieved. However, optimization of cut-off values of these assays may still be possible [106].

It was recently demonstrated that detection of IgG in serum collected from mild cases of MERS-CoV may be challenging with commercial ELISA testing (from Euroimmun) while in-house ELISA (from Erasmus Medical Center, EMC) was more sensitive, specific and correlated well with plaque reduction neutralization test (PRNT90) and S1 microarray results [107]. Notably, both ELISAs detected IgG in all samples from severe MERS-CoV cases, but commercial testing was positive in only two out of six samples from mild cases, while in-house testing was positive in four out of five samples. With both commercial and EMC ELISA tests based on binding to the S1 protein, the seeming contradiction between sensitivities was explained as a possible reduction in antigen coating or a loss of some conformational epitopes in the commercial ELISA during storage. In a concurrent same study comparison of PCR-diagnosed MERS cases, PRNT90 testing showed 100% sensitivity for detecting severe cases after the seroconversion period and for up to one year. In mild cases only 50% of samples had neutralization activity, perhaps highlighting lower, shorter-lived neutralizing responses. Importantly, the authors also showed that in-house, S1-based ELISA is more sensitive and specific than S2 or N-protein-based ELISA.
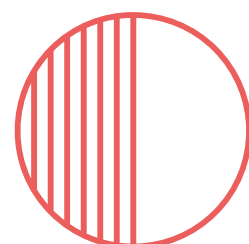
Neutralization Tests

Neutralization is a method for detecting functional anti-MERS-CoV antibody activity via inhibition of infection or replication and tests are typically done by PRNT, microneutralization (MN) and MERS-spike pseudoparticle neutralization test (ppNT) [39]. MN is labor intensive and slow, requiring at least three to five days for results, and neutralization techniques other than ppNT require BSL-3 containment as they involve live-virus cultures.

Comparison of performance of different serological assays [108] stated that the PRNT50 assay was more sensitive than the PRNT90, primarily because it uses the less stringent endpoint of 50% reduction in the plaque count.

Excellent correlation (Table 9) was demonstrated between the PRNT90, MN and MERS-spike ppNT titers, with Spearman's correlations of 0.97-0.98. MERS-CoV S1 ELISA was less strongly correlated with the different neutralization assays, with Spearman's correlation of 0.86-0.87.

Table 9. Proportion of sera positive for antibodies to MERS-CoV in various assays

# of serum samples	# of patients	Proportion of sera with neutralizing antibody titers >1:20				ELISA positive
		PRNT90	PRNT50	ppNT	MN	
7	7	0/7	0/7	0/6a	0/7	0/7
17	11	0/17	0/17	0/17	0/17	0/17
18	17	7/18	9/18	6/18	6/18	7/18
19	17	14/19	15/19	14/18a	14/19	15/19
34	9	33/34	34/34	31/33a	33/34	33/34

ELISA: enzyme-linked immunosorbent assay; MN: microneutralization; PRNT: plaque reduction neutralization test; ppNT: pseudoparticle neutralization test.

a One serum in each of these groups could not be tested in the ppNT assay, thus the denominator for the ppNT differed from the others.

Unlike other neutralization tests that require handling live MERS-CoV in BSL-3 containment, the MERS-spike ppNT assay does not require BSL-3 containment. It was also found to have good correlation with PRNT90 and MN tests. ppNTs have proved to be reliable surrogates for neutralization tests in other infections including avian influenza A(H5N1) [109, 110]. Thus, the MERS-spike ppNT may be usable for large scale seroepidemiology studies to assess the extent of MERS-CoV infection in the general population, to assess risk factors of infection in high-risk groups or when selecting patient sera for plasmapheresis for preparation of convalescent plasma where quantification of neutralizing antibody may be important.

As noted earlier in MERS-CoV Molecular Biology and Structure section, the S protein of MERS-CoV binds to the DPP4 receptor and to sialic acid [27]. Likely because of this sialic acid recognition, and similarly to influenza A virus, MERS-CoV virions (EMC strain) can cause hemagglutination (HA) of human erythrocytes. Notably, soluble S protein did not yield HA, but engineered nanoparticles decorated with multiple copies of S protein arrayed on their surfaces were demonstrated to cause agglutination (HA titer =128). It is not known if erythrocytes from other species would bind better to MERS-CoV. Demonstration of inhibition of HA is a classical method of measuring the level of antibodies produced by a viral infection, such as influenza. One recent paper described the use of HA inhibition testing to characterize MERS-CoV monoclonal antibodies [111], and one hopes that this method may find broader use in the near future for characterization of immune responses to MERS-CoV infection or vaccination.

5. MERS-CoV ANIMAL MODELS

Well characterized, robust animal challenge models are a critical component for development of any vaccine, and as with assays, harmonization or standardization of animal models can accelerate vaccine development by promoting like-versus-like comparisons of vaccine efficacy data between laboratories [112]. In the case of MERS-CoV and other emerging diseases, the refinement and harmonization of animal models takes on additional importance regarding the pathway to vaccine licensure, because animal model study data will most likely be required to enable licensure decisions in the absence of human efficacy data, as per the FDA Animal Rule. Ideally, an animal model should reproduce the hallmarks of human disease as closely as possible in an immunocompetent animal following a realistic dose of challenge virus via an appropriate inoculation route. Table 10 lists clinical symptoms observed in currently used animal models of MERS-CoV infection and Table 11 summarizes benefits and limitations of these animal models.

Table 10. Comparison of clinical symptoms after infection in MERS-CoV animal challenge models

MERS-CoV Animal Challenge Model	Route and dose	Clinical disease	Gross lesions	Microscopic lesions	Ref
NZ white rabbits	IT; IN; 5x106 TCID50	Asymptomatic	Absent	Heterophilic rhinitis, pulmonary interstitial heterophilic infiltration	[113]
Rhesus macaques	IT; 6.5x107 TCID50	Fever	Pulmonary congestion	Interstitial pneumonia	[114]
Common marmosets	OC, oral, IT, IN, or IT only; 5 x 106 TCID50 to 5 x107 PFU	Mild to severe respiratory disease, potentially fatal	Interstitial pneumonia	Interstitial pneumonia to widespread bronchointerstitial pneumonia	[115-117]
hDPP4-transduced mice	IN; 105 PFU	Lack of weight gain or mild weight loss	Mild pulmonary congestion and inflammation	Pulmonary perivascular and peribronchial inflammation, interstitial pneumonia	[64]
hDPP4-transgenic mice	IN; 106 TCID50	Severe respiratory disease, weight loss, 100% fatal	Pulmonary consolidation	Bronchointerstitial pneumonia, perivascular cuffing in the brain	[118]
hDPP4-humanized mice	IN; 2x105 PFU	Asymptomatic	Not reported	Bronchointerstitial pneumonia	[119]
CRISPr-Cas9 modified mice	IN; 5x106 PFU; (MERS-15, mouse-adapted strain)	ARDS, decreased survival, weight loss, signs of end-stage lung disease	pulmonary hemorrhage	severe inflammation and edema in the large airways and alveoli, and hyaline membrane formation	[120]

Experimental infections with MERS-CoV require BSL-3 biocontainment, leading to difficulties in handling large animals and increased housing costs. Dromedary camels, considered to be the reservoir for MERS-CoV, develop a mild URT infection with abundant viral shedding. Being large animals, dromedary camels are neither widely available nor a practical model for vaccine studies, but these animals have been used to demonstrate protection in an adjuvanted vaccine study [121]. Despite the limitations, the camel model will likely be indispensable for the development of veterinary vaccine(s), as per WHO TPP, in demonstrating the efficacy of the eventual vaccine. Another recent study demonstrated experimental infection of Bactrian camels (Bactrians), which are part of the Old World camelids along with dromedary camels. It was shown that clinical signs of the MERS-CoV infection in Bactrians were benign, but shedding of large quantities of MERS-CoV from the URT was observed, indicating both susceptibility to infection and similarity of symptoms to dromedary camels [122]. Alpacas have also been proposed as an animal model for MERS-CoV vaccine studies due to their susceptibility to infection and more "user-friendly size [123], which can perhaps make them better suited for development of a veterinary vaccine.

Table 11. MERS-CoV experimental animal models: advantages and limitations

| Species	Advantages	Limitations	Reference
Rabbit	Readily available and easy to handle	No clinical disease, low viral titers in tissues, only mild pulmonary lesions upon infection; no animal-to-animal transmission	[113]; [30]; [124]
Rhesus macaques	Human-specific reagents available for immunologic analysis; immune and respiratory systems similar to humans; clinical disease similar to humans; useful for confirming vaccine efficacy	Limited availability; expensive; expert husbandry required; no transmission studies; only transient disease; ethical concerns	[32]; [117]
Common marmoset	Model severe/fatal disease; some human-specific reagents cross-react; immune and respiratory systems similar to humans; clinical disease similar to humans; useful for confirming vaccine efficacy	Limited availability; expensive; expert husbandry required; no transmission studies; ethical concerns	[117]; [30]
hDPP4-transgenic mice	Model severe, potentially fatal MERS-CoV infection; easy to handle; reagents available; useful for screening antivirals and vaccines	Exhibit global overexpression of DPP4 in all tissues, unlike human tissue distribution of DPP4 receptor	[118]
hDPP4-transduced mice	Easy to handle and house; can be produced rapidly; reagents and assay widely available	Clinical disease is transient and reliant on transduction efficiency; hDPP4 is expressed transiently and only in lungs	[64]
DPP4 humanized mice	Retain normal DPP4 expression patterns; easy to handle and house; reagents and assays are widely available; useful for screening vaccines	No clinical disease	[119]

FDA Animal Efficacy Rule

Since MERS-CoV outbreaks are sporadic and infect relatively small numbers, performing controlled vaccine efficacy studies in humans becomes very challenging, if not impossible. A potential alternative pathway is the FDA "Animal Rule," a mechanism designed for disease indications for which efficacy studies would be infeasible or unethical. Under this mechanism Phase I/II safety testing is conducted in healthy human subjects, but prediction of clinical benefit is then determined through immuno-bridging to the animal model immunogenicity and efficacy data in a well-established animal model [112] when all of the following four criteria are met:

1) There is a reasonably well-understood pathophysiological mechanism of the toxicity of the substance (pathogen) and its prevention or substantial reduction by the product (vaccine);

2) The effect is demonstrated in more than one animal species expected to react with a response predictive for humans, unless the effect is demonstrated in a single animal species that represents a sufficiently well-characterized animal model for predicting the response in humans;

3) The animal study endpoint is clearly related to the desired benefit in humans, generally the enhancement of survival or prevention of major morbidity; and

4) The data or information on the kinetics and pharmacodynamics of the product or other relevant data or information, in animals and humans, allows selection of an effective dose in humans.

Only three biologics, including one vaccine (against anthrax [125]), have been approved by FDA's Center for Biologics Evaluation and Research under the Animal Rule, and it is clear that the animal model(s) should be highly refined and characterized with regard to understanding of disease pathogenicity, and their performance (along with the appropriate assays) should be standardized as much as possible. It is notable that the European Medicines Agency also has an animal-based licensure pathway.

Major gaps for all animal models include:

· Lack of consensus and availability of the optimal animal model to replicate severe human illness from MERS-CoV infection;

· Limited availability of currently or recently circulating MERS-CoV strains;

· Lack of understanding of clinically relevant symptoms that can be incorporated into clinical scores or used as a signal to begin treatment in animal models; and

· Competition for laboratory space, availability of animals and expertise with other emerging or reemerging infectious diseases.

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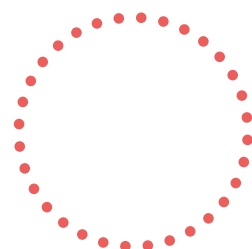
MERS-CoV Challenge Strains

Almost all reviewed papers for this landscape analysis describing animal studies with experimental infection with MERS-CoV used the strain first identified at the Erasmus Medical Center (EMC), Rotterdam, The Netherlands, in 2012, and thus referred to as EMC/2012 [7]. Most publications typically describe the strain as a gift from Bart Haagmans and/or Ron Fouchier, scientists at the EMC, and the virus is always described as propagated in Vero cells. The most likely reasons for such a wide use of one strain are the openness of EMC to collaboration and convenience to end users, but it may also point out to lack of alternatives. It is not clear from the literature if the academic labs at the EMC or elsewhere have well-documented history of the cell line used for MERS-CoV virus propagation. The assay to measure the amount of virus in various labs also appears to be non-standardized.

Briefly, EMC/2012 MERS-CoV challenge virus is known to come from the patient material containing the virus which had been originally subjected to passage in Vero cells four times in the Dr. Soliman Fakeeh Hospital, Jeddah, Saudi Arabia. The MERS-CoV reportedly can be propagated relatively easily in rhesus monkey kidney epithelial cell line LLC-MK2 or in Vero cells. In the EMC LLC-MK2, cells are inoculated with EMC/2012 in minimal essential medium (MEM-Eagle) with Earle's salts, supplemented with 2% serum, 100 U/ml penicillin, 100 mg/ml streptomycin and 2 mM glutamine. Vero cells are inoculated with virus in Dulbecco's modified Eagle medium supplemented with 1% serum, 100 U/ml penicillin, 100 mg/ml streptomycin and 2 mM glutamine. After inoculation, the cultures are incubated at 37°C in a CO_2 incubator and checked daily for cytopathic changes. Three days after inoculation, supernatant from Vero cells is collected. The virus stock that was sequenced was derived from sputum specimen passaged six times in Vero cell culture [126].

While few other strains of MERS-CoV have been isolated since EMC/2012, studies that evaluate phenotypic differences between these strains in animals are currently lacking. Only three studies listed in Table 12 reported use of MERS-CoV challenge strains other than EMC/2012.

With a possibility remaining that some MERS-CoV strain isolation and characterization studies took place without being published, it may be advisable for CEPI to support one or more studies to more vigorously characterize a few more recent strains of MERS-CoV and evaluate their performance in relevant animal models.

MERS-CoV genomes are phylogenetically classified into two clades: A and B [127]. The viral genomes detected in the earliest cases in humans (clade A cluster; EMC/2012 and Jordan-N3/2012) are genetically distinct from the clade B. No clinical differences have been noted between clades [128] and viruses in clade B are further classified into five lineages that have all caused human infections [129]. Some recent data indicate presence of putative clade C (non-A and non-B) in Kenyan camels [38, 130].

Table 12. MERS-CoV strains used for experimental infection of animals

Strain	Animal model of infection	Virus GenBank accession number	References
EMC/2012 (passage 7, human isolate)	Rhesus macaque, camel, pig, rabbit, marmoset, llama	JX869059 (clade A)	[117]; [121]; [20]; [31]; [113];
Jordan-n3/2012 (human)	Marmoset	KC776174 (clade A)	[116]; [131]
Qatar15/2015 (human)	Rabbit (transmission study)	MK280984	[124]
Dromedary_MERS-CoV_Al-Hasa_KFU-HKU13/2013	Alpaca	KJ650295–KJ650297	[123]

MERS-CoV Rabbit Model

New Zealand white rabbits (Oryctolagus cuniculus) have been described as an animal model of asymptomatic MERS-CoV infection [113]. Clinical signs and gross lesions were not observed in rabbits inoculated through intratracheal (IT) and IN routes (other routes were not tested). Microscopically, lesions were detected in the upper and LRT at three and four days post-infection (dpi). In the nasal cavity, there was a mild to moderate infiltration of heterophils that in some cases was accompanied by epithelial necrosis and regeneration. The lungs exhibited mild heterophilic infiltration in alveolar septa and lumina, predominantly around terminal bronchioles. Type II pneumocyte hypertrophy and hyperplasia of bronchus-associated lymphoid tissue were also observed. Virus was detected in tissues in the upper and LRT by real-time quantitative reverse transcription polymerase chain reaction, in situ hybridization and immunohistochemistry. Additionally, virus titration revealed the presence of infectious virus in nasal swabs up to seven dpi.

Despite the fact that rabbits shed MERS-CoV from their URT, it appears that the New Zealand white rabbit model is neither suitable to study MERS-CoV transmission [124], nor is the model appropriate for studying clinical disease progression, given that rabbits remained asymptomatic after MERS-CoV inoculation.

MERS-CoV Nonhuman Primate Models

Three species of NHP have been described as MERS-CoV animal models: the rhesus macaque (Macaca mulatta) [31, 117], common marmoset (Callithrix jacchus) [115-117] and African green monkey (AGM) [132]. Inoculation of these species with MERS-CoV leads to viral replication, but disease is more severe in the common marmoset than in the rhesus macaque or in AGM.
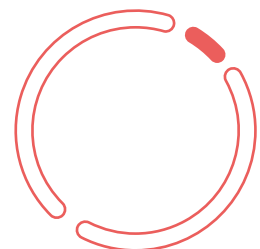
MERS-CoV rhesus macaque model

Rhesus macaque was the first model to be developed for MERS-CoV and was used to confirm the virus as causative agent of MERS. The animals were inoculated with strain EMC/2012 with either a combination of inoculation routes (IT, IN, oral and ocular) or IT only. Observed clinical signs were mild to moderate, appeared within 24 hours and were transient. An increase in body temperature, reduced appetite, increased respiratory rate, cough, piloerection and hunched posture were reported. Radiographic imaging showed varying degrees of localized infiltration and interstitial markings [114, 133, 134].

Although MERS-CoV RNA was detected in nasal swabs, bronchoalveolar lavage samples, oropharyngeal swabs and also in some upper and LRT tissue samples, infectious virus was only isolated from the lungs. MERS-CoV replication occurred in type I and II pneumocytes, and viral antigen co-localized with sites of pneumonia. Macaques represent a useful model to study mild MERS-CoV infection because they develop a transient respiratory disease similar to humans.

MERS-CoV common marmoset model

The increased severity of MERS illness seen in the common marmoset model was an important advance in the ability to evaluate potential vaccines and therapeutic agents against MERS-CoV, as discrimination between successfully treated and control animals was more apparent. In addition, the more closely models recapitulate the disease observed in humans, the more likely findings can be eventually translated into use in humans.

MERS-CoV administered through combined simultaneous ocular, oral, IT, and IN routes caused severe, potentially lethal respiratory disease in common marmosets. Clinical signs included tachypnea, labored or shallow breathing, cyanosis, and hemorrhagic oral discharge, which necessitated early euthanasia of two of nine marmosets [115]. Clinical signs were first noted one dpi, peaked at four to six dpi, and resolved by 13 dpi. Correspondingly, radiographs showed pulmonary interstitial infiltration as early as one dpi; infiltrates were no longer evident by 13 dpi [115, 117]. Similar to macaques, gross findings were present only in the lung and correlated with moderate to severe bronchointerstitial pneumonia [115]. MERS-CoV antigen was detected by immunohistochemistry in both type I and II pneumocytes and alveolar macrophages, but the virus replicated only in type I pneumocytes and macrophages.

However, the outcome of MERS-CoV infection in marmosets has been somewhat controversial after the publication of a study which demonstrated no lethality after IT inoculation [116]. The authors used both EMC/2012 and Jordan-n3/2012 strains for inoculation, and with consistent results between both strains, argued that differences in animal manipulations during the study rather than other differences — such as minute changes observed in S protein sequence — were more likely to contribute to disagreements in their findings vis-à-vis those of Falzarano et al., which used only EMC/2012 strain [115]. It is difficult to conclude whether Johnson et al. study was an outlier, but the authors noted that based on clinical signs and hematology, the animals did not develop systemic clinical disease and thus it may not be surprising that mortality was not demonstrated.

Notably, only three groups published MERS-CoV infection studies in marmosets — (NIAID (Frederick, MD), Chinese Academy of Medical Sciences, and NIAID/NIH Rocky Mountain Lab (Hamilton, MT), and the study of Yu et al. did not follow infected animals for mortality (Table 13).

Since common marmosets appear to represent severe human MERS disease better than other current models, it would be beneficial for CEPI to assemble a working group representing experts from all three scientific groups to examine the issues surrounding lethality outcomes in marmoset model of MERS-CoV infection and agree on common testing parameters for future studies.

Table 13. Comparison of MERS-CoV challenge studies in common marmosets

Organization	Animals challenge dose	Challenge strain and concentration	Clinical signs	Mortality	Ref
Rocky Mountain Laboratories, NIAID/NIH (Hamilton, MT)	Nine marmosets (2-6 years old); 100 µl per nare (IN); 500 µl (oral); 500 µl IT; 50 µl per eye Total dose= 5.2x106 TCID50	EMC/2012 4x106 TCID50/ml	Increased respiration rate, decreased activity, loss of appetite	3 animals were observed for survival and 6 were scheduled for necropsies. 1 of the 6 animals that were not euthanized at the scheduled 3 dpi necropsy had to be euthanized due to severity of disease. 1 of 3 animals followed for survival had to be euthanized (2 survived).	[115]
NIAID (Frederick, MD)	6 marmosets (3-9 years old); 3 per challenge virus; IT	EMC/2012 (5x107 PFU) JOR (5x107 PFU)	No increases in body temperatures above normal ranges were observed; subjects maintained peripheral oxygenation throughout the study; respiratory rates increased above normal range sporadically throughout the study	No mortality was observed.	[116]

Regardless of this controversy, certain hallmarks of severe disease observed in humans, such as consolidation of the lungs and changes in blood chemistry indicative of liver or kidney failure, are reproduced in the marmoset model. The evaluation of antivirals and vaccines might therefore be more predictive in marmosets than in rhesus macaques. However, the relatively small size of the marmoset limits the number of samples that can be taken, and consequently, the data obtained within an experiment. Both models provide important insights into the MERS-CoV infection mechanism and disease progression, and further development is desirable.

MERS-CoV African Green Monkey model

Vero cells support propagation of MERS-CoV, and it would be logical to investigate whether African Green Monkey(AGMs), the original source of Vero cells, could serve as MERS-CoV infection model. A recent abstract described exposure of AGMs to aerosolized MERS-CoV at dosages 103, 104 and 105 plaque-forming units (PFU) [132].

Disease progression was followed with daily health observations, weights, body temperatures, blood and throat swab collection. The lowest-tested dose was associated with minimal disease including lack of fever, lower viral titers and minimal clinical scores over 28 days of observation post-exposure to MERS-CoV. In

contrast, the 104 PFU dose, and especially the 105 PFU dose, were associated with significantly more observable disease signs of MERS-CoV infection, although all AGMs survived for the 28-day duration of the study. It remains to be seen if exposure to higher doses will result in more severe disease.

MERS-CoV Mouse Models

An alternative to adaptation of MERS-CoV to mice is the expression of human DPP4 in murine tissues [135]. The functionality of such a model has been shown in two different ways: transient expression of human

DPP4 in mouse lungs, established by transduction with a recombinant adenovirus encoding human DPP4 [64], and transgenic mice expressing human DPP4 in all tissues [118, 119].

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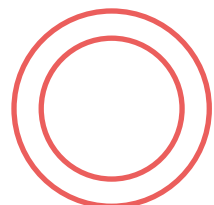
hDPP4-transduced mouse model

In this model, an adenoviral vector was used to induce transient expression of hDPP4 in the lungs of wild-type BALB/c mice, wild-type (WT) C57BL/6 mice, and multiple knockout mouse strains. hDPP4 was expressed only by epithelial cells lining the airways and alveoli [64]. After IN inoculation with EMC/2012 MERS-CoV, clinical signs in the hDPP4-transduced mice were minimal and characterized by lack of weight gain in young mice and mild weight loss in older mice. Mice exhibited mild gross pulmonary lesions, which corresponded to peribronchiolar and perivascular inflammation that developed into interstitial pneumonia. Immunohistochemistry staining (IHC) showed that MERS-CoV antigen was colocalized with hDPP4 expression in the lungs. Virus replication was detected in the lungs by two dpi; however, viral infection was transient. Transduced knockout mice – which had impaired immune systems, such as a lack of type I IFN signaling – typically exhibited earlier, more severe clinical signs and gross and microscopic lesions than infected transduced WT mice. MERS-CoV does not induce significant amounts of IFNα/α expression in vitro, but the role of type-I IFN induction and signaling in vivo is unknown. IFN is induced via RIG-I-like receptors (RLRs) and Toll-like receptors (TLRs) in coronavirus infections. To determine the role of each in MERS-CoV-infected mice, Ad5-hDPP4-transduced mice impaired in RLR [mitochondrial antiviral signaling protein-/- (MAVS-/-)] or TLR [myeloid differentiation primary response gene 88-/- (MyD88-/-)] signaling were infected with MERS-CoV. Infection of MyD88-/- but not MAVS-/- mice resulted in up to 20% weight loss. Without type-I IFN signaling (IFNAR-/-), infection was even more severe than in MyD88-/- mice, with weight loss beginning earlier by two dpi.

In all mouse strains, gross and microscopic lesions and virus replication were not present in organs outside the respiratory tract, which fits with the lack of detectable hDPP4 in these tissues. Since hDPP4-transduced mice exhibited mild transient clinical disease and pulmonary lesions, these mice could be used to model mild MERS-CoV disease [64]. However, this model cannot be used to analyze clinical respiratory disease, since it lacks respiratory clinical signs. An increase in clinical disease severity with earlier onset of gross and microscopic lesions was achieved by transducing immunodeficient knockout mice. Mice deficient in T cells [T cell receptor α-/- (TCRα-/-)], B cells (μMT), or T and B cells [recombination activating gene 1-/- (RAG1-/-) severe combined immunodeficiency (SCID) were transduced with Ad5-hDPP4. Virus was not cleared in mice lacking T cells (TCRα-/-, RAG1-/-), or in SCID mice but was cleared in μMT mice demonstrating the need for functional T cells.

Due to the ease of transducing different strains of knockout mice, this technique could be used to study moderate MERS-CoV disease and the impact of different aspects of the immune system on MERS-CoV infections [135]. However, disease and hDPP4 expression are transient (ca. 17 days) in hDPP4-transduced mouse models, which may interfere with vaccine screening. Additionally, the expression of hDPP4 in these mouse models may not exemplify normal hDPP4 expression, as hDPP4 in these mice is solely expressed in the lungs with widespread expression by airway epithelial cells and pneumocytes and is not present in other cell types or tissues, including the URT. Thus, the physiologic response to MERS-CoV infection may not fully represent that in infected humans. It is tempting to speculate that use of adenovirus serotypes other than Ad5 could achieve a different tissue tropism of DPP4 [136], but that approach would still be unlikely to resolve the transient nature of the target expression.

hDPP4-transgenic mouse model

Using an hDPP4 expression cassette, transgenic mice expressing human DPP4 in all tissues were generated [118]. Upon IN inoculation of transgenic and WT mice with MERS-CoV, mice expressing hDPP4, but not WT mice, started losing weight at two dpi, up to 30% at five dpi. Mortality was 100% at 6 dpi. Viral shedding was not investigated, but viral RNA could be detected in the lungs, brain, heart, spleen and intestine of hDPP4-expressing mice. Infectious virus was isolated from lungs (two and four dpi) and brain (four dpi), but no other investigated tissues. Gross lesions (red to dark red discoloration and multifocal consolidation) were observed in the lungs of hDPP4-expressing mice. Microscopically, a moderate bronchointerstitial pneumonitis was observed two dpi, which progressed to include more intense cellular infiltrates four dpi. No pathological changes were observed in the brain. Viral antigen was detected in type I and type II pneumocytes, brain microglia, astrocytes and neuronal cells. Elevated gene expression of antiviral cytokines, proinflammatory cytokines and chemokines was detected by qRT-PCR [118].

Currently, the hDPP4-transgenic mouse is good small animal model of severe, lethal MERS-CoV infection that develops respiratory disease. However, these mice globally express hDPP4 in all cell types, unlike normal DPP4 expression in humans. The widespread expression of hDPP4 in the transgenic mice resulted in the development of nervous system lesions as well as lesions in the respiratory tract, which is likely not representative of human MERS-CoV infections. Nevertheless, this model could still be used to screen the efficacy of antivirals and vaccines to mitigate or prevent MERS-CoV-induced respiratory disease.

In a different transgenic approach, VelociGene technology was used to replace the mouse DPP4 ORF with the hDPP4 ORF [119]. In these mice, DPP4 was expressed solely by cell types that would normally express mouse DPP4, thus retaining authentic DPP4 expression patterns. After IN MERS-CoV inoculation, DPP4-humanized mice remained asymptomatic yet developed pulmonary lesions consistent with bronchointerstitial pneumonia by four dpi. High levels of MERS-CoV RNA and infectious virus were detected in the lungs. Since the DPP4-humanized mouse model was asymptomatic, these mice could be used to model mild MERS-CoV infections, but they are not an appropriate model for severe MERS-CoV disease.

Yet another variation of the approach utilized CRISPR-Cas9 gene editing to modify the mouse genome to encode two amino acids (positions 288 and 330) that match the human sequence in the DPP4 receptor, making mice susceptible to MERS-CoV infection and replication [120]. Transgenic mice supported efficient infection and replication of the human MERS-CoV strain EMC/2012, the camel MERS strain Dromedary/ Al-Hasa-KFU-HKU13/2013 and a recombinant virus derived from a molecular infectious clone (icMERS) in the lungs but demonstrated no clinical signs of disease. Serial MERS-CoV passage in these engineered mice was then required to generate a mouse-adapted virus that replicated efficiently within the lungs (but not in the brain) and elicited symptoms similar to severe acute respiratory distress syndrome, including decreased survival, extreme weight loss, decreased pulmonary function, pulmonary hemorrhage and pathological signs indicative of end-stage lung disease. The infection with MERS-CoV could be prevented by treatment with neutralizing antibody 3B11 [137] in the same way the antibody was used in the NHP model [138]. This model, despite requiring an adapted mouse challenge virus, appears to better represent severe form of MERS-CoV infection that the hDPP4 model described above [119].

Although both the DPP4-humanized and hDPP4-transgenic mouse models could be used to screen antivirals and vaccines, each model has its own advantages. The DPP4-humanized mouse model may be preferred since these mice do not develop brain lesions and may best simulate hDPP4 expression patterns, whereas the hDPP4-transgenic mouse model may be favored because it is a lethal model, which allows for easier determination of antiviral and vaccine efficacy.

Statement of Support

Acknowledgements
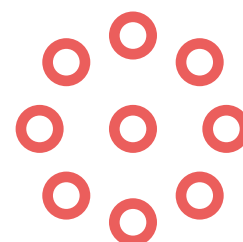
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