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Univ.-Prof. Dr. med. vet. Gerd Sutter

Characterization of recombinant Modified Vaccinia virus Ankara for delivery of Middle East Respiratory Syndrome Coronavirus spike protein antigens

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A journey of a thousand miles must begin with a single step.

Contents

I. INTRODUCTION

Infectious diseases remain a major cause of human deaths worldwide. Against many traditional pathogens significant progress has been made by development of sensitive diagnostic tests, antibiotic and antiviral drugs, and efficient vaccines; whereas emerging and reemerging zoonotic infectious diseases represent still a major challenge for public health prevention strategies.

Modern vaccines against emerging pathogens have to fulfill different requirements: rapid and flexible generation of safe and efficient vaccines with technologies that can facilitate accelerated regulatory review. Different strategies like inactivated vaccines, live-attenuated vaccines, subunit or DNA vaccines, and viral vector vaccines are used to meet these needs. Especially, poxvirus vector vaccines are thought to be among the best experimental means to induce cellular immunity and humoral responses. The Modified Vaccinia virus Ankara (MVA), a highly attenuated strain of vaccinia virus originating from growth selection on chicken embryo fibroblasts, is widely used in experimental vaccine development targeting infectious pathogens as well as cancer. Technologies for genetic engineering, large scale production, and quality control have been established and match today's requirements.

In this work the MVA vector system was used to generate a candidate vaccine against a newly emerging pathogen, the Middle East respiratory syndrome coronavirus (MERS-CoV). This novel coronavirus is causing a severe respiratory disease and deaths in humans and was first described in 2012. The virus is suspected to persist in animal reservoirs and cause zoonotic infections in humans.

MVA expressing the full-length spike protein of MERS-CoV was constructed and characterized by genetic engineering and molecular analysis. Mice vaccinated with the recombinant MVA produced high levels of serum antibodies that neutralized MERS-CoV in tissue culture infections.

II. LITERATURE

1 Emerging infectious diseases

Emerging infectious diseases can be divided into two categories. Newly emerging infectious diseases are recognized in the human host for the first time, and reemerging infectious diseases are diseases that historically have infected humans but continue to reappear either in new locations or in resistant or new forms or reappear after apparent control or elimination (Fauci and Morens, 2012). In the past two decades, humans have faced many new viral infectious agents in emerging infection (Table 1).

Emerging disease	Year identified	Estimated global impact	
		Cases	Death
Human Immunodeficiency Virus / Acquired Immune Deficiency Syndrome (HIV/AIDS)	1981	35.3 million	36 million
H5N1 Influenza (“bird flu”)	1997	637	378
Severe Acute Respiratory Syndrome (SARS)	2003	8096	774
H1N1 (2009) Influenza (“swine flu”)	2009	unknown	>280000
Middle East Respiratory Syndrome (MERS)	2012	153	64
H7N9 Influenza (“bird flu”)	2013	137	45

Table 1 Selected emerging diseases
(from http://kff.org/global-health-policy/fact-sheet/the-u-s-government-global-emerging-infectious-disease-preparedness-and-response**;** modified)

For example the severe acute respiratory syndrome (SARS) was first reported in Guangdong, China in 2002, but within one year the disease quickly spread worldwide (Figure 1). Over all the World Health Organization (WHO) reported more than 8000 infected people and 774 deaths. The etiologic agent was the SARS coronavirus (SARS-CoV), a lineage B betacoronavirus, which jumped to humans from animal reservoirs, especially bats, Himalayan palm civets and raccoon dogs (Graham et al., 2013; Guan et al., 2003; Shi and Hu, 2008). Virus spread in human populations happened firstly by person-to-person transmission in confined spaces, then within hospitals, and finally by human movement between international air hubs (Hsueh and Yang, 2003).

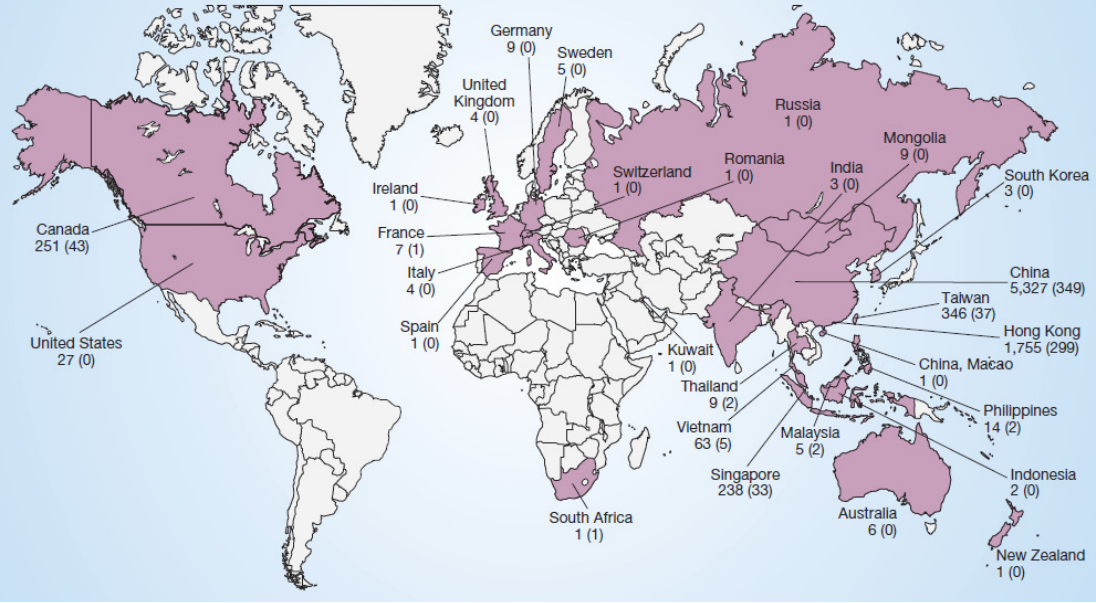

Figure 1 Cases of severe acute respiratory syndrome (SARS) from November 2002 to July 2003. SARS-related deaths indicated in parentheses.
(from Morens et al., 2004, with permission)

The SARS pandemic caused great economic losses, associated with disruptions of trade, travel, investment, interruption of product supply chains and behavior changes in consumers, rather than from medical costs or the loss of human life. The Asian Development Bank calculated a global economic loss of about 59 billion US$ (Baric, 2008; Gupta et al., 2005) (Figure 2).

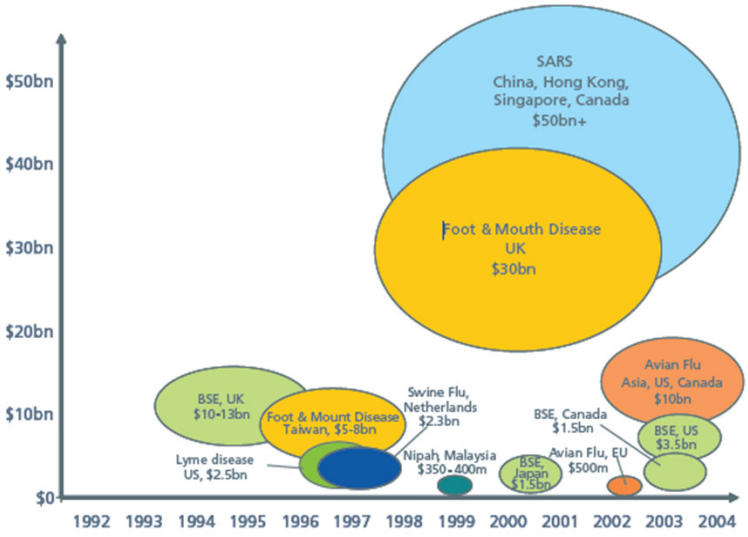

Figure 2 Economic impact of SARS compared with other emerging diseases
(from SARS and the New Economics of Biosecurity, 2003)

Besides the SARS-CoV infection there are many other examples for emerging zoonotic virus diseases. In 1998-1999 nipahvirus emerged from fruit bats in Malaysia and caused an epizootic in herds of intensively bred pigs which in turn served as the animal reservoir from which the virus was passed on to humans causing more than 100 deaths (CHUA, 2000). Only two years later the same virus emerged in India and Bangladesh causing more than 200 deaths to date. Also here fruit bats are the animal reservoir and transmit the nipahvirus to humans through shedding and contaminating fruits and other food (for example palm sap) (Kulkarni et al., 2013; Luby et al., 2009; Luby et al., 2006).

In 2009 the pandemic H1N1 influenza virus emerged also from pigs, after complex genetic reassortment of North American swine, Eurasian swine, North American avian, and human influenza virus strains (Garten et al., 2009; Smith et al., 2009). The H5N1 influenza subtype first infected humans during an outbreak of avian influenza in Hong Kong in 1997. After re-emergence in 2003 and 2004, influenzavirus H5N1 has spread from Asia to Europe and Africa. Until today millions of infections in poultry, about seven hundred human cases, and more than 300 human deaths were the consequence (WHO). H7N9 bird flu is the newest atypical influenza virus infection that has just been reported since early 2013. The emerging of this new disease occurred in China and becomes the presently most closely observed virus concerning the threat of a new influenza A virus pandemic (Wiwanitkit, 2013).

Also when these are only some of the latest examples, it is clear, that infectious diseases have been emerging and reemerging over a long period. There a numerous factors, including genetic, biological, social, political and economic factors, that contribute to emergence and reemergence of infectious diseases (Table 2).

Microbial Agent	Human Host	Human environment
Genetic adaptation and change	Susceptibility to infection	Climate and weather
Polymicrobial diseases	Demographics and behavior	Changing ecosystems
	International trade and travel	Economic development and land use
	Occupational exposures	Poverty and social inequality
		Lack of public health services
		Animal populations

Table 2 Factors involved in the emergence of infectious diseases
(from Morens et al., 2004, modified)

Infectious diseases will continue to emerge and reemerge. Therefore, fast developement of pathogen-specific diagnostic tests and treatment strategies as well as vaccine development are important countermeasures.

2 Vaccines against emerging infectious diseases

Vaccines are among the most cost-effective public health prevention strategies for combating infectious diseases. Marano et al. (2007) illustrated the importance of vaccines for emerging zoonotic infections. For example vaccination of animals can be an important tool for control of zoonotic disease (e.g. West Nile fever and avian influenza). But emerging and reemerging infectious pathogens are a major challenge to vaccine development. On one hand vaccine generation, testing and production must be rapid and flexible, to accommodate the abrupt appearance of the causing pathogen, on the other hand the technologies for vaccine generation and production must be consistent, highly standardized, and reproducible to facilitate accelerated regular review (Leblanc et al., 2012).

Multiple strategies can be used to generate vaccines against emerging virus diseases, which include inactivated vaccines, live attenuated vaccines, subunit vaccines, DNA vaccines and vector vaccines. All types of vaccines have certain advantages and disadvantages. Graham et al. (2013) reviewed different strategies that were used to generate SARS-CoV-specific vaccines. Inactivated virus vaccines were prepared by use of chemicals or radiation. Thus, the virion structure is maintained, but the virus cannot infect, propagate or cause disease. The vaccines were used with or without adjuvant. In most studies high level of neutralizing antibodies was induced, but also adverse side effects like T helper 2-stimulation and interleukin-4-driven inflammatory pathology were observed (Bolles et al., 2011; Roper and Rehm, 2009; See et al., 2006).

Live-attenuated virus vaccines can elicit T cell- and B cell-dependent immune responses (Vignuzzi et al., 2008). For SARS-CoV mutants lacking the envelope protein or the exonuclease ExoN were replication attenuated and were able to protect mice in challenge experiments (Eckerle et al., 2010; Fett et al., 2013; Netland et al., 2010). Nevertheless, recombination events with wild-type coronaviruses might cause reversion to virulence.

For subunit vaccines the antigenic components are generated *in vitro*. The spike and the nucleocapsid proteins served as target antigens for SARS-CoV vaccines (Bisht et al., 2005; Gupta et al., 2006; Liu et al., 2006). Subunit vaccines cannot cause viral disease, but can induce B cell- and T cell-dependent immune responses. Bisht et al. (2005) were able to demonstrate neutralizing antibodies and reduced SARS-CoV loads in spike protein vaccinated mice.

DNA vaccines targeting the spike (S) protein and the nucleocapsid (N) proteins were used for SARS-CoV (Woo et al., 2005b; Yang et al., 2004; Zhao et al., 2005). Here, viral genes that encode the antigenic compound are directly injected or otherwise inoculated into the vaccinee and the antigen is produced in the host cells. DNA vaccines expressing the SARS-CoV-S were able to induce neutralizing antibodies, whereas DNA vaccines that encode the N protein induced cell-mediated immunity but were not protective after high titer challenge. Furthermore, induction of delayed-type hypersensitivity was observed (Zhao et al., 2005).

Vector vaccines rely on a host viral genome that is genetically modified to encode for antigens of the pathogen of interest. Some of the used vector systems have strong inherent adjuvant activities and can efficiently induce innate and B cell- as well as T cell-mediated immune responses. For delivering SARS-CoV-S or -N genes vesicular stomatitis virus (Kapadia et al., 2005), rhabdovirus (Faber et al., 2005), parainfluenza virus (Bukreyev et al., 2004) and vaccinia virus (Bisht et al., 2004; Chen et al., 2005) were used as vector systems. Especially, the modified vaccinia virus Ankara (MVA) has widely been used for the vaccine development against human diseases (Volz and Sutter, 2013).

3 Modified Vaccinia virus Ankara (MVA)

MVA was derived from the vaccinia virus strain Ankara that was used in Turkey for vaccination against smallpox. The virus got attenuated by over 570 serial passages in chicken embryo fibroblast cells (CEF) (Mayr, 1975). Genomic studies demonstrated that long-term passages resulted in the loss of approximately 15% of the MVA genome compared to the parental chorioallantois vaccinia virus Ankara (CVA) strain (Meyer et al., 1991). Six large genomic deletion sites as well as many shorter deletions, insertion and point mutations, resulting in gene fragmentation, truncation, or

deletions of open reading frames (ORFs) (Antoine et al., 1998; Meisinger-Henschel et al., 2007) have been identified. Due to these deletions and disruptions, the host range genes, like K1L and C12L/SPI-1 gene, the immunomodulatory proteins (Blanchard et al., 1998) (such as the functional receptors for TNF, IFN-α, IFN-β/γ and CC chemokine) and also some structural proteins (such as the major protein of the A-type inclusion body) are heavily affected by the genomic changes. All the serial changes contribute to the replication deficiency of the virus in human cells and its avirulence in animals (Mayr et al., 1978).This high-level attenuation was a key property that allowed for an extended use of MVA in human clinical applications.

In addition to the high safety profile, the main reasons for the general appreciation of poxvirus vectors include the following features: (i) high genetic stability of the vector viruses, (ii) the viruses can accommodate large amounts of heterologous DNA, (iii) versatility for expression of many foreign proteins, and (iv) ability to stimulate high levels of humoral and cellular immune responses when used as recombinant vaccine.

High genetic stability of MVA

The genetic stability of a vector virus is determined by the stability of the viral genome and the stability of the recombinant gene sequences. Concerning the viral genome, the five genomic sequences of MVA, which were independently isolated in different laboratories (original description, the Acambis 3000 strain deposited from CDC, MVA-I721 and MVA-BN deposited by Bavarian-Nordic GmbH), are confirmed to be identical (with the exception of the variable-length terminal repeat regions)(Antoine et al., 1998). This result provides confirmation of Anton Mayr`s original conclusion that MVA can be considered genetically stable after 570 passages in CEFs (Mayr et al., 1975).

About the stability of transgene sequences inserted into recombinant MVA, it must be considered recombinant gene products can exert a negative selective pressure on viral growth. Therefore, the rare mutants with reduced or altered expression of the foreign protein should be undetected, like the study about the recombinant MVA-BN (Timm et al., 2006), after 20 passages under selective conditions, RT-PCR results confirmed the transcription of the inserted genes. However, two cases about the instability of HIV Env have been reported independently by two groups (Burgers et al., 2008; Wyatt et al., 2008). To avoid instability of the flanking regions of insertion sites and to prevent frame shifts in recombinant genes observed at consecutive runs of C or

G bases it was proposed to design recombinant MVA using codon optimization by gene synthesis and insertion of heterologous sequences between two essential genes (Wyatt et al., 2009). Also overexpression of a heterologous protein may result in instability, for example because of toxicity of the gene product to the cells. Here, use of the modified H5 promoter has been proposed to improve stability of the MVA vector while high level immunogenicity of the genetically engineered MVA vaccine is maintained (Wyatt et al., 1996).

Packaging capacity for large amounts of heterologous DNA and versatile gene expression

As a member of the orthopoxviruses with double-stranded DNA genomes, MVA encodes for a multitude of proteins required for virus replication. Unlike other DNA viruses, poxviruses exclusively replicate in the cytoplasm of host cells. This specific feature makes the viruses independent from the host cell genome and poxvirus gene expression is typically controlled by virus-specific promoters and virus-specific transcriptional machinery. Therefore, genes do not contain introns and mRNAs are not spliced (Tolonen et al., 2001).

One interesting feature of poxviruses, including MVA, is their genetic plasticity which allows large amounts of foreign DNA (at least 25 kb) to be incorporated without loss of infectivity or reduction of gene expression. Methodologies leading to the generation of recombinant MVA vectors are well established (Kremer et al., 2012). Genomic insertion of heterologous gene sequences is mediated by homologous recombination between the viral DNA genome and the DNA from a shuttle plasmid that carries the gene sequence of interest under the transcriptional control of vaccinia virus (VACV)-specific promoters.

Stimulating high level of humoral and cellular immune responses

The most important feature of the non-replicating MVA vaccines is their astonishing immunogenicity when compared to replication-competent VACV. In addition to the possibility that MVA may be particularly suitable for expression large foreign gene sequences, this observation can be well explained by the particular ability of MVA to present antigens in a favorable environment of infection providing for important innate responses and early activation of the host immune system (Delaloye et al., 2009; Halle et al., 2009; Lehmann et al., 2009).

MVA is known to trigger early migration of leukocytes to the site of infection (Lehmann et al., 2009). Dentritic cells (DC) are expected to be recruited at the inoculation site, to sample MVA-infected dying cells and following maturation to migrate to lymph nodes and activate antigen-specific T cells (Greiner et al., 2006; Pascutti et al., 2011). In this context, Price et al. (2013) recently summarized the strong immunostimulatory capacities of MVA targeting various components of the innate immune system. This data indicates that recombinant MVA vaccines do not just serve for production of antigen but also function as intrinsic adjuvant systems.

An element becoming increasingly important for an efficient presentation is the stability of the expressed immunogen. The capacity of MVA vectors to elicit high levels of both humoral and cellular immune responses was shown upon evaluation of the first recombinant MVA vaccine that delivered the influenza virus HA and NP antigens (Sutter et al., 1994). Gasteiger et al. (2007) proposed that an immunogen produced by MVA as long-lived mature protein is the optimal substrate for efficient priming of antigen specific cytotoxic T cells ($CD8^+$ T cells). The capacity to induce T cell mediated immunity was particularly well established with MVA candidate vaccines directed against immunodeficiency virus infections including human AIDS (Drexler et al., 2004; Sutter and Haas, 2001). Overall these observations strongly support the fact that recombinant MVA can serve as efficient vaccines to induce high T cell responses against various infectious agents and tumor antigens.

Draper et al. (2013) recently summarized the data about the substantial potential of recombinant MVA to induce antigen-specific antibodies in both preclinical and clinical studies. The vaccine regimens tested were often based on heterologous prime-boost schedules with priming immunizations using a DNA vaccine or an adenovirus vector and the booster immunization with recombinant MVA vaccines. It can be assumed that the inherent adjuvant effect of the MVA vector vaccine provides for the strong booster responses and broader specificity of the antibodies as observed in many of these heterologous prime-boost studies.

The history of the use of MVA vaccines in humans

MVA was originally generated as attenuated vaccine against smallpox (Mayr, 1975; Mayr et al., 1978) and has subsequently been developed as vector to induce immune responses against a transgenic antigen (Moss, 1996; Sutter and Moss, 1992; Sutter

and Staib, 2003; Sutter et al., 1994). Until 1980, the non-recombinant MVA has been safely used in Germany to vaccinate more than 100,000 individuals for prophylaxis of smallpox, mostly in combination with conventional smallpox vaccine (Mayr, 1999). In 2013, MVA was licensed as stand-alone third generation smallpox vaccine in Europe (EMA/490157/2013; EMEA/H/C/002596).

In addition to its use as a vaccine against variola virus infections, the high safety profile of the virus spurred the use of MVA as recombinant vaccine in humans for prevention or therapy of important infectious diseases and cancer. The first published clinical studies with recombinant MVA targeted malaria, AIDS or breast cancer (Cosma et al., 2003; Moorthy et al., 2003; Rochlitz et al., 2003). Current clinical trials include various phase I and phase II studies also targeting other important human diseases such as hepatitis B, hepatitis C, and influenza (Table 3) (Kreijtz et al., 2013). Until now, several thousand individuals including children have been vaccinated with various recombinant MVA vaccines and no serious adverse events have been reported (Gilbert, 2013; Gomez et al., 2013).

Target disease	Antigen	Stage (phase)	Safe	Immunogenic	Reference
AIDS	HIV-1 Env HIV A gag/pol	I/II	yes	yes	Bakari et al., 2011
	HIV-1 Nef	I/II	yes	yes	Cosma et al., 2003
	HIV-B env/gag/pol/nef	I/II	yes	yes	Gomez et al., 2011 Garcia et al., 2011
Hepatitis B	HBV S	I	yes	yes	Cavenaugh et al., 2011
Hepatitis C	HCV NS3, NS4 and NS5B	II	yes	yes	Wedemeyer H, 2011
Human papilloma	HPV16 E6 and E7	II	yes	yes	Albarran et al., 2007
Influenza	NP and M1	I/II	yes	yes	Berthoud et al., 2011 Lillie et al., 2012 Antrobus et al., 2012

Table 3 Clinical evaluation of recombinant MVA vaccines for viruses
(from Kreitz et al., 2013; modified)

4 Middle East Respiratory Syndrome Coronavirus (MERS-CoV)

In June and September 2012, almost ten years after the SARS pandemic, health authorities recognized two human patients from the Middle East with severe respiratory illness reminding of the pathogenesis of SARS. Both patients succumbed to acute pneumonia and renal failure. The causing agent was not SARS-CoV but a novel human coronavirus (Bermingham et al., 2012). The complete genome sequence of the novel coronavirus was obtained by the group of Ron Fouchier at the Erasmus Medical Center (EMC) in Rotterdam, The Netherlands. Therefore the new virus was first named “human coronavirus EMC” (hCoV-EMC)(van Boheemen et al., 2012). In May 2013, the Coronavirus Study Group of the International Committee on Taxonomy of Viruses (ICTV) renamed the virus “Middle East respiratory syndrome coronavirus” (MERS-CoV)(de Groot et al., 2013). In the meantime the newly discovered virus became an important emerging pathogen in the Middle East (Figure 3).

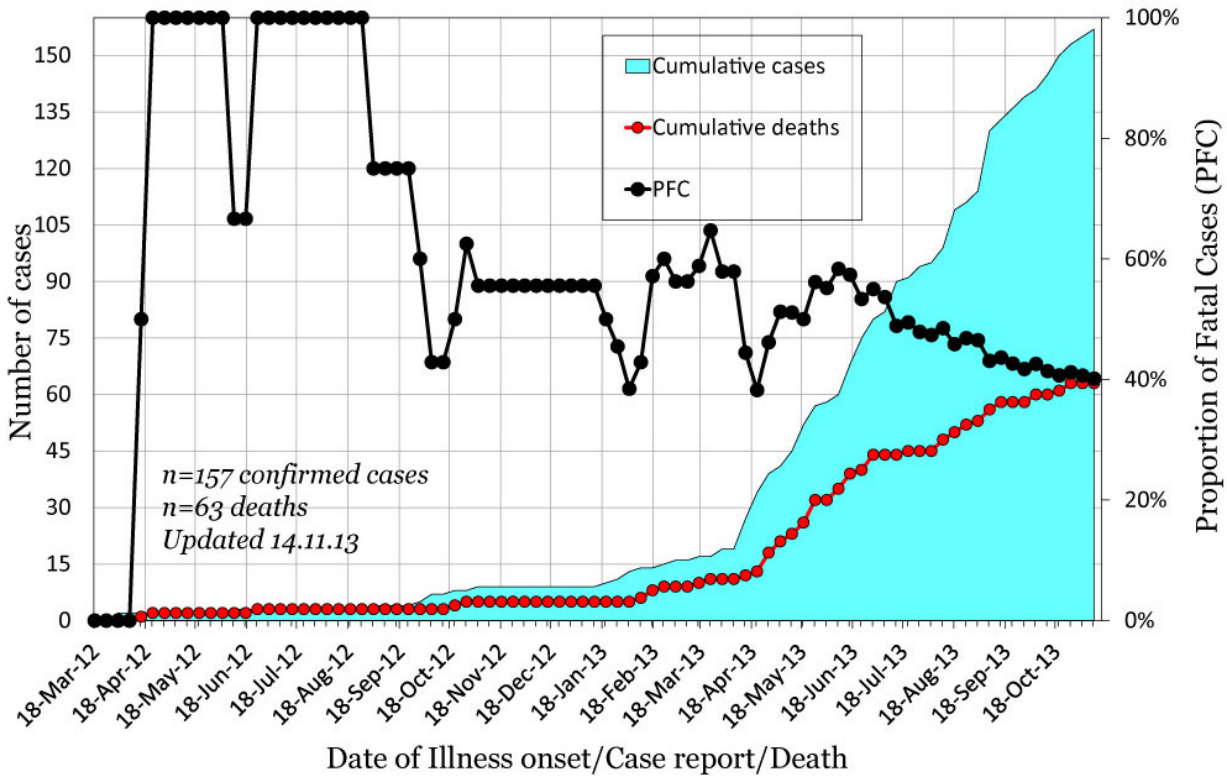

Figure 3 Cumulative MERS-CoV cases and deaths and proportion of fatal cases (http://virologydownunder.blogspot.de)

Globally, from September 2012 to the time of writing, WHO has been informed of a total of 189 laboratory-confirmed cases of infection with MERS-CoV, including 82 deaths (http://www.who.int/csr/don/2014_03_12/en/). MERS-CoV infection causes atypical pneumonia, an acute respiratory distress syndrome (ARDS) and sometimes renal failure, which often causes the death of the patient. Because of the low number of confirmed cases, the mortality rate cannot be accurately estimated. There are also reports that MERS-CoV infection can even be asymptomatic or causes only mild disease (Assiri et al., 2013a; Assiri et al., 2013b; Memish et al., 2013).

Virological characteristics of MERS-CoV

Coronaviruses are pleomorphic, enveloped, single-stranded, positive sense RNA viruses. They are widespread in many different species of birds and mammalians and may cause respiratory, enteric, hepatic, or neurologic disease, with variable severity in various animal species (Perlman and Netland, 2009).

Based on genome sequence analysis, the ICTV has divided the family coronaviridae into four genera, named alpha-, beta-, gamma-, and deltacoronavirus. Furthermore, the genus alphacoronavirus contains two (a and b) and the genus betacoronavirus contains four (a, b, c, d) different lineages. The two human coronaviruses hCoV-229 and hCoV-NL63, which were identified in the 1960s and caused mild common colds, belong to the genus alphacoronavirus. SARS-CoV is a lineage B betacoronavirus and the lineage A of genus betacoronavirus includes hCoV-OC43 and hCoV-HKU1, which were discovered subsequently after the SARS-CoV (Fouchier et al., 2004; van der Hoek et al., 2004; Woo et al., 2005a). The genera gamma- and deltacoronavirus contain only viruses that infect animals. However, the phylogenetic analysis performed by Zaki et al. (2012) showed that, together with the bat coronavirus BtCoV-HKU4 and BtCoV-HKU5, the MERS-CoV belongs to the lineage c of betacoronavirus (Figure 4).

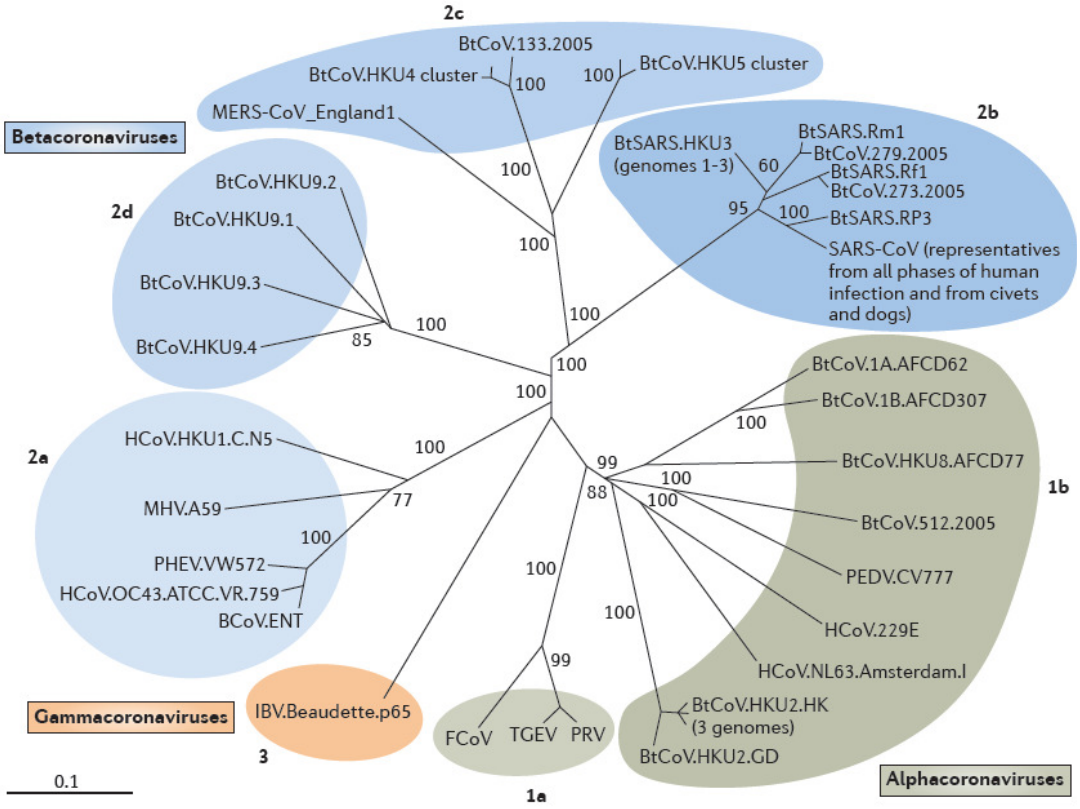

Figure 4 Phylogenetic tree (maximum likelihood) of representative coronaviruses
(from Graham et al., 2013, with permission)

The MERS-CoV genome contains 30119 nucleotides and at least ten open reading frames (ORFs) (Figure 5). The whole genome includes ORF1a and ORF1b as well as (at least) nine downstream ORFs. ORF1a and ORF1b are two large ORFs and responsible for the production of the polyproteins (pp1a and pp1ab), which occurs via ribosomal frame shifting at the junction between the two ORFs. Downstream of ORF1b the genome harbors the small ORFs encoding for the structural proteins spike (S), envelope (E), membrane (M), and nucleocapsid (N). Between the S and E genes, four additional small ORFs are located, named 3a, 3b, 3c, and 3d and presumed to, encode for non-structural proteins. This genome organization was found to be similar to that of the bat coronaviruses BatCoV-HKU4 and BatCoV-HKU5, once more confirming the similarity among these three viruses (Hofmann et al., 2005; Li et al., 2003).

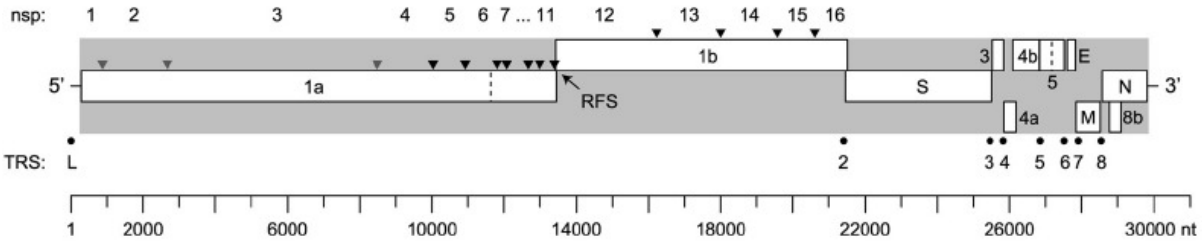

Figure 5 Genome organization of MERS-CoV
(from Boheemen et al., 2012, with permission)

The MERS-CoV virion structure is shown in Figure 6. The spike (S) protein of coronaviruses is responsible for the binding to the host cell receptor and determines the viral host and tissue tropism (Belouzard et al., 2012). The cell surface molecule DPP4 (dipeptidyl peptidase-4, also known as CD26), has been identified as the functional cellular receptor for MERS-CoV (Raj et al., 2013). This seems to be a virus-specific receptor, in contrast to the angiotensin-converting enzyme 2 (ACE2), being used by the SARS-CoV and hCoV-NL63 (Hofmann et al., 2005; Li et al., 2003). After the DPP4 was confirmed as the receptor of MERS-CoV, the receptor binding domain (RBD) has also been mapped (Jiang et al., 2013; Mou et al., 2013). The RBD-S interaction falls with residues 358-662 of the S1 domain and antibodies to this domain were observed to efficiently neutralize MERS-CoV infection. Upon binding, the viral entry of MERS-CoV involves virus-cell fusion, which can be activated by type II transmembrane serine proteases TMPRSS2 (Shirato et al., 2013).

The envelope (E) and membrane (M) proteins are small transmembrane proteins associated with the envelope of all coronaviruses. Like the conserved proteins encoded by ORF1ab, the nucleocapsid gene (N) gene is also another common target for phylogenetic analysis. Due to its immunogenicity, it is also a common target for cloning and generation of recombinant proteins for serological assays (Woo et al., 2010).

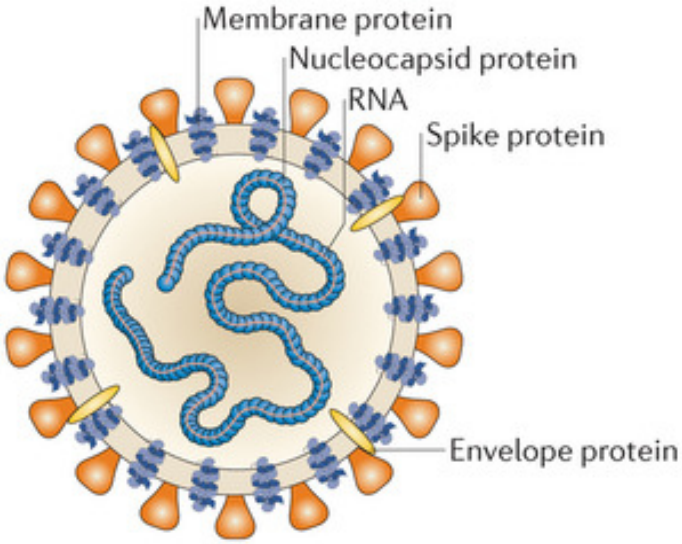

Figure 6 Coronavirus virion structure and proteins
(from Graham et al., 2013, with permission)

Epidemiology of MERS-CoV

MERS-CoV infections have been reported from countries in the Middle East including Jordan, Kingdom of Saudi Arabia (KSA), the United Arab Emirates (UAE), Kuwait, Oman, and Qatar. In Europe affected countries include France, Germany, the United Kingdom (UK), Italy and North Africa Tunisia (CDC; http://www.cdc.gov/coronavirus/MERS/) (Figure 7).

Overall, the average age of MERS-CoV confirmed cases is 50 years, ranging from 14 months to 94 years, and 64% of patients were male. Among all confirmed cases, there were 18 asymptomatic cases, 30% had non-severe disease, while 64% of patients were reported to suffer from severe pneumonia with the ARDS, septic shock and multi-organ failure, even resulting in death. The development of the disease usually starts with fever and cough, chills, sore throat, myalgia and arthralgia, followed by dyspnea and rapidly progresses to pneumonia, often requiring automated ventilation and other organ support (Assiri et al., 2013a; Assiri et al., 2013b).

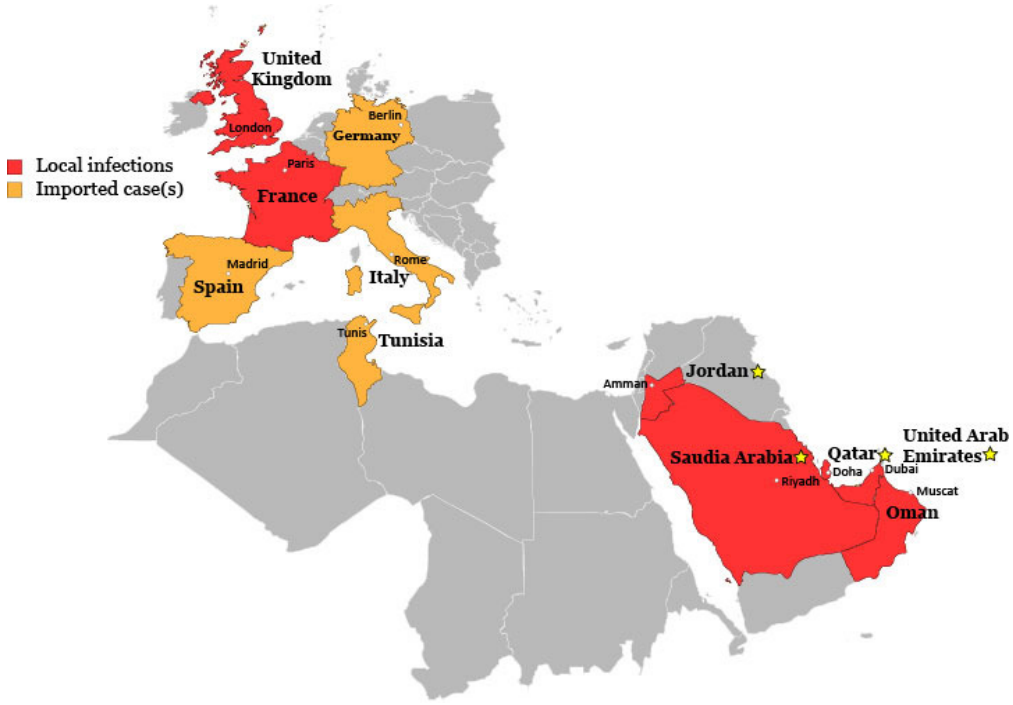

Figure 7 Geographic distribution of human MERS-CoV infections
(from http://www.uq.edu.au/vdu/VDUMERSCoronavirus.html)

The confirmation of virus reservoir and infection source is the key for emerging infection prevention and control. The fact that the reported MERS-CoV infections appear to be sporadic and epidemiological unlinked suggests that MERS-CoV is a zoonotic virus, as reported for other known coronaviruses (To et al., 2013). The phylogenetic proximity of the virus with the bat coronavirus further supports this suggestion.

Until now, the pathways used by MERS-CoV for interhuman transmission are still unknown. Generally, coronavirus are transmitted among humans via aerosol droplets and/or through direct contact with other secretions (stool, urine *etc.*) (Danielsson et al., 2012). Several case investigations have suggested that airborne transmission seems to be the mostly likely route (Guery and van der Werf, 2013; Mailles et al., 2013; Memish et al., 2013). Furthermore, given the high concentration of the virus in the lower respiratory tract of infected patients, airway suctions or the use of bronchoscopes could also serve as a source of transmission (Guberina et al., 2014). The fecal-oral route is likely also important for transmission because of the infectivity in urine and stool samples of patients and given the fact that patients from the same cluster of infection had been sharing toilet rooms during hospitalization. Transmission

via blood should also be considered as a possible route since some reports claim that the virus might be present in blood. This observation might be correlated to the reported person-to-person transmission in hemodialysis unit of a hospital in Saudi Arabia (Assiri et al., 2013b).

When investigating the source of MERS-CoV infection the high similarity of MERS-CoV to BtCoV-HKU4 and BtCoV-HKU5 was recognized upon first phylogenetic analyses of virus genome sequences. Therefore, it was assumed that bats could be the source of infection, especially since the Arabic peninsula harbors a number of various bat species. The observation that MERS-CoV was able to infect cells derived from bat family support this theory. However, direct contact between humans and bats is rather unusual and no such contact has been reported for one of the MERS patients (Chan et al., 2013b). The DPP4 residues responsible for contact with the RBD of the MERS-CoV S protein are highly conserved among different species, including macaques, pigs, and rabbits. This data would imply that MERS-CoV is able of infecting multiple host organisms (Wang et al., 2013). This hypothesise was further supported by the fact that MERS-CoV can also infect primate and porcine cell lines *in vitro* (Bosch et al., 2013).

Based on the above observations, scientists are currently in search of a possible intermediate host that can be the direct source of MERS-CoV infection. In a recent study, sera of sheep, cattle, goat, camels and other camellia species from Middle East, Spain, Netherlands, and Chile were tested for the presence of antibodies against the S protein of MERS-CoV. Interestingly, it was found that 100% of the camels from the Middle East and 14% of the Spanish camel sera were positive and with high titers. No anti-MERS-CoV antibodies were found in any of the other animal species tested (Reusken et al., 2013). Indeed, for some of the MERS patients, contact with camels had been reported (Chan et al., 2013b; Drosten et al., 2013). Most recently Alagaili et al. (2014) detected MERS-CoV in nasal and rectal swabs of juvenile dromedary camels from the Kingdom of Saudi Arabia, indicating their role as potential reservoir for human transmission.

In order to assess the transmissibility of the virus, two groups estimated the basic reproduction number R0, which is the number of secondary cases that one case would produce in a completely susceptible population. The results of this study suggested

that the transmission rate of MERS-CoV among humans is still low and that the virus does not have pandemic potential yet (Breban et al., 2013; Cauchemez et al., 2014).

Diagnosis of MERS-CoV Infection

With no MERS-CoV-specific test established, the samples from the index case from Kingdom of Saudi Arabia were tested by a pan-coronavirus PCR (Zaki et al., 2012). This assay targets highly conserved regions of the RNA-dependent RNA polymerase gene, and is used to detect all known and unknown coronavirus (Vijgen et al., 2008). After the MERS-CoV was identified, different real time RT-PCR assays for routine detection of MERS-CoV have been developed within a short time, targeting the upE region, the ORF1b and the Orf1a (Chan et al., 2013a; Corman et al., 2014). All these assays were confirmed by excluding cross-reactivity with the other known human coronaviruses. In addition, the US Centers for Disease Control and Prevention (US CDC) has developed RT-PCR assays targeting the MERS-CoV nucleocapsid (N) protein gene, which can complement the other assays for screening and confirmation.

The selection of the appropriate clinical specimens that will be tested for the presence of the virus is of high importance. However, it is not yet clear, when MERS-CoV shedding peaks or which body fluids are most likely to yield enough viruses to be tested positive. Therefore, based on the fact that MERS-CoV preferably replicates in cell lines which derived from the lower respiratory tract and the clinical image of the MERS-CoV patients indicates lower respiratory tract involvement (Chan et al., 2013a; Chan et al., 2012), the CDC recommends that lower respiratory tract specimens (sputum, broncho alveolar lavage fluid, or endotracheal aspirate) should be tested for MERS-CoV. This recommendation was further supported by the fact that nasopharyngeal swabs of suspected MERS-CoV patients were found negative, while lower respiratory tract specimens obtained from the same patients were positive (Guery et al., 2013).

Serology can provide valuable information on rates of infection in populations, and serological surveys, particularly among known risk groups or populations. Corman et al. (2012) proposed to detect antibody response against MERS-CoV by immunofluorescence microscopy based on the fact that putative anti-MERS-CoV antibodies in convalescent patient serum could recognize and bind to viral antigens inside MERS-CoV-infected cell lines. However, Chan et al. (2013c) reported that the

presence of cross-reactive neutralizing antibodies against MERS-CoV in the serum of convalescent SARS patients made the specificity of this assay questionable. Therefore, this assay cannot be used independently and has to be confirmed by neutralization test. A highly specific assay for detection of MERS-CoV antibodies using protein microarray technology has also been developed by Reusken et al. (2013). This assay can detect the IgM and IgG antibodies against the receptor binding spike domain S1 and has no cross-reactivity within all known human coronavirus assay. Therefore, this serological assay is available and with great value for human and animal population screening, both of which are necessary to gain insight into the epidemiology of the novel coronavirus. In addition, US CDC has developed a two-stage approach to detect antibodies against MERS-CoV, based on a screening test using a recombinant nucleocapsid (N) protein-based indirect enzyme-linked immunosorbent assay (ELISA), followed by a confirmatory test using a whole-virus indirect fluorescent antibody (IFA) test or neutralization test.

Treatment of MERS-CoV infection

Even ten years after the SARS pandemic there are currently no approved antiviral treatments for human coronavirus infections, including SARS-CoV and MERS-CoV (Graham et al., 2013). So, the current patient management largely depends on provision of organ support, and vigilance for and prevention of complication. In specific circumstances, additional interventions have included empiric use of broad-spectrum antimicrobial agents, antivirals and anti-fungal agents to minimize risk of co-infections with opportunistic pathogens (Chan et al., 2012).

The high mortality associated with MERS-CoV drives the research on potential therapeutic agents. So far, studies have evaluated the effect of interferon (IFN), receptor binding domain (RBD) and DPP4 on the viral replication (Table 4). The fact that MERS-CoV infection does not induce IFN response in human cell lines spurred further investigations (Chan et al., 2013c; Zielecki et al., 2013). The anti-viral effect of IFN-α, IFN-λ3, pegylated IFN-α, and IFN-β has been investigated in vitro by different groups (de Wilde et al., 2013; Falzarano et al., 2013; Kindler et al., 2013). It was concluded that high concentrations of each of the interferon compounds alone are needed to mediate an anti-viral effect (demonstrated by reduced cytopathic effect (CPE) and viral protein levels). Combinations of interferons, however, can yield an anti-viral effect even when lower concentrations are used.

The viral attachment and viral membrane fusion with the host cell membrane are essential for the cellular entry of enveloped viruses and are therefore targets of anti-viral therapeutic strategies. The spike protein has been showen to mediate the binding with the receptor of cells for all coronavirus. Since the RBD was mapped at the C-terminal part of S1 subunit, Chen et al. (2013) and Du et al. (2013a) investigated the possible therapeutic implication of this domain, and showed that the RBD can efficiently inhibit the MERS-CoV entry. A previous study reported that DPP4 serves as the functional receptor for MERS-CoV (Raj et al., 2013). Raj et al. also described that the adenosine deaminase (ADA), a DPP4 binding protein, could compete for virus receptor binding. Thus ADA might be used as a natural antagonist for MERS-CoV infection (Raj et al., 2014).

Drug candidate	Target	In vitro effect	Reference
IFN-α	Interferon	Reduction of MERS-CoV replication in pseudo-stratified HAE cultures	Kindler et al., 2013
Pegylated IFN-α		Inhibition of MERS-CoV induced CPE and reduction of the viral RNA levels in human lung epithelial and monkey kidney cell lines	de Wilde et al., 2013
IFN-β		Reduction of the viral load in MERS-CoV infected human lung epithelial and monkey kidney cell lines	Zielecki et al., 2013
IFN-λ3		Reduction of MERS-CoV replication in pseudo-stratified HAE cultures	Kindler et al., 2013
IFN-α2b		Reduction of the MERS-CoV induced CPE and the viral protein levels in monkey kidney cell lines	Falzarano et al., 2013
Adenosine deaminase (ADA)	Dipeptidyl peptidase-4 (DPP4)	ADA blocks MERS-CoV binding and infection	Raj et al., 2014
MERS-CoV RBD	Receptor binding domain (RBD)	Reduction of the viral load in a MERS-CoV infected monkey kidney cell lines	Chen et al., 2013

Tabelle 4 Drug candidates with *in vitro* effects on MERS-CoV replication

Although some studies already showed promising anti-MERS-CoV results *in vitro*, the experience with SARS-CoV teach us that it may take a long time until an etiological treatment is available. Therefore, the development of safe, stable vaccines is necessary (Graham et al., 2013).

III. OBJECTIVES

Due to the very recent emergence of the Middle East respiratory syndrome coronavirus there is no etiological treatment or vaccine available. Therefore this work describes:

(i) the generation of a recombinant MVA expressing the spike protein of MERS-CoV,

(ii) genetic analysis of the recombinant MVA,

(iii) analysis of the expressed spike protein,

(iv) vaccination experiment in mice

(v) characterization of induced neutralizing antibodies.

IV. RESULTS

The manuscript is presented in the form accepted for publication.

It has its own reference section formatted in the style of the journal. References and abbreviations from the manuscript are not included in the relevant section at the end of this document.

Middle East respiratory syndrome coronavirus (MERS-CoV) spike protein delivered by Modified Vaccinia virus Ankara (MVA) efficiently induces virus-neutralizing antibodies

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Middle East respiratory syndrome coronavirus (MERS-CoV) has recently emerged as a causative agent of severe respiratory disease in humans. Here, we constructed recombinant modified vaccinia virus Ankara (MVA) expressing full-length MERS-CoV spike (S) protein (MVA-MERS-S). The genetic stability and growth characteristics of MVA-MERS-S make it a suitable candidate vaccine for clinical testing. Vaccinated mice produced high levels of serum antibodies neutralizing MERS-CoV. Thus, MVA-MERS-S may serve for further development of an emergency vaccine against MERS-CoV.

Middle East Respiratory Syndrome Coronavirus (MERS-CoV), a novel infectious agent causing severe respiratory disease and deaths in humans, was first described in 2012 (1-3). To date a total of 108 cases of infection with MERS-CoV have been confirmed, including 50 deaths (http://www.who.int/csr/don/2013_08_30/en/index.html). Most infections were geographically linked to the Middle East, i.e. Jordan, Saudi Arabia, Qatar, and United Arab Emirates, but cases also occurred in the United Kingdom, Germany, France and Italy. The epidemiology of MERS-CoV infection remains unclear. The virus is suspected to persist in animal reservoirs and cause zoonotic infections in humans (4, 5). The MERS-CoV spike (S) protein, a characteristic structural component of the virion membrane, forms large protruding spikes on the surface of the virus; its S1 domain mediates binding to dipeptidyl peptidase 4, which serves as the host cell receptor of MERS-CoV (6). Importantly, the S protein is considered a key component of vaccines against coronavirus infection, including severe acute respiratory syndrome (SARS) (7, 8).

Modified vaccinia virus Ankara (MVA), a highly attenuated strain of vaccinia virus originating from growth selection on chicken embryo fibroblasts (CEF), shows a characteristic replication defect in mammalian cells (9, 10, 11). At present, MVA serves as one of the most advanced recombinant poxvirus vectors in preclinical research and human clinical trials for developing new vaccines against infectious disease and cancer (12, 13, 14).

Here, we show that the full-length S protein of MERS-CoV, expressed by MVA, is produced as an ~210-kDa N-glycosylated protein that is specifically recognized by antibodies in Western blot analysis. Further studies suggest cleavage of the mature full-length S glycoprotein into an amino-terminal domain (S1) and an ~85-kDa

carboxy-terminal domain (S2) that is putatively anchored to the membrane. When tested as a vaccine in mice, recombinant MVA expressing the S protein induced high levels of circulating antibodies that neutralize MERS-CoV in tissue culture infections.

Construction and characterization of recombinant MVA. cDNA containing the entire gene sequence encoding MERS-CoV S (GenBank accession no. JX869059) was obtained by DNA synthesis (Invitrogen Life Technology, Regensburg, Germany) and modified by introducing silent mutations that remove three termination signals (TTTTTNT) for vaccinia virus transcription (MERS-S). Furthermore we generated a second version containing a tag sequence encoding nine amino acids (YPYDVPDYA) from influenza virus hemagglutinin (HA tag) attached at the C-terminus of S (MERS-S_{HA}). MERS-S and MERS-S_{HA} were cloned under the transcriptional control of the vaccinia virus early/late promoter PmH5 (15) and introduced by homologous recombination into an existing deletion site (deletion III) in the MVA genome (Fig. 1A).

MVA expressing MERS-S or MERS-S_{HA} (MVA-MERS-S or MVA-MERS-S_{HA}, respectively) were obtained using standard methods to generate recombinant MVA vaccines suitable for clinical testing, as described previously (13). Briefly, transient coproduction of the fluorescent marker protein mCherry (under control of the vaccinia virus late promoter P11 (16) was used to isolate clonal recombinant viruses by screening for fluorescent cell foci during repetitive plaque purification. At this stage, immunostaining of infected cell cultures with anti-HA tag monoclonal or polyclonal antibodies from MERS-CoV-infected macaques suggested synthesis of the recombinant S_{HA} and S proteins in CEF and Vero cells (ATCC CCL-81) (Fig. 2). MVA-MERS-S and MVA-MERS-S_{HA} were genetically stable and replicated efficiently in CEF, but not in human HeLa or HaCat cells (Fig. 1 B and C). The latter findings confirmed that the recombinant viruses could be handled under biosafety level 1 conditions.

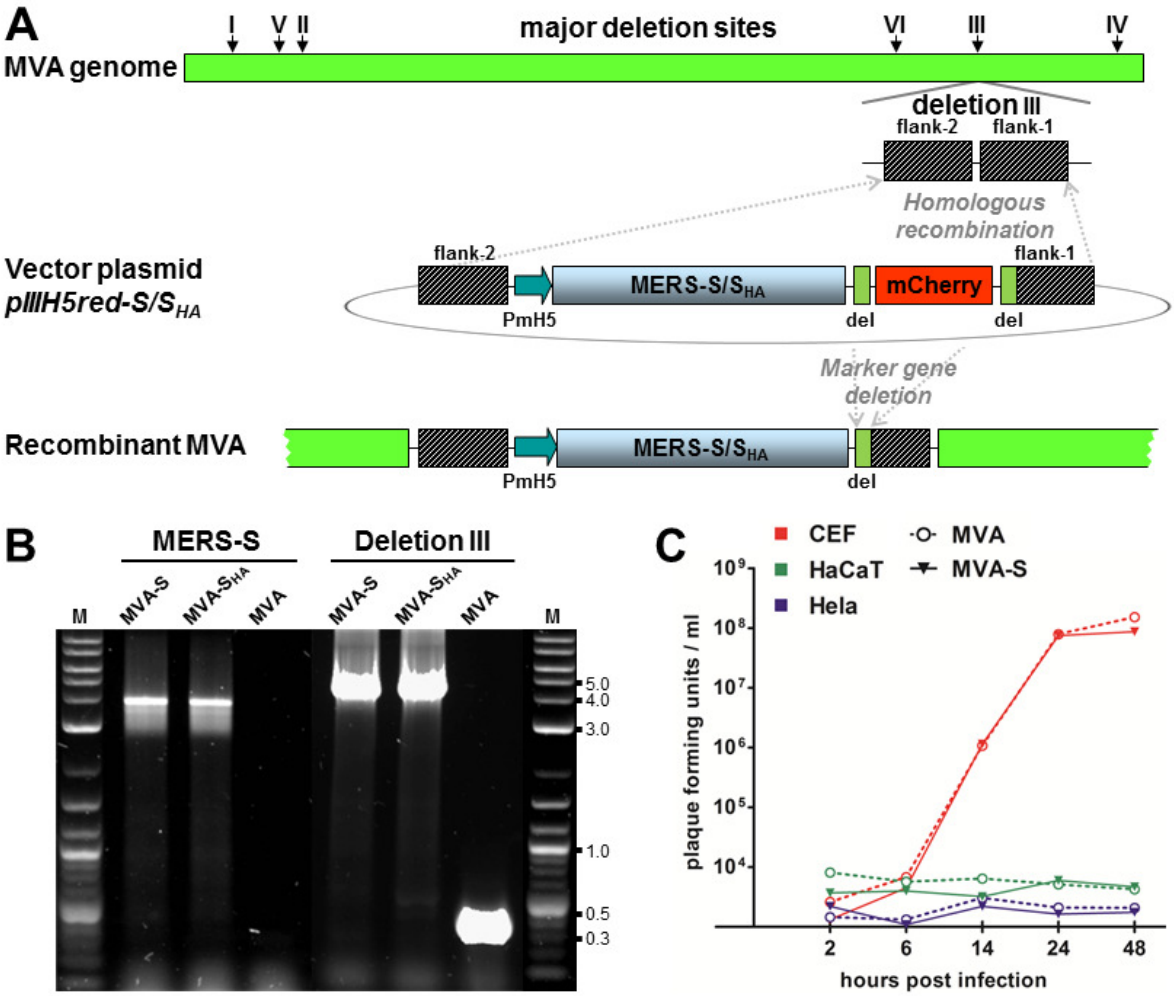

FIG 1 Generating and characterizing recombinant MVA. (A) Schematic diagram of the MVA genome and the locations of major deletion sites I to IV, with deletion III being the site used to insert the MERS-CoV S gene sequences. Flank-1 and flank-2 refer to MVA DNA sequences adjacent to deletion site III which were originally prepared by PCR and cloned into MVA transfer plasmids targeting deletion site III for insertion of recombinant genes. In MVA vector plasmids pIIIH5red-S and -S_{HA}, the S coding gene sequences (MERS-S/S_{HA}) are placed under transcriptional control of the vaccinia virus promoter PmH5 and introduced by homologous recombination between the flanking sequences in the vector and the MVA genome. MVA-MERS-S and MVA-MERS-S_{HA} were isolated in plaque passages by screening for transient co-expression of the fluorescent marker gene mCherry under transcriptional control of the vaccinia virus late promoter P11. Repetitive sequences (del) are designed to remove the mCherry marker by intragenomic homologous recombination (marker gene deletion). (B) Genetic integrity and genetic stability of MVA-MERS-S and MERS-S_{HA}. PCR analysis of genomic viral DNA using oligonucleotide primers to confirm the identity (MERS-S) and proper insertion (deletion III) of S gene sequences. (C) Multiple-step growth analysis of recombinant MVA-MERS-S. Recombinant MVA (MVA-S) and wildtype MVA (MVA) can be efficiently amplified in CEF (multiplicity of infection [MOI,]0.1) but fail to productively grow in HeLa and HaCat human cell lines.

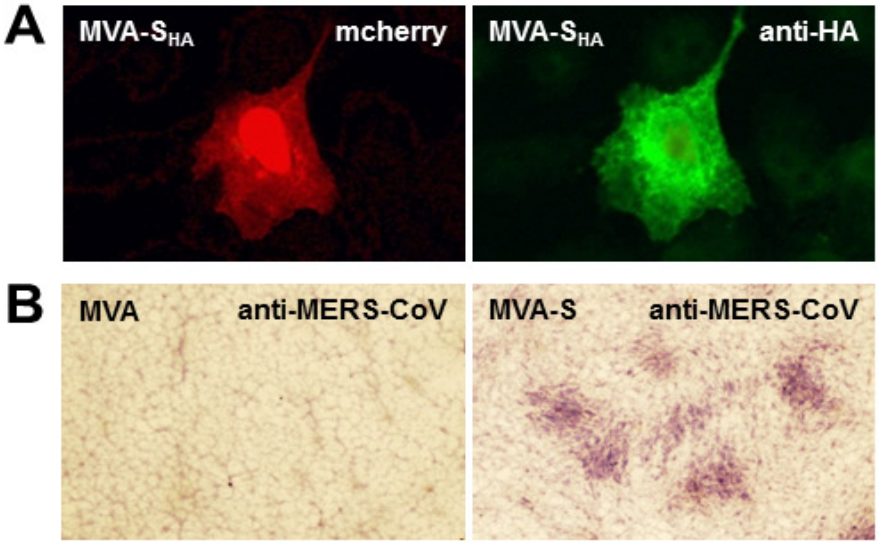

FIG 2 Immunostaining of S proteins in recombinant MVA-infected cells. (A) Transient expression of the marker protein mCherry served to localize single virus infected cells (left panel). Monoclonal antibody directed against the HA-tag (anti-HA) (right panel) reveals the presence of S_{HA} in Vero cells infected (MOI, 0.1) with MVA-MERS-S_{HA} (MVA-S_{HA}). (B) Polyclonal antibodies from a MERS-CoV-infected cynomolgous macaque (anti-MERS-CoV) detected S-producing cell foci in CEF infected with MVA-MERS-S (MVA-S; MOI, 0.1) but no foci when CEF were infected with nonrecombinant MVA (MVA).

Characterization of MERS-CoV S expressed by recombinant MVA. We specifically detected a protein with an estimated molecular mass of about 200 kDa in lysates from MVA-MERS-S and MVA-MERS-S_{HA} infected Vero cells using sera from MERS-CoV infected macaques in Western blots (Fig. 3A, upper panel). Further immunoblot analysis with monoclonal anti-HA tag antibody (Roche, Penzberg, Germany) confirmed the production of a protein doublet of 200 to 210 kDa in cells infected with MVA-MERS-S_{HA} as well as a second protein of about 85 kDa (Fig. 3A, lower panel).

The molecular masses of the 200- to 210-kDa polypeptides detected by SDS/PAGE were significantly higher than the 149 kDa predicted for MERS-CoV S protein based on its nucleotide sequence. NetNGlyc server analysis suggested at least 17 likely N-glycosylation sites (Asn-X-Ser/Thr) for co- and post-translational modification. Therefore, we investigated the glycosylation pattern of the expressed S_{HA} protein using treatment with peptide-N-glycosidase F (PNGase F) or endoglycosidase H (Endo H) (both from New England Biolabs) followed by Western blot analysis (Fig. 3B). S_{HA} produced in MVA-MERS-S_{HA} infected Vero cells was treated with PNGase F, which removes all N-linked oligosaccharide chains from glycoproteins. This treatment reduced the ~210-kDa protein doublet to a sharp protein band of 150-kDa, a value closely matching the predicted mass of unmodified MERS-CoV S protein.

Similarly, PNGase F treatment converted the 85-kDa protein fragment to a polypeptide with 55 kDa. Endo H cleaves N-linked high-mannose oligosaccharides synthesized in the endoplasmic reticulum, but not those matured to more complex oligosaccharides in the Golgi apparatus. Western blot analysis of S_{HA} digested with endo H revealed a band of 150 kDa proteins, indicating complete hydrolysis of N-linked oligosaccharides. An additional subpopulation of S_{HA} migrated with the original size of about 210 kDa indicating resistance to endo H treatment. Of note, the 85 kDa protein subpopulation remained largely unaffected by endo H digestion.

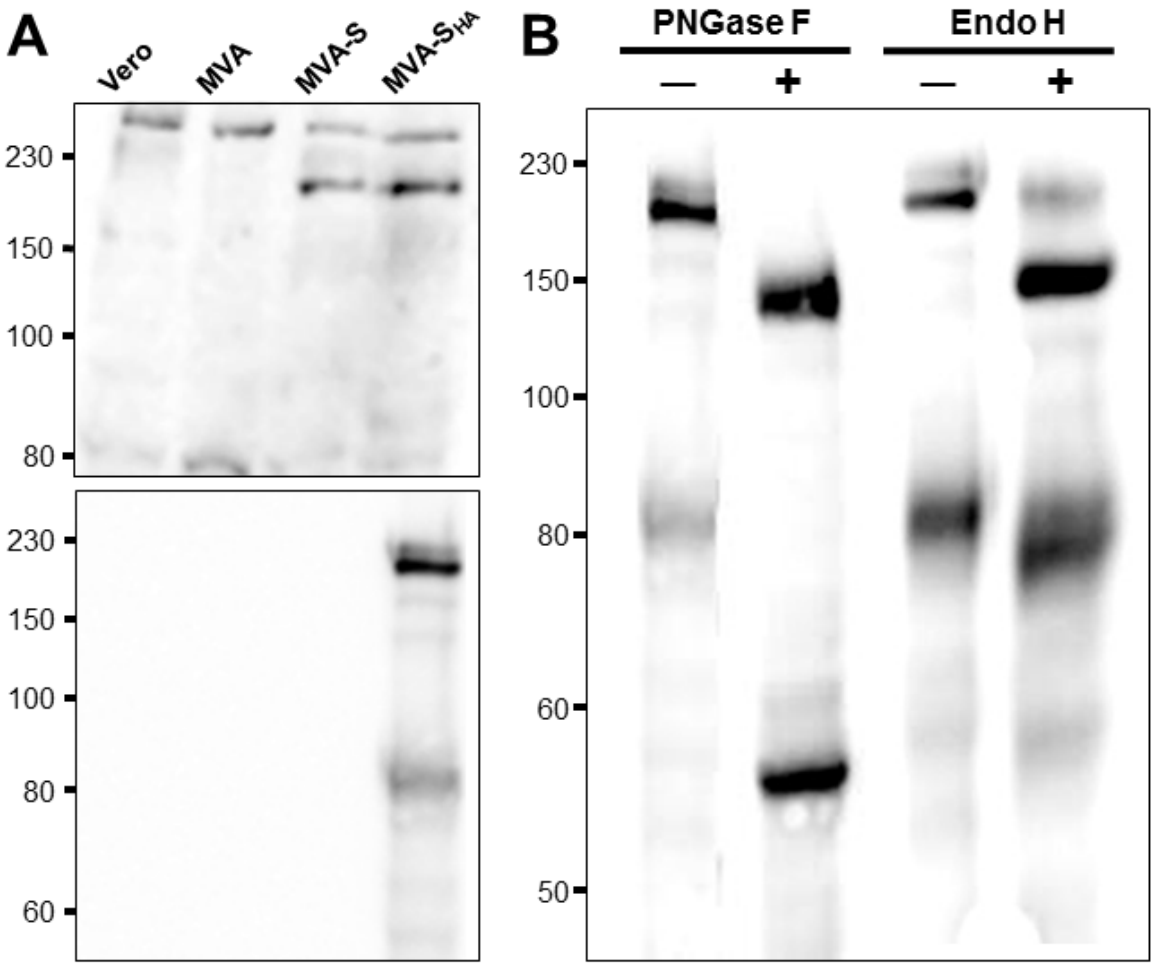

FIG 3 Synthesis of full-length S glycoprotein in recombinant MVA-infected cells. (A) Western blot analysis of cell lysates from MVA-MERS-S (MVA-S)- or MVA-MERS-S_{HA} (MVA-S_{HA})-infected Vero cells 24 h postinfection. Polypeptides were analyzed by SDS-PAGE and immunoblotting using serum from a MERS-CoV-infected macaque (1:1000, upper panel) or monoclonal rat anti-HA-tag antibody (1:50; lower panel). Lysates from uninfected (Vero) or wild-type MVA-infected (MVA) cells served as controls. (B) Western blot analysis of MVA expressed S_{HA} following treatment with glycosidases. Vero cells were infected with MVA-MERS-S_{HA} for 24 h. Cell lysates were incubated with (+) or without (-) glycosidase PNGase F or endo H and analyzed by SDS-PAGE and immunoblotting with monoclonal rat anti-HA-tag antibody (1:50). Numbers on the left indicate molecular masses of marker proteins in kilodaltons.

Immunogenicity of MVA-MERS-S in mice. BALB/c mice were vaccinated intramuscularly with 10^8 pfu MVA-MERS-S at 0 and 3 weeks. Twenty days after the first immunization, and ten days after the second immunization sera samples were tested for their capacity to neutralize MERS-CoV (EMC isolate) in tissue culture infections using 200 50% tissue culture infective doses ($TCID_{50}$) in Vero cells or 100 $TCID_{50}$ in Huh-7 cells. Even a single application of MVA-MERS-S induced low levels of virus-neutralizing antibodies in all eight animals tested (Fig. 4A). After booster immunization all vaccinated animals produced high levels of circulating antibodies that neutralized MERS-CoV (Fig. 4B and C). In contrast, neutralizing antibodies were not detected in sera samples from control animals inoculated with non-recombinant MVA or saline (phosphate buffered saline [PBS]). The specificity of the induced MVA-MERS-S antibodies for MERS-CoV was confirmed by the absence of detectable neutralization against SARS coronavirus (SARS-CoV) (strain HKU39849) (Fig. 4D). In addition, the MERS-CoV-neutralizing activity of these sera could be only partly blocked by preincubation with proteins that encompass the receptor binding domain (RBD) (Fig. 4E and F). The latter observation is consistent with the fact that besides the RBD other parts of the spike protein are able to induce neutralizing antibodies (17).

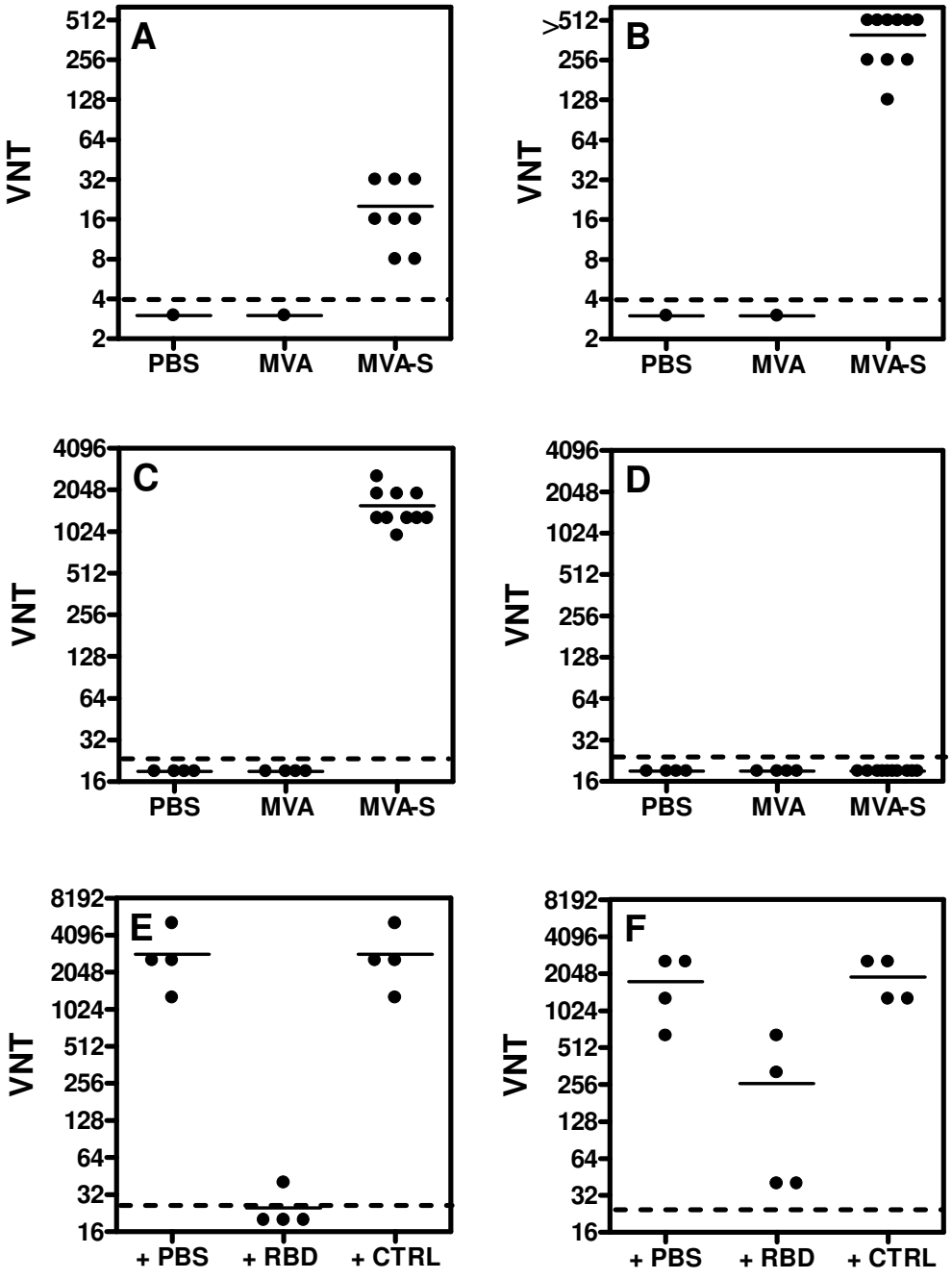

FIG 4 Antibody responses after intramuscular immunization with recombinant MVA-MERS-S. BALB/c mice (*n* = 10) were vaccinated twice within a 21-day interval with 10^8 pfu MVA-MERS-S (MVA-S). Groups of mice (*n* = 4) vaccinated with wild-type MVA (MVA) or saline (PBS) served as controls. MERS-CoV virus-neutralizing titers (VNT) were determined 20 days after primary immunization (A) and 10 days after the second immunization (B and C) using Huh7 (A and B) and Vero cells (C). Sera obtained after the second boost were also tested against SARS-CoV on Vero cells (D). Serum from a rabbit (17) immunized with the MERS-CoV receptor binding domain (RBD) (E) or sera from mice obtained after the second MVA-S boost (F), were preincubated with PBS, the RBD, or a SARS-CoV-derived control protein (CTRL) at 5 µg/ml for 1 h before incubation with MERS-CoV, followed 1 h later by inoculation on Vero cells.

Conclusions. Our objective was to use MVA vectors compatible with clinical evaluation to express mostly native S antigen to induce antibodies that would neutralize MERS-CoV. The S gene of MERS-CoV expressed by recombinant MVA produced a glycoprotein migrating at a molecular mass of about 210 kDa. Glycosidase treatment to remove all N-linked carbohydrates resulted in a polypeptide of 150 kDa, closely corresponding to the molecular mass predicted from the S gene nucleotide sequence. A subpopulation of protein apparently acquired endo H resistance, indicating trafficking of S to the Golgi apparatus, which correlates with observations made for SARS-CoV S protein (18, 19, 20).

In addition, we obtained evidence for putative S1 and S2 cleavage of full-length S as predicted recently (6, 21). The HA-tag fused to the C-terminus of S_{HA} enabled us to detect two glycoprotein subpopulations, full-length S (~210 kDa) and subdomain S2 (~85 kDa), by anti-HA Western blot analysis (Fig. 3). We considered furin cleavage sites because spike proteins of some betacoronaviruses and all gammacoronaviruses are typically activated by intracellular furin-dependent cleavage (22, 23; for review see references 24, 25); the ProP1.0 server indicated three possible furin cleavage sites at amino acid positions 751, 887, and 1113 of MERS-CoV S (data not shown). We favor putative cleavage at amino acid 887 since this cleavage would produce a predicted S2 subdomain of 54.5 kDa for the non-glycosylated protein matching our Western blot data (Fig. 3). Moreover, prominent endo H resistance of the 85-kDa S2 subdomain suggests that S cleavage occurs predominantly during or after passage through the Golgi apparatus.

Since biochemical characterization of the MVA-expressed S suggested synthesis of a mature and properly folded spike antigen, we investigated whether MVA-MERS-S would elicit virus-neutralizing antibodies. Indeed, mice immunized with MVA-MERS-S via an intramuscular route developed circulating antibodies that neutralized MERS-CoV infections in highly permissive tissue cultures. Interestingly, compared to results of previous studies eliciting neutralizing antibodies to SARS-CoV (18), MVA-MERS-S induced relatively high levels of antibodies that efficiently block MERS-CoV infection. This observation may be explained by MERS-CoV-specific differences in receptor usage and entry mechanisms, as discussed previously (18). Previous work with SARS-CoV showed that S-specific neutralizing antibodies correlated with the protective capacity of vaccination in various animal models (18, 26, 27; for a review see reference 28).

Future studies will be necessary to monitor for S antibody-dependent enhancement of MERS-CoV infections, as previously discussed for SARS-CoV and feline coronavirus infections (28, 29, 30). Furthermore, the safety and protective capacity of MVA/MERS-S immunization should be tested in animal models that reproduce MERS-CoV infections in humans. However the current absence of suitable preclinical models recommends the development of an MVA vaccine delivering MERS-CoV-S.

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V. DISCUSSION

In the light of the pandemic threat caused by MERS-CoV, the availability of sufficient doses of safe and effective vaccines is considered a priority. Our objective of this study was to express spike protein antigens in a native state to induce antibodies that would neutralize MERS-CoV by using the live-attenuated vector virus MVA. The newly constructed recombinant MVA-MERS-S can express the full-length MERS-CoV S protein coding sequences with the strong poxvirus-specific promoter PmH5. The recombinant virus MVA-MERS-S was genetically stable and was able to replicate in primary CEF cells to high titers (10^8 PFU/ml). Moreover, the maintained replication deficiency of the vector on mammalian cells support the view that MVA-MERS-S could serve as a safe vaccine for human and animal use. In addition, vaccinated mice produced high levels of serum antibodies neutralizing MERS-CoV. Thus, MVA-MERS-S may serve for further development of an efficacious emergency vaccine against MERS-CoV.

Why is a vaccine needed for prophylaxis against MERS-CoV ?

Middle East respiratory syndrome coronavirus (MERS-CoV) is a newly isolated human coronavirus which caused outbreaks of a SARS-like illness in the Middle East. Until now, WHO has been informed of 189 confirmed cases, including 82 deaths (a case fatality rate >40%) (http://who.int/csr/don/2014_03_12/en). Cases were reported mainly from four Middle East countries (Saudi Arabia, Jordan, Qatar and the United Arab Emirates), three European countries (Frances, Italy, and the United Kingdom), and one African country (Tunisia). Among all confirmed cases, eight clusters MERS-CoV infectionshave been identified.The largest cluster of infections consisted of 25 patients including 14 deaths. This finding of cluster infections strongly suggests the possibility of inter-human transmission of MERS-CoV. So far, the efficiency of human-to-human transmission of MERS-CoV seems lower than the one observed during the SARS epidemic. However, MERS-CoV may gain increased human-to-human transmissibility after its further adaptation and evolution in human or as-yet unknown intermediate hosts. Therefore, MERS-CoV is threatening to eventually cause a global pandemic and thus, the epidemiology of MERS-CoV is closely watched by WHO and possible counter-measures are being considered by public health institutions.

To date, there are no approved antiviral treatments or vaccines against human coronaviruses, including SARS-CoV. Clinical management of MERS-CoV infected patients is mainly supportive placing emphasis on organ support for respiratory and renal failure. Vaccines are among the most cost-effective public health measurements and vaccines are core components of any preventive service package targeting infectious diseases. In consequence, the development of safe and efficacious vaccines against MERS-CoV and other emerging coronaviruses is highly desirable. In particular, it must be reflected that these viruses persist in heterogeneous pools, likely in various host species, and future vaccines would ideally be broad-spectrum or rapidly adaptable to newly emerging coronavirus pathogens.

The spike protein of MERS-CoV - a suitable target antigen for vaccine development.

Like other coronaviruses, the MERS-CoV virion utilizes a large surface spike (S) glycoprotein for interaction with and entry into the target cell. It has been reported that MERS-CoV uses a cell surface amino peptidase, DPP4, as a functional receptor (Jiang et al., 2013; Raj et al., 2013). Another group systematically characterized the MERS-CoV HR1/HR2 complexes and presented a 1.9 Å crystal structure of the viral fusion core. This result demonstrated that the spike protein of MERS-CoV can mediates the membrane fusion in a similar way as reported for other coronaviruses (Gao et al., 2013). The roles of the S protein in both receptor binding and membrane fusion indicate that vaccines based on the S protein could induce antibodies to block virus binding and virus entry through fusion inhibition. Antibodies with such properties are well known for their capacity to neutralize virus infections. Thus, among all structural proteins of coronaviruses, the S glycoprotein is considered the main antigenic component that is responsible for inducing host immune responses, neutralizing antibodies and/or protective immunity against virus infection (Du et al., 2009). S proteins have therefore been selected as preferred targets for the development of new coronavirus-specific vaccines and anti-virals.

Several vaccines that are based on the full-length S protein of SARS-CoV have been reported. Yang et al. showed that a DNA vaccine encoding the full-length S protein of the SARS-CoV Urbani strain induced both T-cell and neutralizing-antibody responses, as well as protective immunity in a mouse model (Yang et al., 2004). Other groups have also shown that vaccination of mice or monkeys with highly attenuated modified

vaccinia virus Ankara (MVA) which encodes the full-length S protein of the SARS-CoV Urbani or HKU39849 strain elicited S-specific neutralizing antibodies and protective immunity as evidenced by decreased virus loads in the respiratory tracts of animals after challenge infections with homologous SARS-CoV (Bisht et al., 2004; Chen et al., 2005).

These reports suggest that the full-length S protein is highly immunogenic and induces protection against SARS-CoV challenge and that neutralizing antibodies alone may be able to suppress virus proliferation. Moreover, these data further supported the rationale that efficacious coronavirus-specific vaccines can be developed based on the S protein as the only antigen.

Coronavirus spike protein can be expressed in a native state by MVA vectors.

The spike protein of coronavirus is a type I transmembrane protein and highly glycosylated. Spike proteins assemble into trimers on the virion surface to form the distinctive “corona” or crown-like appearance. The ectodomain of all coronavirus spike proteins share the same organization in two domains: An N-terminal domain named S1 that is responsible for receptor binding and a C-terminal S2 domain responsible for fusion (Belouzard et al., 2012).

The secretory pathway of the cell has an important quality control function and the trafficking of a protein from the endoplasmic reticulum to the plasma membrane is a sign of proper secondary modification and folding. The N-linked oligosaccharide pathway is frequently used for tracking protein movement. Addition of N-linked oligosaccharides occurs in the endoplasmic reticulum and the conversion of the high mannose form to complex endo H-resistant N-linked chains occurs on transport from the cis to the medial Golgi compartment (Figure 8).

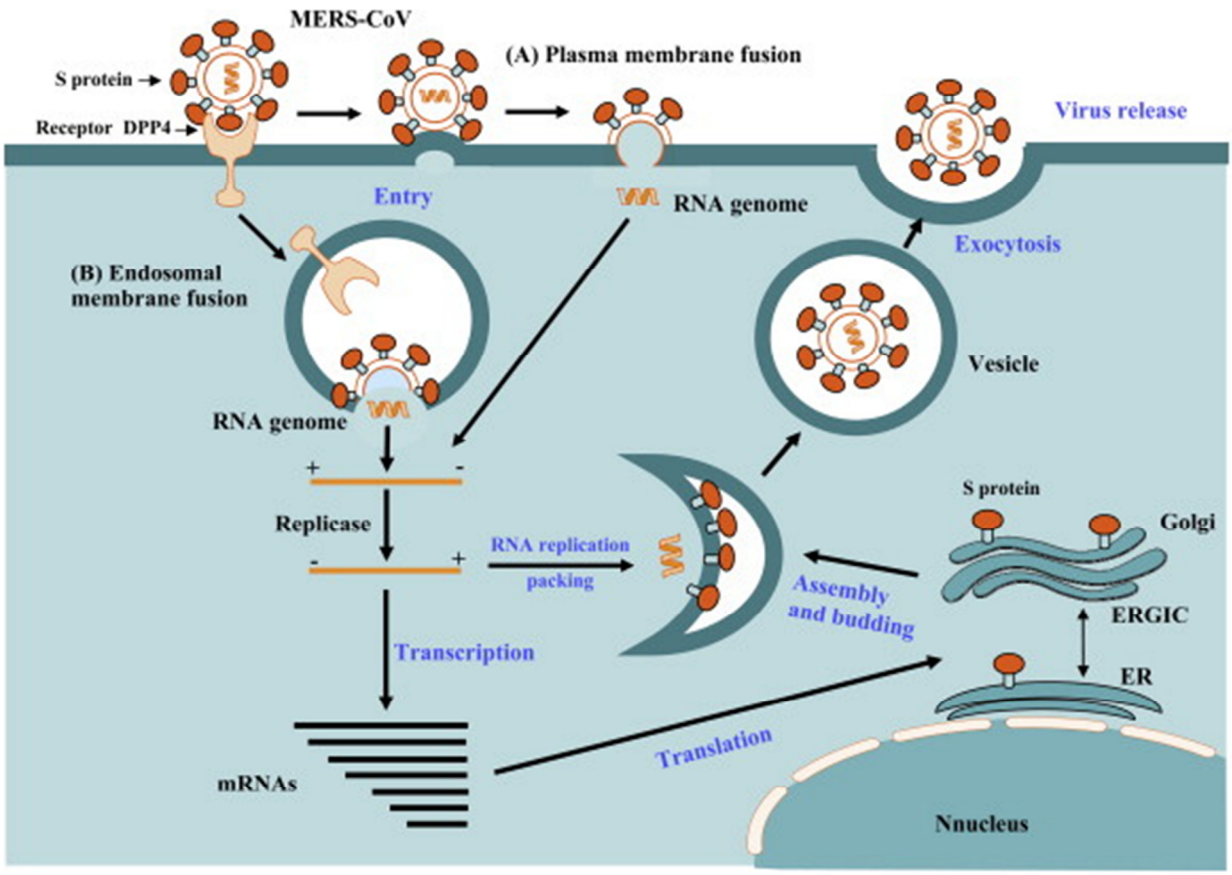

Figure 8 The life cycle of MERS-CoV
(from Lu et al., 2013, with permission)

We specifically detected a protein with an estimated molecular mass of about 200 kDa in lysates from MVA-MERS-S- and MVA-MERS-SHA-infected Vero cells by using sera from MERSCoV-infected macaques in Western blot analysis. Furthermore, the use of the monoclonal anti-HA tag antibody confirmed the production of a protein doublet of 200 to 210 kDa in cells infected with MVA-MERSSHA. The molecular masses of the 200- to 210-kDa polypeptides detected by SDS-PAGE were significantly higher than the 149 kDa predicted for MERS-CoV S protein based on its nucleotide sequence. The treatment of lysates with glycosidase specific for N-linked carbohydrates reduced the 210-kDa protein doublet to a sharp protein band of about 150 kDa a value closely matching the predicted mass of unmodified MERS-CoV S protein. A subpopulation of S protein molecules which was resistant to endo H indicated that the S protein was successfully trafficked to medial Golgi apparatus. Furthermore, the detection of two bands was consistent with cleavage of the S protein (200 kDa) into an N-terminal (80kDa) and a C-terminal (not detected) indicated that S protein could be cleaved into functional S1 and S2 subunits. In this context, Gierer et al found the serine transmembrane protease TMPRRS2 to activate the fusion between viral and host cell

membrane by cleaving the S protein into S1 and S2 subunits. Importantly, MERS-CoV S protein mediated fusion was also revealed using immunostaining of MVA vector infected cell cultures with anti-HA tag monoclonal or polyclonal antibodies from MERS-CoV-infected macaques (Figure 9). These results suggested synthesis of functional recombinant MERS-CoV S_{HA} and S proteins in MVA vector infected avian and mammalian cells.

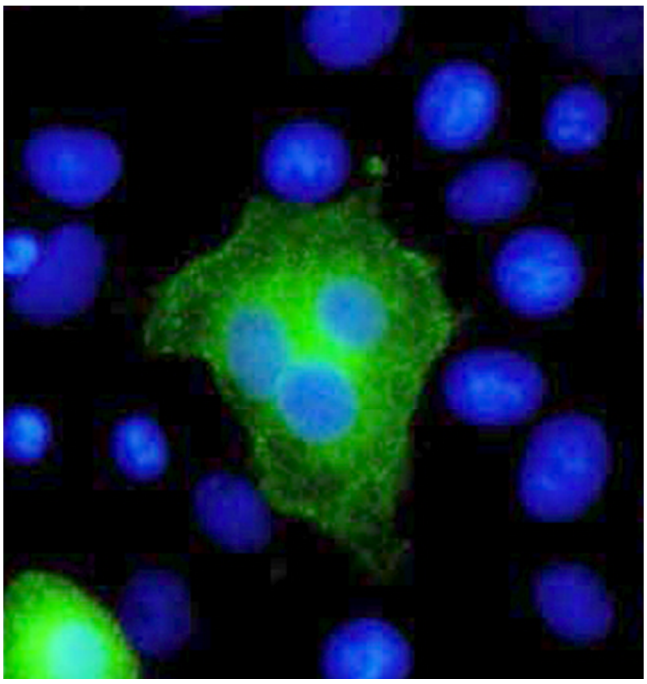

Figure 9 Fusion of MVA-MERS-S infected Vero cells

MVA-MERS-S is safe and replication deficient vector virus.

The growth kinetics of MERS-CoV on CEF cells showed that the recombinant viruses were able to replicate in CEF cells to high titers (10^8 PFU/ml) and can be readily amplified at larger scale. Importantly, the MVA-MERS-S recombinant viruses were unable to productively grow in defined human cell cultures (cell lines HeLa and HaCaT). This data confirmed the maintenance of replication-deficiency and biological safety of the MVA vector virus. Moreover, this conclusion from *in vitro* experiments is well supported by preliminary data from vaccination experiments in mouse models. The MVA-MERS-S vaccine was well tolerated in all vaccinated animals with no adverse reactions being observed. Overall, these results from the preclinical characterization of the recombinant viruses strongly supported the usefulness of MVA-MERS-S for further development as candidate vaccine for humans and animals.

MVA-MERS-Sinduces MERS-CoV neutralizing antibody responses.

First immunizations of mice with MVA-MERS-S demonstrated the induction of high levels of neutralizing antibodies. The IC50 for MERS-CoV neutralization reached almost 2000after two applications of MVA-MERS-S vaccine. This high titer of neutralizing antibodies also supported that the MVA vector produces an authentic and immunogenic MERS-CoV S protein for in vivo antigen presentation and immune recognition. In addition, the prime/boost vaccination protocol may have also contributed to the increased production of neutralizing antibodies. It was reported that MVA-based booster immunizations can modulate the elicited antigen-specific immune response towards a mixed Th1/Th2 response pattern and contribute to optimize the cell mediated responses (Schulze et al., 2008). Moreover, homologous prime/boost immunizations with an MVA candidate vector vaccine against tuberculosis showed that highly persistent and protective immune responses can be elicited for at least 16 months after the initial vaccination (Kolibab et al., 2010).

Sequence and modeling analysis using data from several human coronaviruses allowed to reveal the potential receptor-binding domain of MERS-CoV in the S glycoprotein (Jiang et al., 2013; Raj et al., 2013). The MERS-CoV-neutralizing activity of sera from MVA-MERS-S vaccinated mice could be partly blocked by preincubation with recombinant proteins that encompass the MERS-S receptor binding domain. This observation confirmed the proposed site of the receptor binding domain in the MERS-S protein and also indicated the other parts of the S protein might be targets of MERS-CoV neutralizing antibodies.

Chan et al. have reported that sera collected from convalescent SARS patients might contain cross-reactive antibodies to MERS-CoV as detected by both immunofluorescent and neutralizing antibody tests (Chan et al., 2013c). In response to this observation, the specificity of the antibody response induced by MVA-MERS-S was confirmed by the absence of any detectable neutralization of the SARS coronavirus (SARS-CoV). Also another study showed that monoclonal antibodies specific for the receptor binding domain of the SARS-CoV S protein exhibited no cross-reactive or cross-neutralizing activity to MERS-CoV. This data suggests that the receptor binding domains of S proteins from SARS-CoV and MERS-CoV may not contain significant epitopes for inducing cross-reactive antibody responses (Du et al., 2013b).

Future directions in developing an MVA-MERS-S vaccine.

The initial immunogenicity testing of the MVA-MERS-S vaccine in the mouse model demonstrated its ability to induce MERS-CoV specific antibodies. However, additional studies will be needed for further development as realistic candidatevaccine against MERS-CoV infections. In addition to neutralizing antibodies, Xu et al. reported that SARS-CoV-specific cytotoxic T lymphocyte responses were detected in patients following recovery from SARS (Xu and Gao, 2004). In conclusion, it was suggested that both humoral and cellular immune responses are important for clearance of SARS-CoV and survival of infection. Thus, as SARS-CoV is considered to be a close relative of MERS-CoV the requirement for inducing a strong T cell response may be also important for an efficacious MERS-CoV vaccine. It is encouraging that in previous studies the SARS-CoV S protein proved to be a suitable antigen for induction of strong T cell responses (Czub et al., 2005. Du et al., 2008) . Moreover, the MVA vector platform has been repeatedly shown to be highly suitable for the development of vaccines that induce T cell immunity against various infectious agents and cancer associated antigens (Drexler et al. 2004). Therefore, the future analysis of MERS-CoV specific cytotoxic T lymphocytes may reveal additional advantageous properties of this MVA vaccine candidate.

In MERS patients, MERS-CoV infection was reported to cause severe pneumonia with the replication of MERS-CoV being largely restricted to the lower respiratory tract. Thus, for optimized protection of lung tissue against infection and pathology it should be essential to stimulate robust mucosal immune responses to MERS-CoV. Therefore, further work must aim at the comparative evaluation of different vaccination protocols for capacity to elicit humoral and cellular immune responses at systemic and mucosal levels.

Furthermore, preclinical testing must include the assessment of the protective capacity of MVA-MERS-S immunization in animal models of MERS-CoV infection. However, it was reported that MERS-CoV cannot replicate in commonly used laboratory animals hampering the development of convenient infection models (de Wit et al., 2013; Coleman et al. 2014). DPP4 was identified as the surface receptor employed by the MERS coronavirus to gain entry into host cells including those of human origin. Interestingly, although mouse DPP4 is highly similar (92% identity) to the human DPP4, mouse DPP4 does not serve as a functional receptor for MERS-CoV infection

(Cockrell et al., 2014). Therefore, MERS-CoV fails to productively infect normal or immunodeficient mice including BALB/c, C57B/6, 129SvEv, or STAT1 knockout mice on the 129SvEv background. Recently, Zhao and coworkers described a novel approach to developing a mouse model for MERS-CoV infection. The transduction of mice with recombinant adenovirus allowed for the *in vivo* expression of the human DPP4 receptor, and, most excitingly, upon MERS-CoV infection these mice developed interstitial pneumonia (Zhao et al., 2014). This model provides encouragement for the development of transgenic mouse model with human DPP4 receptor.

VI. SUMMARY

About 10 years after the outbreak of SARS, Middle East respiratory syndrome coronavirus (MERS-CoV), a novel human coronavirus that caused outbreaks of a SARS-like illness in the Middle East, is now considered a threat to global public health. Since the first report, WHO had been informed of 189 laboratory-confirmed cases of infection with MERS-CoV including 82 deaths (a case fatality rate >40%).

In this study, we generated a MERS vaccine candidate by using the live attenuated modified vaccinia virus Ankara (MVA) as a vector. The full-length MERS-CoV spike (S) glycoprotein gene was introduced into the MVA genome (MVA-S). A second version containing a c-terminal epitope tag (influenza hemagglutinin; MVA-S_{HA}) was generated using the same technology. Growth kinetics of MVA and MVA-S in CEF, HeLa, and HaCaT cells showed that the newly generated recombinant MVA-S is replication incompetent in mammalian cells. Cells infected with MVA-S or MVA-S_{HA} synthesized a 200-kDa protein which was recognized by sera from MERS-CoV-infected macaques. Further immunoblot analysis of MVA-S_{HA} infected cells demonstrated a second protein of about 85 kDa. Further studies indicated that S was N-glycosylated and migrated in SDS polyacrylamide gels with an apparent mass of approximately 150 kDa and 55 kDa after treatment with peptide N-glycosidase F. The acquisition of resistance to endoglycosidase H indicated trafficking of S to the medial Golgi compartment.

Intramuscular inoculations of BALB/c mice with MVA-MERS-S produced high levels of serum antibodies neutralizing MERS-CoV. The specificity of the induced MVA-MERS-S antibodies for MERS-CoV was confirmed by the absence of detectable neutralization against SARS coronavirus (SARS-CoV). In addition, the MERS-CoV-neutralizing activity of these serum samples could be only partly blocked by preincubation with proteins that encompass the receptor binding domain of the S protein.

Overall, these results support the further development of MVA-MERS-S as candidate emergency vaccine against MERS-CoV.

VII. ZUSAMMENFASSUNG

Ein Jahrzehnt nach der SARS (severe acute respiratory syndrome)-Pandemie alarmiert ein neues humanes Coronavirus, das Middle East Respiratory Syndrom Coronavirus (MERS-CoV), die Gesundheitsbehörden. Die durch schwere respiratorische Symptomatik charakterisierte Krankheit wurde bisher in 189 Fällen von der WHO bestätigt. Bei 82 Patienten endete die Infektion tödlich.

Im Rahmen dieser Arbeit wurde ein möglicher Impfstoff gegen das MERS-CoV entwickelt, der auf dem Modifizierten Vaccinia virus Ankara (MVA) als Vektor basiert. Hierzu wurde das Gen für das Spike (S)-Protein des MERS-CoV in eine Deletionsstelle des MVA-Genoms inseriert (MVA-S). In einer zweiten Variante wurde das Spike-Protein um eine Tag-Sequence (Epitop des Influenzavirus-Hämagglutinin) ergänzt (MVA-S_{HA}). MVA-S oder MVA-S_{HA} infizierte Zellen synthetisierten ein ca. 200 kDa großes Protein, das im Immunoblot spezifisch mit Serum eines MERS-CoV-infizierten Makakenaffen reagierte. Weitere Untersuchungen mit dem MVA-S_{HA} zeigten ein zweites Protein von ca. 85 kDa Größe. Durch Behandlung mit Petide N-Glycosidase F reduzierte sich die Masse der nachgewiesenen Proteine auf ca. 150 und 55 kDA und bestätigte die Glykosilierung des S-Proteins. Die nachgewiesene Resistenz des Glycoproteins gegenüber der Endoglycosidase H deutet auf die Passage des Trans-Golgi-Netzwerkes hin. Die rekombinanten Viren MVA-S und MVA-S_{HA} vermehren sich in Hühnerembryofibroblasten nicht aber in humanen Zellen. Ein Befund der die biologische Sicherheit der rekombinanten MVA unterstreicht.

BALB/c Mäuse bildeten nach intramuskulärer Injektion mit MVA-S hohe Titer MERS-CoV neutralisierender Antikörper. SARSCoV wurde hingegen nicht neutralisiert. Die Präinkubation der Seren mit der Rezeptor-bindenden-Domäne des S-Proteins führte zur Reduktion der MERS-CoV-neutralisierenden Wirkung. Diese Ergebnisse bestätigten die Bedeutung der Rezeptor-bindenden-Domäne als Antigenstruktur und sprechen gleichzeitig für das Vorhandensein anderer Epitope für MERS-CoV neutralisierende Antikörper.

Das hier generierte und untersuchte rekombinante Virus MVA-MERS-S ist daher ein vielversprechender Impfstoffkandidat, der für weitere präklinische und klinische Untersuchungen zur Verfügung steht.

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IX. ABBREVIATIONS

ACE	Angiotensin-converting enzyme 2
ADA	Adenosine deaminase
AIDS	Acquired immune deficiency syndrome
ARDS	Acute respiratory distress syndrome
cDNA	complementary DNA
CEF	Chicken embryo fibroblast cells
CPE	Cytopathic effect
CVA	Chorioallantois vaccinia virus Ankara
DC	Dentritic cell
DNA	Desoxyribonucleic acid
DPP4	Dipeptidyl peptidase-4
E	Envelope
ELISA	Enzyme-linked immunosorbent assay
EMC	Erasmus Medical Centre
Endo H	Endoglycosidase H
HA	Hemagglutinin
HIV	Human immunodeficiency virus
ICTV	International Committee on Taxonomy of Viruses
IFN	Interferon
IFA	Indirect fluorescent assay
Ig	Immunoglobulin
kb	Kilobase
kDa	Kilodalton
KSA	Kingdom of Saudi Arabia
M	Membrane
MERS	Middle East respiratory syndrome
MERS-CoV	Middle East respiratory syndrome coronavirus
ml	Milliliter
MOI	Multiplicity of infection
mRNA	messenger ribonucleic acid
MVA	Modified vaccinia virus Ankara
N	Nucleocapsid
ORF	Open reading frame
PAGE	Polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PCR	Polymerase chain reaction
PFU	Plaque forming unit
PNGase F	Peptide-N-glycosidase F
RBD	Receptor binding domain
S	Spike protein
SARS	Severe acute respiratory syndrome
SARS-CoV	Severe acute respiratory syndrome coronavirus
SDS	Sodium dodecyl sulfate
TMPRRSS2	Type II transmembrane serine proteases
TNF	Tumor necrosis factor
UAE	United Arab Emirates
UK	United Kingdom
VACV	Vaccinia virus

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