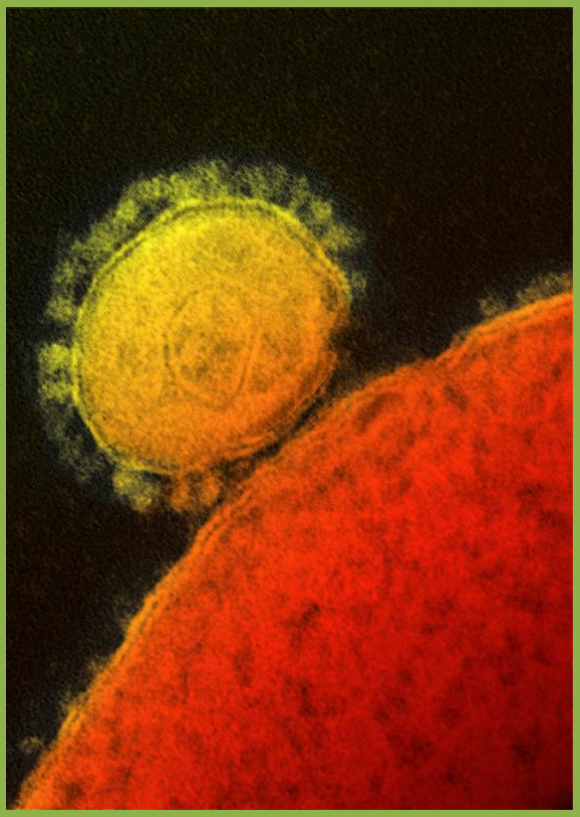
Middle East Respiratory Syndrome coronavirus (MERS-CoV): A Review



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Cover image: Electron microscope image of a MERS-CoV particle, available by the National Institute of Allergy and Infectious Diseases-Rocky Mountain Laboratories.

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Abstract

Ten years after the outbreak of Severe Acute Respiratory Syndrome coronavirus (SARS-CoV), which caused the first large-scale epidemic of the 21st century, a novel human coronavirus becomes identified, posing another threat to global public health. The virus, discovered in Saudi Arabia, in September 2012, causes infections with a clinical manifestation similar to that of SARS-CoV, although its transmissibility among humans seems to be lower. Within a year, 130 cases have been reported in Europe, Africa and Asia, 58 of which were fatal. Due to the fact that the majority of these cases appeared to be linked with the Middle East, the virus was named Middle East Respiratory Syndrome coronavirus (MERS-CoV). Although certain aspects related to this novel virus have already been unraveled, our knowledge of its source, pathogenesis and ways of transmission remains limited. This thesis summarizes the literature that is currently available on MERS-CoV and discusses ways to combat its spread and prevent another epidemic.

Introduction

Coronaviruses are large enveloped viruses with a single-stranded positive-sense RNA genome. They can infect humans, as well as a variety of animals, such as bats, mice, birds, dogs, pigs, and cattle, causing mainly respiratory and enteric diseases[1]. Before the 21st century, it was believed that human coronaviruses, represented by the viruses hCoV-OC43 and hCoV-229E, can only cause mild respiratory symptoms –known as the common cold[2]. This notion changed after the outbreak of the severe acute respiratory syndrome (SARS) in 2002-2003, when a previously unknown human coronavirus, named severe acute respiratory syndrome coronavirus (SARS-CoV), caused the first coronavirus-associated human epidemic, infecting approximately 8000 and killing 774 people[3]. In the years that followed, two additional human coronaviruses were discovered, namely hCoV-NL63 and hCoV-HKU1[4,5]. All known human coronaviruses are believed to have a zoonotic origin, with bats playing a major role in the interspecies transmission[6]. Today, ten years after the SARS outbreak, a novel human coronavirus has come to light, posing the threat of another epidemic. This thesis presents the current literature on several aspects related to this virus and discusses measures that need to be taken for the prevention of an outbreak.

On June 13, 2012, a 60-year-old man from Jeddah, Saudi Arabia, was hospitalized presenting fever, cough, expectoration, and shortness of breath. The patient developed acute pneumonia and renal failure and died after 11 days of hospitalization. When the patient's sputum sample was used to inoculate cells *in vitro*, cytopathic effect (CPE) was observed, suggesting viral replication. The pan-coronavirus real-time reverse transcription polymerase chain reaction (RT-PCR) assay[7] yielded the expected size of the PCR fragments, revealing that the patient had been infected by a coronavirus. Further analysis and comparison to other known coronaviruses revealed that this was a novel coronavirus[8]. On September 20, 2012, the discovery of this novel coronavirus was reported on the Program for Monitoring Emerging Diseases (ProMEDmail) by Dr. Ali Moh Zaki, the virologist that first isolated the virus[9].

On September 11, 2012, a 49-year-old man from Qatar, with a history of travel to Saudi Arabia, was transferred to the United Kingdom with symptoms of severe respiratory illness. Lower respiratory tract samples of the patient were found positive after a pan-coronavirus RT-PCR assay. Comparison of the sequence of the PCR fragments with the ones obtained in the case of the Saudi patient revealed that they share 99% similarity, suggesting infection by the same virus[10].

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, where the virus was named "human coronavirus EMC" (hCoV-EMC)[11]. In May 2013, the Coronavirus Study Group of the International Committee on Taxonomy of Viruses renamed the virus "Middle East respiratory syndrome coronavirus" (MERS-CoV)[12].

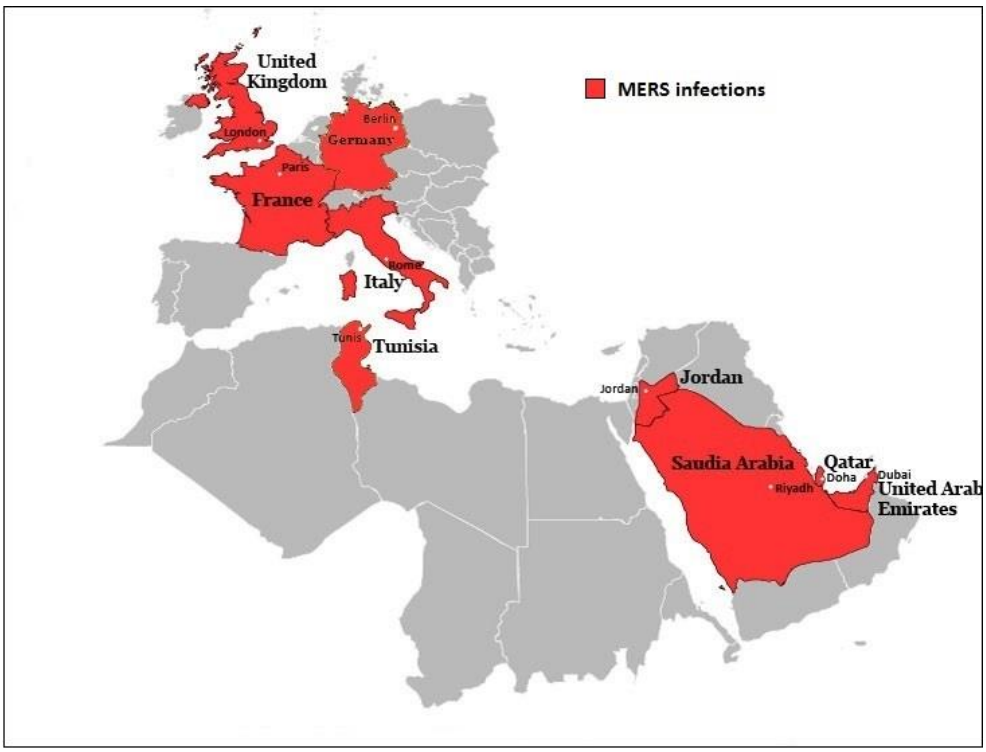

Figure 1. A map of the countries where laboratory-confirmed cases of MERS-CoV infection have been reported (countries highlighted with red) (adapted from http://www.uq.edu.au/vdu/VDUMERSCoronavirus.htm).

As of September 20, 2013, 130 laboratory-confirmed cases of MERS-CoV infection have been reported to the World Health Organization (WHO), among which 58 fatal[13]. So far, infections have occurred in several countries, including Saudi Arabia, Qatar, Jordan, the United Arab Emirates, the United Kingdom, France, Germany, Italy and Tunisia[13] (**Fig. 1**). All infections are linked to the Middle East, since the described cases either traveled or had been in close contact with people that recently traveled to that region[13]. The common symptoms of a MERS-CoV infection include fever, cough, shortness of breath, acute pneumonia and acute renal failure[14].

Virus Classification

Coronaviruses form the subfamily Coronavirinae within the family Coronaviridae of the order Nidovirales. Based on genome sequence analysis, the International Committee on Taxonomy of Viruses (ICTV) has divided the family Coronaviridae into four genera, named *Alpha*-,

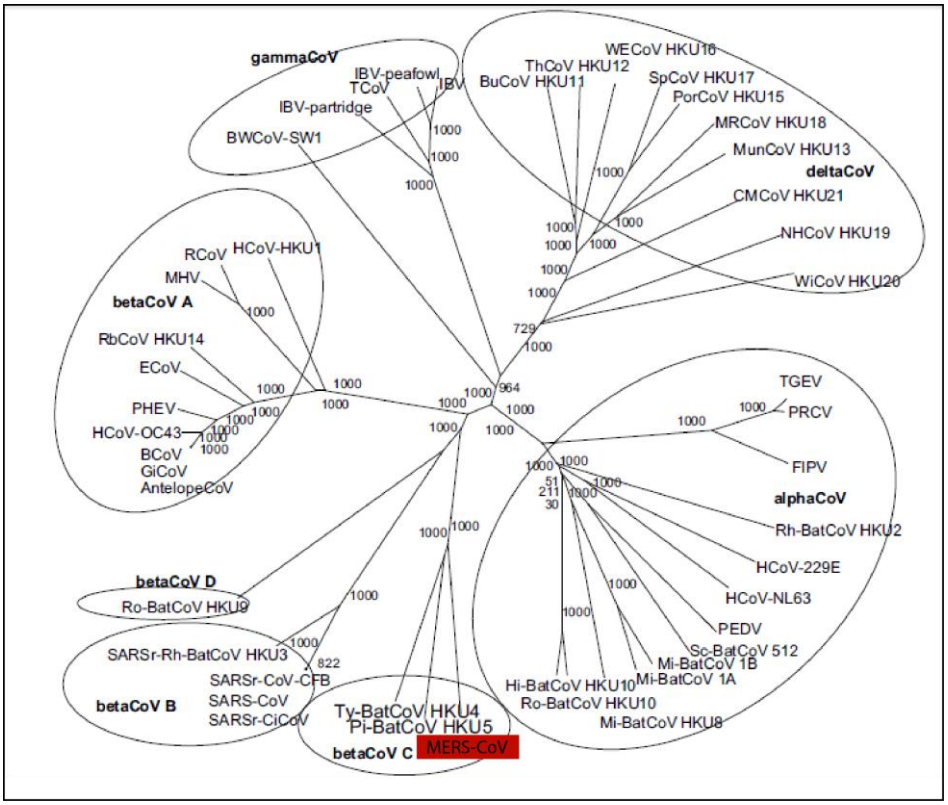

Figure 2. Phylogenetic tree of MERS-CoV and other coronaviruses (adapted from Chan *et al.*[15]) ALCCoV: Asian leopard cat coronavirus, AntelopeCoV: sable antelope coronavirus, BCoV: bovine coronavirus, BuCoV HKU11: bulbul coronavirus HKU11, BWCoV-SW1: beluga whale coronavirus SW1, CCoV: canine coronavirus, CMCoV HKU21: common moorhen coronavirus HKU21, ECoV: equine coronavirus, FIPV: feline infectious peritonitis virus, GiCoV: giraffe coronavirus, HCoV-229E: human coronavirus 229E, HCoV-HKU1: human coronavirus HKU1, HCoV-NL63: human coronavirus NL63, HCoV-OC43: human coronavirus OC43, Hi-BatCoV HKU10: Hipposideros bat coronavirus HKU10, IBV: infectious bronchitis virus, IBV-partridge: partridge coronavirus, IBV-peafowl: peafowl coronavirus, MERS-CoV: Middle East respiratory syndrome coronavirus, MHV: murine hepatitis virus, Mi-BatCoV 1A: Miniopterus bat coronavirus 1A, Mi-BatCoV 1B: Miniopterus bat coronavirus 1B, Mi-BatCoV HKU8: Miniopterus bat coronavirus HKU8, MRCoV HKU18: magpie robin coronavirus HKU18, MunCoV HKU13: munia coronavirus HKU13, NHCoV HKU19: night heron coronavirus HKU19, PEDV: porcine epidemic diarrhoea virus, PHEV: porcine haemagglutinating encephalomyelitis virus, Pi-BatCoV-HKU5: Pipistrellus bat coronavirus HKU5, PorCoV HKU15: porcine coronavirus HKU15, PRCV: porcine respiratory coronavirus, RbCoV HKU14: rabbit coronavirus HKU14, RCoV: rat coronavirus, Rh-BatCoV HKU2: Rhinolophus bat coronavirus HKU2, Ro-BatCoV-HKU9: Rousettus bat coronavirus HKU9, Ro-BatCoV HKU10: Rousettus bat coronavirus HKU10, SARS CoV: SARS-related human coronavirus, SARSr-CiCoV: SARS-related palm civet coronavirus, SARSr CoV CFB: SARS-related Chinese ferret badger coronavirus, SARSr-Rh-BatCoV HKU3: SARS-related Rhinolophus bat coronavirus HKU3, Sc-BatCoV 512: Scotophilus bat coronavirus 512, SpCoV HKU17: sparrow coronavirus HKU17, TCoV: turkey coronavirus, TGEV: transmissible gastroenteritis virus, ThCoV HKU12: thrush coronavirus HKU12, Ty-BatCoV-HKU4: Tylonycteris bat coronavirus HKU4, WECoV HKU16: white-eye coronavirus HKU16, WiCoV HKU20: wigeon coronavirus HKU20.

Beta-, *Gamma- and Deltacoronavirus.* The genus *Betacoronavirus* contains four different lineages, A, B, C and D (**Fig.2**). The human coronaviruses hCoV-229 and hCoV-NL63 belong to the genus *Alphacoronavirus*, while hCoV-OC43 and hCoV-HKU1 belong to the lineage A of the genus *Betacoronavirus*. SARS-CoV belongs to lineage B of the same genus. The genera *Gamma-* and *Deltacoronavirus* contain only viruses that infect animals[16].

Phylogenetic analysis performed by Zaki *et al.*[8] after the isolation of MERS-CoV from the Saudi patient suggested that the virus belongs to the lineage C of the *Betacoronavirus* genus, together with the bat coronaviruses BtCoV-HKU4 and BtCoV-HKU5, which have been isolated from the species *Tylonycteris pachypus* and *Pipistrellus abramus*, respectively[17]. As stated by the ICTV, viruses that present a >90% sequence identity in their replicase domains belong to the same species[16]. To investigate whether the newly identified virus is the prototype of a novel virus species, the amino acid sequence of the replicase gene –obtained by sequencing of the PCR fragments that the pan-coronavirus PCR yielded- was aligned with the respective sequences of its closest relatives, BtCoV-HKU4 and BtCoV-HKU5. The comparison showed that the identity the viruses shared was less than 80%, suggesting that the discovered virus represents a novel *Betacoronavirus* species, the first human coronavirus described in lineage C of this genus. These results were repeated by the group of Ron Fouchier, after they obtained the complete genome sequence of the virus[11].

Genome Analysis

The sequencing of MERS-CoV genome revealed its organization and expression strategy. The genome contains 30119 nucleotides and at least ten open reading frames (ORFs) (**Fig. 3**). Downstream of the 5'-UTR, there are two big ORFs, ORF1a and ORF1b, responsible for the production of the polyproteins pp1a and pp1ab, which occurs via ribosomal frame shifting at the junction between the two ORFs. Following ORF1b, ORFs encoding for the spike (S), envelope (E), membrane (M) and nucleocapsid (N) structural proteins are located in the genome, followed by the 3'-UTR. Between the S and E genes, there are four small ORFs, named 3a, 3b, 3c, 3d, encoding for non-structural proteins[11]. This gene order was found to be similar to that of the bat coronaviruses BatCoV-HKU4 and BatCoV-HKU5, highlighting once more the similarity among the three viruses[14]. Furthermore, the discovery of the viral genome organization provided useful information for the development of diagnostics that are based on viral RNA detection[18,19].

Cell-virus Interplay

The interaction between coronaviruses and the host cell is mediated by the binding of the viral spike (S) protein to specific cell receptors of the host. This step is a major determinant of the viral host and tissue tropism[20]. So far, two receptors have been identified for human coronaviruses: CD13, used by hCoV-229E[21] and ACE2, used by hCoV-NL63 and SARS-CoV[22,23]. Soon after the first cases of MERS-CoV were reported, scientists attempted to identify the cell receptor that is utilized by this novel virus. Müller *et al.*[24] performed a series of experiments to

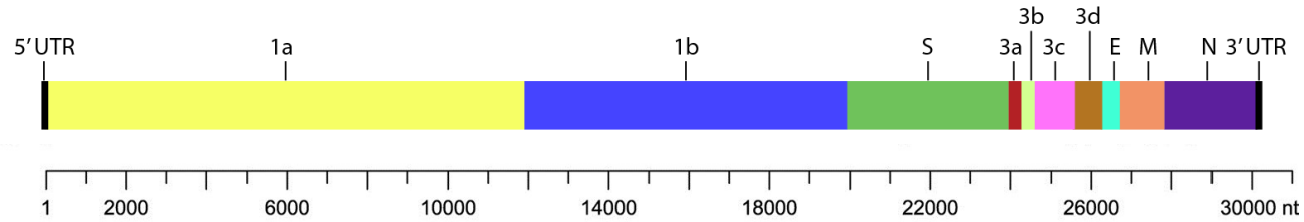

Figure 3. Genome organization of MERS-CoV. The ten open reading frames (ORFs) and the 5' UTR and 3' UTR are labeled.

investigate whether MERS-CoV uses the same receptor as SARS-CoV, i.e. ACE2, for cell entry. The group generated a baby hamster kidney cell line expressing human ACE2 (hACE2). As expected, SARS-CoV successfully replicated in these cells. However, this was not the case for MERS-CoV, suggesting that the presence of human ACE2 is not sufficient for the cell entry of the novel virus. In a subsequent experiment using monkey, human and swine kidney cells, both SARS-CoV and MERS-CoV displayed successful replication. However, the use of anti-hACE2 antibodies blocked only the infection by SARS-CoV and not MERS-CoV. The same was observed after pre-incubation of each virus with a soluble form of the receptor. Taken together, these results led to the conclusion that MERS-CoV does not require the same receptor as SARS-CoV for cell entry. Similar results were obtained by Gierer *et al.*[25], who showed that neither the two known receptors for human coronaviruses (ACE2 and CD13), nor the murine receptor CEACAM-1, used by the murine hepatitis virus (MHV), facilitates the entry of MERS-CoV, by overexpressing these receptors in a human embryonic kidney cell line and evaluating the effect on the viral entry.

The receptor in question was eventually found to be the dipeptidyl peptidase 4 (DPP4), an exopeptidase also known as CD26. Raj *et al.*[26], using monkey kidney and human liver cell lines, identified DPP4 as a cell surface protein that bound to MERS-CoV S protein during affinity assays. This binding was also observed with a soluble form of DPP4, but not ACE2. Furthermore, soluble

DPP4 was able to inhibit the infection of the monkey kidney cells by MERS-CoV and expression of this receptor in otherwise non-susceptible cells induced binding of MERS-CoV S protein to the cell surface. DPP4 is 766aa-long type II transmembrane glycoprotein, forming dimers on the cell surface. It appears to have multiple functions, depending on the cell type in which it is expressed. It has a role in the immune response, glucose homeostasis, cell adhesion and apoptosis[27,28]. The fact that MERS-CoV has shown high infectivity in cell lines with undetectable expression of the DPP4 receptor implies that the infectivity of the virus might not be directly associated with the expression levels of DPP4 or even that MERS-CoV might utilize alternative receptors, additionally to DPP4, for cell entry[29].

Apart from the cell receptor, scientific interest was also gained by the S protein of MERS-CoV, since it is the one that mediates the binding of the virus to the host receptor and the membrane fusion, the two essential steps of the viral entry[20]. The S protein of MERS-CoV is a 1353aa-long type I membrane glycoprotein. It is present in trimmers that form the peplomers (or spikes) on the surface of the virus. The N-terminal part of the protein, named S1 (residues 1-751), is responsible for the first step of the viral entry, i.e. the binding to the host receptor, while the C-terminal part, named S2 (residues 752-1353), is responsible for the second step, i.e. the membrane fusion. In all coronaviruses, the binding to the host receptor is mediated by a ~150-300aa-long receptor binding domain (RBD), located in the S1 subunit[20,30].

In order to identify the RBD of MERS-CoV on the S1 subunit, Mou *et al.*[30], fused different S1 truncations with the Fc-region of human IgG and tested their ability to interact with a soluble form of DPP4 (sDPP4) in a co-purification assay. The truncation containing the residues 358-588 of S1 successfully bound to sDPP4, suggesting that the RBD of MERS-CoV is located within these residues. This region is homologous to the RBD of SARS-CoV[31]. The same truncation was able to bind to human embryonic kidney cells expressing DPP4, with similar efficiency to that of the full-length S1 protein. In a follow-up experiment, cells of a human liver cell line were pre-incubated with the S1 truncations, in order to test their ability to block MERS-CoV infection. The 358-588 variant was able to inhibit the infection, further supporting the notion that the MERS-CoV RBD is located within these residues. In agreement with these results, Du *et al.*[32] mapped the MERS-CoV RBD within the residues 377-662 of the S1 subunit. The group had previously predicted that the RBD domain lies within these residues[31]. To prove their hypothesis, they fused this region with the Fc-region of human IgG and confirmed its binding specificity to the DPP4 receptor via a series

of experiments, similar to those performed by Mou *et al.*[30]. A third study showed that the RBD of MERS-CoV is located within the residues 367-588, in concordance with the other two studies[33].

Several research groups attempted to unravel the crystal structure of the virus-receptor binding interface. Jiang *et al.*[31] presented a predicted crystal structure of the MERS-CoV RBD domain, based on the structure of the respective domain of SARS-CoV, using the Swiss-Model Workplace homology modeling server. Subsequently, the group suggested putative conformations of this domain in complex with the DPP4 receptor, the structure of which is already known[34,35]. Few months later, three independent groups published their data after successful crystallization of the RBD, two of which in a complex with DPP4[33,36,37]. The structures proposed by all three studies were similar. The results suggest that, like SARS-CoV, the MERS-CoV RBD is composed of a core subdomain and a long extended loop[38] (**Fig. 4**). The core subdomain consists of five antiparallel β-sheets and several short α-helices, while the loop contains four

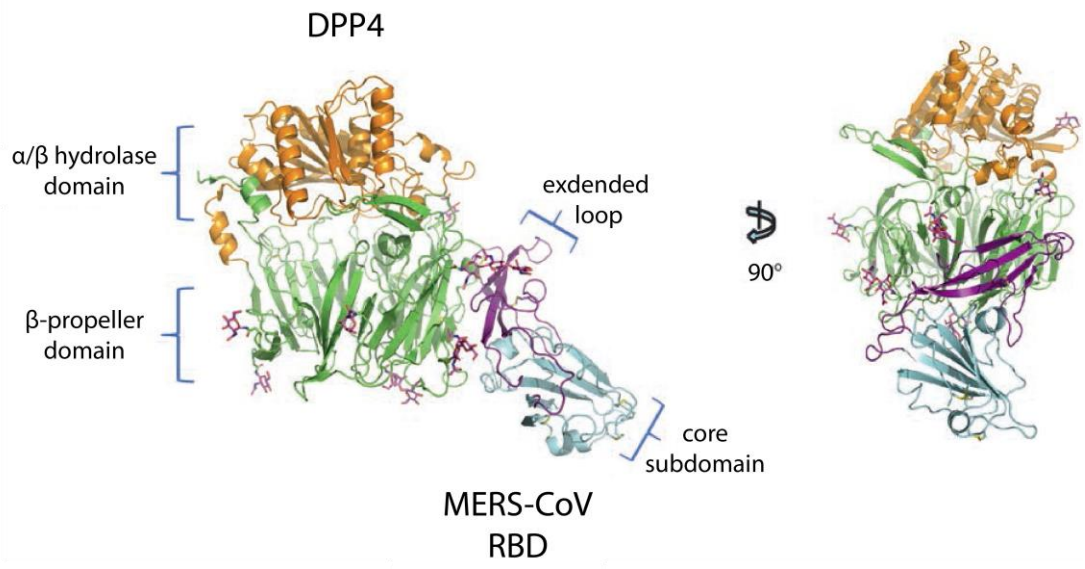

Figure 4. Crustal structure of the MERS-CoV RBD-DPP4 receptor complex. The RBD consists of a core subdomain (light blue) and a long extended loop (purple). The extracellular domain of the DPP4 receptor contains a β-propeller domain (green) and an α/β hydrolase domain (orange). The RBD-receptor contact is mediated via the extended loop of the RBD and the β-propeller domain of the receptor (adapted from Wang *et al.*[36]).

antiparallel β-sheets. The receptor binding motif (RBM), i.e. the part of the RDB that mediates its contact with the virus-binding site of the DPP4 receptor, was predicted to be part of the extended loop. While the core subdomain seems to be conserved between MERS-CoV and SARS-CoV, the loop region is highly variable, with the one of MERS-CoV being much longer. This can

explain the fact that the two viruses use different host receptors for cell entry[24]. The extracellular domain of the DPP4 receptor contains a β-propeller domain –comprising of eight blades– and an α/β hydrolase domain[35] (**Fig. 4**). The receptor contacts the MERS-CoV RBM utilizing blades 4 and 5. Therefore, the contact site is far from the hydrolase domain, implying that the peptidase activity is independent of the virus-receptor interaction. This is in agreement with the findings of Raj *et al.*[26], who showed that DPP4 inhibitors do not affect the MERS-CoV entry[36].

Defining the major components that mediate the interaction between the virus and the host cell –here the MERS-CoV RBD and the DPP4 receptor– sets the basis for a series of follow-up investigations. Most importantly, it provides the information needed in order to propose drug candidates that would target the virus-cell interaction and could be potentially used for anti-viral intervention. Additionally, knowing the receptor that MERS-CoV utilizes for cell entry, it is possible to predict the animal reservoir of the virus, by evaluating its ability to infect organisms with orthologous receptors.

Virus origin and Natural reservoir

The fact that the reported MERS-CoV infections appear to be sporadic and epidemiologically unlinked suggests that MERS-CoV is a zoonotic virus, in concordance with what has been suggested for the rest of the known human coronaviruses[6]. The phylogenetic proximity of the virus with the bat coronaviruses further supports this suggestion. Additionally, contact with animals before the symptom onset has been reported for some of the identified cases[39,40]. Coronaviruses have a potential ability of interspecies transmission, via adaptation of their S protein to receptors of other species. Such adaptation is facilitated by several factors that induce the characteristic diversity of coronaviruses, including the infidelity of the RNA-dependent RNA polymerase (RdRp) and the large size of the viral genomes, which allow for high rates of RNA recombination[16]. Adaptation to new hosts can have dramatic consequences, as exemplified by the SARS outbreak. It is believed that the SARS-CoV existed in horseshoe bats, which served as a natural reservoir and it was eventually transmitted to humans, using civets as intermediate hosts[3,41].

When the first phylogenetic analyses were performed and the high similarity of MERS-CoV with BtCoV-HKU4 and BtCoV-HKU5 was revealed, it was assumed that bats could be the source of infection, especially since the Arabic peninsula harbors a big number of bat species[8]. This was further supported by the observation that MERS-CoV was able to infect cells isolated

from a series of bat families in *in vitro* cultures[24]. According to the obtained phylogenetic trees, MERS-CoV seems to share common ancestries with BtCoV-HKU4 and BtCoV-HKU5[8,11,42]. A study that performed phylogenetic analysis in an attempt to identify the precursors of MERS-CoV, suggested a European Vespertilionidae bat ancestry for this virus, based on the observation that MERS-CoV would always cluster with viruses identified in bats of this family[42] (of note, the *Tylonycteris* and *Pipistrellus* genera, where BtCoV-HKU4 and BtCoV-HKU5 were identified, respectively, also belong to the Vespertilionidae family). Although these results support thr notion that MERS-CoV have originates from bat viruses, they do not prove that bats are the source of infection. Moreover, direct contact between humans and bats is rather unusual and no such contact has been reported by any of the MERS patients[39]. Therefore, without ruling out that bats can be part of the natural reservoir, it is possible that the virus has a wider host tropism, existing in additional animal species that serve as intermediate hosts, from which it has jumped to humans -in a way similar to SARS-CoV. Supporting this suggestion, molecular clock analysis has shown that BtCoV-HKU4 and BtCoV-HKU5 are unlikely the direct ancestors of MERS-CoV, since MERS-CoV seems to have diverged from the two bat viruses centuries ago[43]. Furthermore, the DPP4 residues responsible for contacting the RBD of MERS-CoV display high conservation among different species, including macaque, pig and rabbit, implying that MERS-CoV is able of infecting multiple organisms[36,44]. This was subsequently proven by susceptibility studies performed by two independent groups, which showed that, apart from bat cells, MERS-CoV can also infect primate and porcine cell lines *in vitro*[24,45]. The most recent of these studies demonstrated that the virus can additionally infect cell lines obtained from civets and rabbits[45].

Based on the above observations, scientists are currently in search of a possible intermediate host that can be the direct source of MERS-CoV infections. Although the cell line susceptibility studies pointed out animal species that could potentially serve as intermediate hosts, they did not provide evidence. For some of the MERS cases, contact with farm animals or camels had been reported[39,40]. In a recent study, scientists tested the sera of animals from the Middle East and other regions (Spain, Netherlands, Chile) for the presence of antibodies against the S protein of MERS-CoV, followed by neutralization studies. Sera were isolated from sheep, cattle, goats, camels and other camelid species. Interestingly, it was found that 100% of the camels from the Middle East were positive for such antibodies (with quite high antibody titers), while this was the case for only 14% of the Spanish camels. No anti-MERS-CoV antibodies were found in any of the other tested animals[46]. Although the presence of these antibodies increases

the chances that camels are the intermediate host, further investigation is required, in order to find out whether the virus that infected those camels is the same as the one that infects humans and not just a MERS-CoV-like virus. The research group claimed that they did not try to isolate and sequence the virus from the camels, because they believe it is rather unlikely to be present in animals with so high antibody titers[47].

Tissue tropism & Pathology

Susceptibility studies testing the ability of MERS-CoV to infect cell lines derived from different organs provided indications about the tissue tropism of the virus. MERS-CoV was found to infect cells of the human respiratory tract, kidney, intestine and liver[25,26,45,48–50]. Of note, the above tissues are among the tissues where the DPP4 receptor is primarily expressed[26–28]. More specifically, the target of MERS-CoV in the respiratory tract was the non-ciliated cells of the epithelium -in contrast to the majority of the other respiratory viruses, which mainly infect the ciliated cells-, and the type II pneumocytes of the lung tissue[26,49,50]. Additionally, it was revealed that MERS-CoV replicated successfully in cells of the lower respiratory tract, while cells from the upper respiratory tract did not support viral replication[45]. The suggested tropism of MERS-CoV for cells of the respiratory tract, kidney and intestine is correlated with the detection of the virus in respiratory swabs, tracheal aspirates, sputum, urine and stool of MERS patients[8,10,40,51]. This broad range of human tissue tropism displayed by MERS-CoV (broader than that of all the other human coronaviruses) could possibly explain the high mortality of the infected patients[45].

Apart from the tissue tropism, the studies that performed *in vitro* infection of human cell lines with MERS-CoV evaluated the pathological effect of the infection at the cellular level. Cytopathic effect (CPE) was observed after inoculation of several cell lines, manifested as syncytia formation, cell rounding and detachment, as revealed by light microscopy[45,52]. Additionally, there was formation of membranous structures that support viral RNA synthesis, such as double-membrane vesicles (DMVs) and convoluted membranes (CMs)[52]. Two independent studies reported extensive apoptosis, based on detection of characteristic apoptotic markers, including nuclear margination, chromatin condensation, formation of apoptotic bodies and, finally, positive staining for caspase-3, as revealed by immunohistochemistry[49,53]. This latter finding suggests that the prominent mechanism of the observed CPE is caspase-dependent apoptosis. One of the two studies performed additional staining for viral antigens, which revealed that apoptosis occurred in cells other than the ones

directly infected by the MERS-CoV, suggesting that apoptosis was probably induced via paracrine mechanisms[49].

Additional studies that performed transcriptomic analysis in human lung epithelial cells infected by MERS-CoV revealed that, unlike SARS-CoV, MERS-CoV infection did not induce inflammatory cytokine or type I and III interferon (IFN) responses[48,49,50]. Furthermore, genes within the antigen presentation pathway, such as type I and II major histocompatibility complex (MHC) genes, were down-regulated upon infection of the same type of cells[54]. Another category of genes that underwent down-regulation were the ones related to metabolism[54]. Genes that were up-regulated were mostly related to viral recognition[54].

The findings of the above studies can be linked to some of the clinical manifestations of MERS-CoV infections. The ability of the virus to infect cells of the respiratory tract *in vitro* is in agreement with the occurrence of severe pneumonia, one of the most common symptoms among MERS patients[14]. The fact that the virus was found to infect type II pneumocytes is consistent with the observed severe lung pathology, since these cells play a crucial role in the regeneration of the alveolar epithelium after injury caused by infection[49]. Furthermore, the development of rapid clinical deterioration with lower respiratory tract involvement that has been reported for a number of patients is correlated to the *in vitro* findings of Chan *et al.*[45], who showed that MERS-CoV replicated only in cell lines derived from the lower respiratory tract. Another dominant clinical feature of MERS-CoV infections is acute renal failure[14] and it is in agreement with the ability of MERS-CoV to infect human kidney cell lines *in vitro*. Respectively, the *in vitro* infectivity of MERS-CoV in cells of the intestine is linked to the gastrointestinal symptoms, such as anorexia, abdominal pain and diarrhea, that have been reported for a small number of patients[55]. Despite the significant *in vitro* infection of liver cells by MERS-CoV, no hepatitis has been reported for any of the cases –except of the first case, where elevated levels of hepatic parenchymal enzymes were detected[8]–, although this might be due to under-reporting[45]. The induction of CPE as early as the first day of *in vitro* MERS-CoV infection of human cell lines, which is much earlier than in the case of SARS-CoV, could possibly explain the more severe symptoms and the higher fatality rate observed in the MERS-CoV infections[45]. The inability of MERS-CoV to induce cytokine or type I and III interferon (IFN) responses in human lung epithelial cells suggests that the bronchial epithelium is unable of inducing a strong innate immune response upon infection. However, in the case of SARS-CoV, viral infection induced cytokine production by other cell types that are responsible for innate immunity in the lung, such

as dendritic cells or macrophages[56]. Therefore, the effect of MERS-CoV infection in these cell types should also be evaluated, before final conclusions are drawn[57] (successful replication of MERS-CoV in human macrophages *in vitro* has already been reported[25]). Finally, the down-regulation of genes related to the antigen presentation pathway suggests that, apart from the innate immune response, MERS-CoV infection has an inhibitory effect on the adaptive immune response as well, which could explain its virulence.

In the study of Renee *et al.*[49] immunohistochemistry of MERS-CoV-infected *ex vivo* lung tissue cultures revealed the presence of the virus in endothelial cells within interstitial blood vessels of the lung, suggesting that the virus might spread systematically and infect several organs[49]. This could explain the complicated clinical image of the MERS-CoV infections that implies the impairment of multiple tissues. This is in agreement with the findings of Guery *et al.*[51], who suggest that MERS-CoV is present in blood. A possible scenario could be that the infection starts from the lungs and it is later spread -via blood- to the kidneys and the intestine, based on the observation that almost all reported patients developed acute pneumonia, while fewer developed renal or intestinal impairment. However, although the virus has been detected in urine and stool, it remains to be confirmed that it causes direct infection of the renal or intestinal tissue and that the observed impairment is not a secondary symptoms. Performing autopsies and collecting samples from several organs would probably give an answer to this question. It should be noted that blood-mediated viral dissemination would imply infection of the blood cells, given that the DPP4 receptor is expressed on immune cells, including T-cell lymphocytes[28]. In support of the above speculation, hematological abnormalities, such as lymphocytopenia, thrombocytopenia and neutropenia have been reported in some of the MERS cases[55] –although these might be the result of an indirect effect.

Interhuman transmission

While the introduction of MERS-CoV to the human species from an animal reservoir seems to be the reason for the initial infections, the occurrence of clusters suggests that the virus has adapted to human-to-human transmission. It seems that the first cluster occurred already before the discovery of the virus, in a hospital in Jordan, in April 2012[58]. Eleven people, among which eight health care workers, developed severe pneumonia, which, until the discovery of MERS-CoV, remained of unknown etiology. Two of the patients died. Stored samples of all patients were tested for the presence of MERS-CoV upon its discovery and the ones of the two

deceased patients were found positive. Additional clusters were reported in the Middle East, either within hospitals or among family members[39]. However, as long as the clusters kept occurring in that region, it could not be excluded that the cluster patients happened to be exposed to a common source, present in the Middle East. Stronger evidence for human-to-human transmission was provided by the occurrence of a family cluster in the United Kingdom, in the beginning of 2013. The index case had returned from Pakistan and Saudi Arabia 10 days before the onset of the symptoms, however, the other two cases had no history of travel to the Middle East and admitted contact with the index case[59]. Similar clusters were reported in the months that followed, further supporting the ability of the virus to transmit among humans[39,48,57,61].

Generally, coronaviruses are transmitted among humans via aerosol droplets and/or through direct contact with other secretions (stool, urine etc.)[62]. Currently, the pathways used by MERS-CoV for interhuman transmission remain unknown. Several case investigations have suggested that airborne transmission seems to be the most likely route[51,63,64]. Furthermore, given the high concentration of the virus in the lower respiratory tract of infected patients, airway suctions or use of bronchoscopes could also serve as a source of transmission[65]. The infectiousness of urine and stool is currently under investigation, since the virus has been detected in urine and stool samples of patients and based on the fact that cluster patients had been sharing toilet rooms during hospitalization. Transmission via blood should also be considered a possible route, since scientists claim that the virus might be present in blood[51]. This could be correlated to the reported person-to-person transmission in hemodialysis units of a hospital in Saudi Arabia[66].

In order to assess the transmissibility of the virus, a recent study estimated the basic reproduction number (R_0), which represents the number of secondary cases per index case in a fully susceptible population[67]. 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 a pandemic potential yet. Nevertheless, scientists worldwide remain alerted, since possible mutations might increase the transmissibility of the virus and lead to an outbreak.

Current diagnostics

The detection of MERS-CoV in the first reported case was performed by a pan-coronavirus RT-PCR assay[8]. This assay targets the gene of the RNA-dependent RNA polymerase

(RdRp) of coronaviruses, encoded within ORF1b[11], and it is used for the detection of all coronaviruses, known and unknown[7,]. However, for detection of MERS-CoV in particular, alternative RT-PCR assays are required, detecting certain targets that have been described to be specific for the virus.

As early as after the reporting of the first two cases of MERS-CoV infection, Corman *et al*.[18] proposed two RT-PCR assays for the detection of the virus, each one targeting different parts of the viral genome. The first assay targets a region upstream of the envelope (E) gene (upE assay), while the second assay targets part of ORF1b (ORF1b assay), which does not overlap with the target of pan-coronavirus assay. The upE assay was found to be more sensitive (3.4 RNA copies per reaction) in comparison to the ORF1b assay (64 RNA copies per reaction). This could be because fragments of subgenomic RNA, including the target of the upE assay, are released upon cell damage, according to *in vitro* experiments[18]. Thus, the use of the upE assay is recommended for screening, while the ORF1b assay can be used for confirmation. The specificity of both assays was confirmed by excluding cross-reactivity with the other known human coronaviruses. A third assay, optimized for sensitivity, was described by the same group, this time targeting ORF1a[19]. Overall, a combination of the upE and ORF1a assay seems to be the optimal approach for MERS-CoV detection.

The selection of the appropriate clinical specimens that will be tested for the presence of the virus is of high importance. Successful replication of MERS-CoV preferably in cell lines derived from the lower respiratory tract and the clinical image of the MERS-CoV patients that indicates lower respiratory tract involvement suggest that specimens obtained from this area should be collected[15,45]. Such specimens are sputum, bronchoalveolar lavage, endotracheal aspirate and lung tissue[15]. This speculation was further supported by the fact that nasopharyngeal swabs of suspected MERS-CoV patients were found negative in an RT-PCR assay, while lower respiratory tract specimens obtained from the same patients were positive[51,68].

An alternative diagnostic approach is the detection of an antibody response against MERS-CoV, by immunofluorescence microscopy. A relevant protocol has been proposed by Corman *et al.*[19] and it is based on the fact that putative anti-MERS-CoV antibodies in convalescent patient serum would recognize and bind to viral antigens inside MERS-CoV-infected cell lines. However, the specificity of this assay is questionable, since antibodies against Betacoronaviruses are known to cross-react within the genus[19]. This was further demonstrated by Chan *et al.*[69], who suggested the presence of cross-reactive neutralizing antibodies against

MERS-CoV in the serum of convalescent SARS patients. Another disadvantage of this assay is that it allows viral detection only at the convalescent and not the acute phase[39]. Therefore, serological testing should preferably be used to complement RT-PCR findings and not independently.

Drug candidates for MERS therapy

So far, there are no effective anti-viral agents against infections by human coronaviruses, including SARS-CoV[15]. Therefore, the current clinical management of MERS-CoV infections is primarily supportive, focusing on organ support for respiratory and renal failure, the two main clinical manifestations of the infection[15]. In the case of acute respiratory failure, use of extracorporeal membrane oxygenation (ECMO) has been shown to significantly reduce mortality rates[15,70], while for renal failure, continuous veno-venous hemofiltration is commonly used in intensive care units[15]. Use of broad-spectrum antibacterial and antiviral agents, such as a regimen consisting of a b-lactam, a macrolide and an antiviral against influenza, has also been recommended[15].

In order to identify potential therapeutic agents against MERS-CoV infection, a plethora of studies have evaluated the effect of several compounds on the viral replication (**Table 1**). Many of these studies investigated the anti-viral effect of interferon (INF), based on the fact that MERS-CoV infection does not induce interferon responses in human cell lines[48,49,50]. Treatment with interferon has also been suggested as a promising therapeutic strategy against SARS-CoV infections[71]. Kindler *et al.*[50] showed a beneficial effect by INF-α and INF-λ3, based on the observation that pretreatment with any of the two compounds reduced MERS-CoV replication in pseudo-stratified human airway epithelium (HAE) cultures, obtained from three different donors. The effect of pegylated IFN-α (PEG-IFN) treatment on MERS-CoV (and SARS-CoV) replication was investigated by de Wilde *et al.*[52], where it was shown that addition of PEG-INF inhibited MERS-CoV-induced CPE and reduced the viral RNA levels, in human lung epithelial and monkey kidney cell lines. Interestingly, the sensitivity of MERS-CoV to the treatment was much higher than that of SARS-CoV. In a different study, addition of INF-β was found to reduce the viral titers in human lung epithelial and monkey kidney cell lines infected by MERS-CoV or SARS-CoV[48]. In agreement with the results of de Wilde *et al.*[52], MERS-CoV exhibited once more a much more pronounced sensitivity, compared to SARS-CoV. The anti-viral effect of INF-α and INF-β against

Table 1. Compounds that have been suggested as possible drug candidates against MERS-CoV infections

Drug candidate	Observed effect	Study
INF-α	Reduction of MERS-CoV replication in pseudo-stratified HAE cultures	Kindler *et al.* (2013)[50]
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)[52]
INF-β	Reduction of the viral load in MERS-CoV-infected human lung epithelial and monkey kidney cell lines	Zielecki *et al.* (2013)[48]
INF-λ3	Reduction of MERS-CoV replication in pseudo-stratified HAE cultures	Kindler *et al.* (2013)[50]
INF-α2b	Reduction of the MERS-CoV-induced CPE and the viral protein levels in monkey kidney cell lines (more efficient when combined with Ribavirin)	Falzarano *et al.* (2013)[72]
Ribavirin	Reduction of the MERS-CoV-induced CPE and the viral protein levels in monkey kidney cell lines (more efficient when combined with INF-α2b)	Falzarano *et al.* (2013)[72]
Corticosteroids	Significant improvement of the respiratory condition of a MERS-CoV patient (no direct effect has been proved)	Guberina *et al.* (2013)[65]
Cyclosporin A	Inhibition of the MERS-CoV-induced CPE in monkey kidney and a human liver cell lines	de Wilde *et al.* (2013)[52]
SB203580	Reduction of the viral load in a human lung epithelial cell line	Josset *et al.* (2013)[54]
ADS-J1	Inhibition of MERS-CoV pseudo-virus infection in human liver and mink lung cell lines	Zhao *et al.* (2013)[29]
HP-HAS	Inhibition of MERS-CoV pseudo-virus infection in human liver and mink lung cell lines	Zhao *et al.* (2013)[29]
MDL28170	Inhibition of MERS-CoV-S-mediated transduction of a human fetal lung fibroblast cell line	Gierer *et al.* (2013)[25]
NH_4Cl	Inhibition of MERS-CoV-S-mediated transduction of a human fetal lung fibroblast cell line	
Camostat	Inhibition of MERS-CoV-S-mediated transduction of a human colon cell line	Gierer *et al.* (2013)[25]
N3	Inhibition of the proteolytic activity of MERS-CoV 3CLpro	Ren *et al.* (2013)[73]
CE-10	Inhibition of the proteolytic activity of MERS-CoV 3CLpro	Kilianski *et al.* (2013)[74]
MERS-CoV RBD	Reduction of the viral load in a MERS-CoV-infected monkey kidney cell line	Chen *et al.* (2013)[33]

MERS-CoV was confirmed by another study, where it was demonstrated that addition of any of the two molecules one hour after infection suppressed viral replication, in *ex vivo* cultures of human lung tissue[49]. Finally, Falzarano *et al.*[72] investigated a possible therapeutic effect by interferon-α2b and ribavirin, using monkey kidney cell lines. The group concluded that, while each of the compounds alone displays an anti-viral effect –demonstrated as reduced CPE and

viral protein levels- only in high concentrations, their combination yields a similar result, even when lower concentrations are used.

Given the beneficial effect of interferon administration, Guberina *et al.*[65] further supported the contribution of immunomodulatory therapy in MERS treatment, proposing the use of corticosteroids as a therapeutic strategy. Their suggestion was based on the fact that corticosteroids have already been successfully used n SARS-CoV patients, alleviating the symptoms of the infection, and on the observation that the respiratory condition of a MERS patient reported in their study improved significantly only after treatment with corticosteroids[65,75]. Whether such improvement was indeed due to the corticosteroids and whether this drug has a therapeutic effect on MERS-CoV infection requires further investigation.

The use of another immunomodulatory drug, cyclosporin A (CsA), was investigated by de Wilde *et al.*[52]. CsA is an immunosuppressant and it was previously shown to inhibit the replication of SARS-CoV and other coronaviruses[76]. The research group showed that CsA completely inhibited the CPE in monkey kidney and human liver cell lines infected by MERS-CoV, while it did not affect the viability of either the infected or the intact cells. However, some of the infected cells escaped the inhibitory effect, in agreement to what had been previously reported for other coronaviruses[76]. It should be noted that the use of immunosuppressive drugs for the treatment of SARS was believed to be successful due to the fact that SARS-CoV infection induces a cytokine storm, which can be potentially fatal[77]. The cell response to a MERS-CoV infection is completely different, since no cytokine response is induced upon *in vitro* infection[48,49,50]. Therefore, despite the promising results of de Wilde and his colleagues, the administration of immunosuppressants to MERS patients requires careful consideration.

Based on the results of their transcriptomic analysis of MERS-CoV infected cells, Josset *et al.*[54] tried to predict potential anti-viral agents that would reverse the phenotype induced by the infection. Focusing on a set of 207 genes that were found to be dysregulated early and permanently upon MERS-CoV infection, the group looked for compounds that were known to down-regulate the genes that were up-regulated and vice versa. SB203580, a kinase inhibitor, was among the top predicted regulators and its ability to block viral replication was tested using a human lung epithelial cell line. The results suggested that pretreatment with SB203580 might have a therapeutic effect, since it significantly reduced the viral titer in the infected cells.

Additional anti-MERS-CoV agents were proposed by Zhao *et al.*[29], who tested known HIV entry inhibitors for their inhibitory activity on MERS-CoV, using a pseudo-virus-based assay and

cell lines derived from human liver and mink lung. The screen pointed out ADS-J1, a small inhibitor targeting the HIV glycoprotein 41 (gp41)[78] and the 3-hydroxyphthalic anhydride-modified human serum albumin (HP-HAS), which was previously found to target the HIV-1 glycoprotein 120 (gp120) and the HIV-1 receptor, CD4[79]. Both molecules significantly inhibited infection of the cell lines by a MERS-CoV pseudo-virus.

The fusion of the viral membrane with the host cell membrane is essential for the cellular entry of enveloped viruses and is therefore a target of anti-viral therapeutic strategies. Successful membrane fusion often requires proteolytic activation of viral proteins by host cell proteases, such as the pH-dependent endosomal cysteine proteases cathepsin B/L or the type II transmembrane serine protease TMPRSS2, which are responsible for the activation of SARS-CoV spike (S) protein[25,80,81]. For this reason, inhibitors of these proteases have been proposed as therapeutic agents in SARS therapy[80,82]. Gierer *et al.*[25] revealed that cathepsin B/L and TMPRSS2 are responsible for activating the S protein of MERS-CoV as well, since inhibitors of these proteases blocked MERS-CoV-S-mediated transduction of cells in culture. The inhibitors that were tested for cathepsin B/L were MDL28170 and ammonium chloride and the inhibitor tested for TMPRSS2 was camostat. A human fetal lung fibroblast and a human colon cell line were used, respectively. The ability of these inhibitors to block the entry of MERS-CoV remains to be evaluated.

Replication of coronaviruses depends on the proteolytic activation of the replicase polyprotein by the viral papain-like protease (PLpro) and the 3-chymotrypsin-like protease (3CLpro), also named main protease (Mpro)[1]. Therefore, these proteases are another target for anti-viral intervention and, since they are present only in the virus and not in the host cell, this therapeutic strategy appears to be much safer than the one that targets host cell proteins[83,73]. Inhibitors of the viral proteases that block SARS-CoV infection have already been discovered and, since the sequence and structure of the 3CLpro shows high similarity between SARS-CoV and MERS-CoV, they could also be utilized as therapeutic agents against MERS-CoV infection[83,73]. Two independent studies have already investigated the possible beneficial effect of two of these inhibitors. Ren *et al.*[73] showed that the anti-coronavirus inhibitor N3 blocks the proteolytic activity of MERS-CoV 3CLpro and they presented the crystal structure of the N3 in a complex with the protease, concluding that N3 blocks the function of MERS-CoV 3CLpro in a similar way it does to the protease of other coronaviruses. Kilianski *et al.*[74] evaluated the ability of several antiviral drugs that were optimized for inhibition of SARS-CoV 3CLpro to block MERS-CoV 3CLpro activity

and found that the antiviral inhibitor CE-10, a 5'-chloropyridine ester, resulted in successful inhibition. The proposed inhibitory effect of these agents against MERS-CoV replication remains to be validated.

Chen *et al.*[33], one of the groups that solved the crystal structure of the MERS-CoV RBD, investigated the possible therapeutic implications of this domain. After the RBD was mapped at the C-terminal part of the S1 subunit of the virus (residues 367-588), its ability to block MERS-CoV infection of a monkey kidney cell line was evaluated. Measurement of the viral titers indicated that the RBD can efficiently inhibit the MERS-CoV entry. Same results were obtained using a pseudo-virus-based assay.

The approaches described in the above studies can be used as an example for identification of additional anti-MERS-CoV drugs. Thus, candidates proposed for SARS therapy should continue being tested for their potential in the treatment of MERS, given the similarity of MERS-CoV and SARS-CoV. The genome-based drug prediction suggested by Josset *et al.*[54] also appears to be a promising approach and it should be considered a tool for further identification of drug candidates. Moreover, knowing the molecular and cellular biology of the MERS-CoV entry and replication, several steps of these processes can be used as targets, in order to prevent disease. Thus, in the same way that Chen *et al.*[33] predicted that the MERS-CoV RBD could block the interaction of the virus with the host cells, by competing for receptor binding, drugs can be designed to interfere with the membrane fusion, viral internalization, intercellular transmission etc.

The RBD of MERS-CoV as a vaccine candidate

Although numerous measures should be taken in order to prevent the spread of MERS-CoV, vaccination remains the most powerful tool. Based on the results obtained by Gierer *et al.*[25], showing the presence of MERS-CoV S-specific neutralizing antibodies in MERS patients and the fact that the RBD of SARS-CoV was found to be a promising candidate for anti-SARS-CoV vaccines[84], several studies investigated the potential of MERS-CoV S protein as a vaccine candidate.

The immunostimulatory effect of MERS-CoV S protein was tested using the Modified Vaccinia virus Ankara (MVA), an attenuated strain of vaccinia virus that is currently one of the most advanced recombinant vectors used for the development of new vaccines[85,86]. The full-length S protein was expressed in MVA and biochemical characterization showed that the

product was a mature and properly folded protein. Subsequent vaccination of mice with the recombinant MVA elicited antibodies that were able to neutralize MERS-CoV infection in human liver and monkey kidney tissue cultures, probably by blocking the interaction between the S protein and the DPP4 receptor[86]. According to this study, MVA-expressed MERS-CoV S protein induces higher immunogenicity than the S protein of SARS-CoV, which was expressed in the same system in a previous study[87].

The immunogenicity of MERS-CoV S protein was also evaluated in a different system, where the RBD (residues 377-662 of the S1 subunit) was fused to the Fc fragment of human IgG[32]. The MERS-CoV RBD-Fc was used to immunize mice and the collected sera were tested *in vitro* for their neutralizing activity against MERS-CoV, after the presence of antibodies specific for the MERS-CoV RBD was confirmed. The sera displayed successful neutralization of MERS-CoV infection in two different monkey kidney cell lines. The group compared their findings with those of a similar study evaluating the immunogenicity of the SARS-CoV RBD-Fc and they concluded that MERS-CoV RBD-Fc induces lower levels of neutralizing antibodies[88]. This is opposed to the findings of Song *et al.*[86], although this might be due to the different expression system used in each study. The ability of the MERS-CoV RBD-Fc to elicit neutralizing antibodies against MERS-CoV infection was confirmed by two additional studies, with minor differences in the residues used[29,30]. These results indicate that, having a much higher biosafety profile and demonstrating comparable efficiency, the RBD-Fc fusion could be used as an alternative to attenuated virus-based vaccines.

Given that the detailed crystal structure of the MERS-CoV RBD is already revealed, it is possible to design structure-based vaccines with enhanced performance, as proposed by Chen *et al.*[33]. For example, additional glycosylation sites can be introduced to the surface of the core subdomain, in order the antigenicity to be focused to the receptor binding motif (RBM). Furthermore, alternative disulfide bonds can be introduced to the RBD and certain loops can be modified, in order to enhance the stability of the RBD. The beneficial effect of these suggested modifications remains to be experimentally validated.

Discussion

MERS-CoV, discovered last year in Saudi Arabia, constitutes the sixth known human coronavirus and the first human coronavirus in lineage C of the *Betacoronavirus* genus[8]. The clinical manifestation of MERS-CoV infections is similar to that of SARS, the known international

pandemic caused by the SARS-CoV in 2002-2003[3], and it includes fever, cough, shortness of breath, acute pneumonia and acute renal failure[14]. However, the transmissibility of MERS-CoV among humans is currently much lower than that of SARS-CoV[67]. Nevertheless, the scientific community remains concerned, since small genome mutations could render the virus highly transmittable and lead to another epidemic.

Within a year from its discovery, MERS-CoV has infected 130 people, 58 of whom died[13], showing a fatality rate (~45%) much higher than SARS-CoV (~15%)[3]. However, it has been suggested that the high mortality attributed to MERS-CoV infections is due to under-reporting, since mild or asymptomatic cases "escape" diagnosis[51,60]. The possibility that mild or asymptomatic cases can occur in the human population was proposed based on the molecular clock analysis performed by Cotten *et al.*[42], which revealed that MERS-CoV had been circulating in the human population for more than a year prior its discovery, suggesting that most of the initial infections caused only mild or no symptoms. Proof was obtained when MERS-CoV cases that had developed only mild respiratory symptoms were detected among contacts of MERS patients that were screened for the presence of the virus[61].

Currently, the reason why some of the MERS-CoV-infected people develop severe symptoms and others do not remains unclear. For many of the severe cases that required hospitalization, additional health-related issues were reported. Some of these patients were receiving immunosuppressive treatment for an underlying condition, e.g. malignancy, while others had been previously diagnosed with diabetes[51,59,89]. In addition, co-infection by another respiratory virus, such as influenza A or type 2 parainfluenza virus, was reported for some of the patients[59]. Consequently, one could hypothesize that underlying conditions/infections might support the development of severe symptoms. To further validate this hypothesis, detailed medical history should be requested from all individuals infected by the virus, despite the severity of their symptoms, and the presence of additional viruses should be tested, in order to detect a possible pattern. An alternative scenario could be that patients that develop severe disease might have been affected with a MERS-CoV strain that is more virulent. As shown by Wang *et al.*[36], a single amino acid substitution can alter the infectivity of MERS-CoV by >50%, if it occurs at one of the RBD residues that contact the DPP4 receptor. In order to evaluate the validity of this assumption, strains isolated from a number of patients with different clinical image should be sequenced and aligned, so that probable alterations in key residues are detected.

While the transmissibility of MERS-CoV among humans remains low, the main concern of the scientific community should be to restrain its spread, so that a potential epidemic is prevented. A zoonotic origin has been attributed to MERS-CoV and, since epidemiologically unlinked cases continue to occur in the Middle East[13], it seems that the animal source of infection is still active. Therefore, identifying and isolating the animal hosts of the virus is a key step in preventing its spread. Although it was initially thought that the MERS-CoV jumped from bats to humans, given its phylogenetic proximity with the bat coronaviruses BtCoV-HKU4 and BtCoV-HKU5[8], a more likely scenario is that the virus was first transmitted from the bats to other species that serve as intermediate hosts. In order these hosts to be identified, samples from animals of the Middle East and elsewhere should be collected and tested for the presence of MERS-CoV by detection of the viral genome. Focus should be given on the animals that have been predicted to be the most susceptible to MERS-CoV infection, based on the sequence similarity between their DPP4 receptor (particularly the residues that contact the RBD of MERS-CoV) and the human one, such as macaque, pig and rabbit[36,44]. Additionally, detailed history of contact with animals should be requested for every confirmed MERS case.

So far, MERS patients have admitted contact with camels and farm animals, turning the focus of scientists towards these species. In a recent study, Reusken *et al.*[46] showed that high titers of MERS-CoV neutralizing antibodies were present in the sera of camels from the Middle East, suggesting that camels might be the intermediate host of the virus. This would explain the link of the virus with the Middle East, considering that dromedary camels are among the main sources of meat and milk in that region[90]. A proposed scenario is that camels got infected by eating dates that were contaminated by bat droppings, although there is no evidence for that at the moment[91]. The suggestion of camels being the intermediate host has raised arguments. It has been suggested that the observed result could be a false positive, since camels possess an alternative type of antibodies, consisting of only two heavy chains, which makes them able to recognize a broad spectrum of antigens[47,92]. This however would not explain why, in the same study, these antibodies did not react with SARS-CoV[46]. Since serological assays seem to be insufficient, further investigation is required, before conclusions are drawn about the role of camels in the spread of MERS-CoV. A sequence alignment of the human and camel DPP4, as well as evaluation of the viral replication in camel cells *in* vitro, could provide more information. Furthermore, despite the fact that scientists doubt the presence of the virus in animals with so high antibody titers, detection of the viral genome in the screened camels should be attempted.

Apart from the interspecies transmission of MERS-CoV, human-to-human transmission should also be eliminated for successful prevention of an outbreak. Although challenging, identification and monitoring of all MERS-CoV infections would be a crucial step. Fortunately, diagnostic assays specific for the virus were developed soon after its discovery and they seem to be efficient[18,19]. However, these assays can be beneficial only if they are picked up by the majority of countries that are currently at risk. A survey performed in December 2012, showed that diagnostic assays were available only in approximately half of the responding countries in the WHO European Region[93], although the numbers might have changed in the months that followed. A new survey should be performed to evaluate the capability of countries to monitor MERS-CoV infections at present.

In order to identify all MERS-CoV cases and isolate them before they cause further infections, all individuals with clinical manifestations similar to those of MERS should be tested for the presence of the virus, by collection of lower respiratory tract samples, even if an alternative diagnosis has been made. This should be applied even for patients with mild symptoms, given that mild MERS-CoV cases have been described[61]. Moreover, all contacts of a confirmed case should be tested as soon as possible, since it is currently unknown whether the patients are able to transmit the virus before they develop symptoms. It is essential that these contacts will be self-isolated and followed-up for a time period as long as the incubation period of the virus. Since May 2013, it is believed that this period is 9-12 days[51,64]. An initially negative result should be confirmed with collection of new samples, during this period. Additionally, considering that many of the reported clusters have occurred in health care facilities[39], it is important that health care workers are well-informed and educated, so that they take all possible measures of protection, until the exact way of transmission is identified. However, even with such strict manipulations, it is inevitable that asymptomatic cases will escape detection (unless they are among the contacts of a confirmed case) and will further spread the virus.

Another essential step for the control of an outbreak is the surveillance of the mass gatherings in the Middle East. Every year, hundreds of thousands of pilgrims from all over the world gather in Mecca, Saudi Arabia, where they remain in a confined area, during the week of the Hajj. Despite the fact that last year's Hajj did not result in an increased number of MERS-CoV infections, the fear that this year's gathering might augment the spread of the virus internationally is still present. The local medical authorities recommend that general travel health precautions should be taken[94], although the efficiency of these precautions is

questionable, as long as the exact ways of viral transmission remain unknown. Screening the pilgrims for detection of MERS-CoV, on the other hand, would be a more efficient approach for monitoring possible infections. The screen could be performed upon arrival at the home countries. In that case, analysis of flight itinerary data to predict population movements out of the Middle East should be performed, as exemplified by Khan *et al.*[95], so that countries can act accordingly to their estimated potential for importation of the virus. Alternatively, travelers could be screened before their departure from the Middle East. Based on the findings of a study that evaluated the entry and exit screening of airline travelers during the pandemic of H1N1 in 2009[96], Khan *et al.*[95] suggested that exit screening of the pilgrims prior to their departure would be sufficient, while higher efficiency would be achieved if the screening was focused on few pivotal airports. However, in both of the above cases, a large number of individuals would have to be screened simultaneously, implying that such approach would require time, money and decent organization.

A study performed in December 2012 proposed the use of recombinase polymerase amplification (RPA) as an alternative method for the detection of an animal coronavirus, the bovine coronavirus, which was previously performed mainly via RT-PCR[97]. Given that a big number of animals have to be screened in short time, RT-PCR is not considered the optimal technique for field detection of the bovine coronavirus, due to its relatively high costs and the need for laboratory equipment. RPA is also method for amplification and detection of nucleic acids, but in contrast to RT-PCR, it is performed at a constant temperature. Amplification is detected in real-time via a portable scanner and a laptop computer[98]. Consequently, RPA is a simple, fast and low-cost alternative to RT-PCR, maintaining its sensitivity and specificity and it is therefore considered the optimal approach for on-site screening[97]. Because of these characteristics, RPA could be a valuable tool for the detection of MERS-CoV, since it would simplify its diagnosis a) at airports, during exit screens of travelers that depart from the Middle East and b) in the field, when herds of animals need to be screened in order possible hosts of the virus to be predicted.

Preventing a possible epidemic would also require the development of safe and effective therapeutics and vaccines. Several drug candidates have already been proposed for the treatment of MERS by a number of studies, mainly based on results of *in vitro* experiments (**Table 1**). Concerning vaccine candidates, the RBD domain of the S1 subunit of MERS-CoV has shown good potential, since multiple independent studies demonstrated its ability to elicit

antibodies with *in vitro* neutralizing activity against MERS-CoV[29,30,32]. Of note, the *in vitro* experiments required for the evaluation of both drug and vaccine candidates, could be simplified using pseudovirus-based assays, as exemplified by Zhao *et al.*[29], since assays that employ live viruses require biosafety level 3 (BSL-3) facilities, which are not available to all researchers. Zhao and his colleagues have already shown that both types of assays yielded consistent results[29].

Despite the promising *in vitro* results, *in vivo* evaluation of the drug and vaccine candidates is necessary. This highlights the need for an animal model of MERS-CoV infection. In April 2013, Munster *et al.*[99] reported a nonhuman primate disease model. The group inoculated six rhesus macaques with MERS-CoV, which resulted in development of mild to moderate pneumonia in all animals. The virus was re-isolated from lung tissue, fulfilling Koch's postulates. The team is currently testing the effect of INF-α and ribavirin in the infected monkeys, as a follow-up of their *in vitro* studies[72,100]. However, the macaque model is not as practical as a small animal model and it is not widely used by research groups. Additionally, the infections described by Munster and his colleagues are not completely representative of the clinical image of MERS patients, since neither severe respiratory illness nor renal disease was developed in the macaques. Thus, additional animal models are required. Researchers have already tried to infect other lab animals, such as mice, ferrets and hamsters, without successful outcome[101]. This could be due to the fact that the MERS-CoV contact residues of the DPP4 of these animals show little conservation when compared to those of the human DPP4, while in the case of the macaque there is 100% similarity[44]. Expression of the human DPP4 in rodents could possibly overcome this issue[102]. Alternatively, researchers could try to infect rabbits, given that their DPP4 sequence is quite similar to the human one. Developing a successful animal model for MERS-CoV infection not only will enable the evaluation of anti-viral strategies and immunization, but it will also allow the detailed characterization of the tissue tropism and pathogenesis of the virus, as well as the identification of the possible ways of viral transmission.

As long as research on MERS-CoV continues and new aspects related to this virus become elucidated, the chances for a successful prevention of an epidemic increase. However, it is essential that novel research findings become available to the scientific community. A recent incident in the Netherlands reminds us that this can often be hampered. Virologist Ron Fouchier, leader of the team that obtained the complete genome sequence of MERS-CoV in the Erasmus Medical Center (EMC) in Rotterdam, initiated a legal battle with the Dutch government, after the decision of the latter to ban the publication of Fouchier's findings on the transmission of H5N1,

claiming that they could be used for bioterrorism[103]. Fouchier and other virologists, though, stated that the obtained results could aid the prevention of an influenza pandemic. The threat of bioterrorism should definitely be taken seriously. However, dissemination of the scientific knowledge remains the most powerful tool for dealing with any possible threat towards the global public health and therefore it should never be constrained.

Conclusions

MERS-CoV, identified last year in the Middle East as a pathogen with the ability to cause illness similar to that caused by SARS-CoV, is now considered a threat to global public health. Our current knowledge of the virus is limited, since many important epidemiological and clinical aspects remain unknown. Although MERS-CoV displays lower transmissibility among humans than SARS-CoV, the possibility that future mutations will render the virus highly transmittable, with a devastating outcome, cannot be discarded. In order to combat the spread of the virus, scientists should aim at the identification of its animal hosts, as well as the early detection and isolation of the majority of MERS-CoV cases. Surveillance of the mass gatherings in the Middle East would be a key step. Additionally, focus should be given on the development of efficient therapeutics and vaccines, something that requires the availability of a successful animal model. The global health and scientific community should remain vigilant and not underestimate the ability of this novel virus to cause a new pandemic. Examples should be taken from the outbreak of SARS-CoV, which proved that lack of anticipation can have dramatic consequences. Let's hope that the lesson was learned.

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Summary

In September 2012, a previously unknown virus was discovered in a patient from Saudi Arabia, later named Middle East Respiratory Syndrome coronavirus (MERS-CoV). MERS-CoV is a close relative of Severe Acute Respiratory Syndrome coronavirus (SARS-CoV), which caused the international SARS epidemic in 2002-2003. The two viruses cause similar symptoms upon infection, although MERS-CoV seems to be less transmittable among humans. Despite its lower transmissibility, this novel virus is now considered a threat to global public health, mainly due to the fact that important aspects, such as its source, pathogenesis and way of transmission are currently unknown. This thesis summarizes the literature that is currently available on MERS-CoV and discusses ways to control its spread and prevent a possible epidemic.