DPP4 IN MERS-COV TRANSMISSION AND PATHOGENESIS

Widagdo

The research described in this thesis was mainly conducted at the Department of Viroscience, Erasmus MC, Rotterdam, The Netherlands within the framework of the Erasmus Post Graduate School of Molecular Medicine. The research presented in this thesis was financially supported by Nederlandse Organisatie voor Wetenschappelijk Onderzoek (TOP project Grant 91213066) and Zoonotic Anticipation and Preparedness Initiative (Innovative Medicines Initiative grant 115760), with assistance and financial support from Innovative Medicines Initiative and the European Commission and contributions from European Federation of Pharmaceutical Industries and Associations partners. The financial support for printing of this thesis by Viroclinics Biosciences B.V. and Cirion Foundation is gratefully acknowledged.

Cover and lay-out design : Nadia Ayu Lestari
Curriculum Vitae Photograph : Aditya Perkasa
Print : ProefschriftMaken (www.proefschriftmaken.nl)
ISBN : 978-94-6380-349-6

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DPP4 in MERS-CoV transmissie en pathogenese

Thesis

to obtain the degree of Doctor from the
Erasmus University Rotterdam
by command of the
rector magnificus

Prof.dr. R.C.M.E. Engels

and in accordance with the decision of the Doctorate Board.

The public defence shall be held on

Tuesday 18 June 2019 at 9.30 hours

by

Widagdo
born in Cirebon, Indonesia

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This thesis is dedicated to my grandmothers that taught me hope, sacrifice, and faith – and to my beloved parents who empower their children through education.

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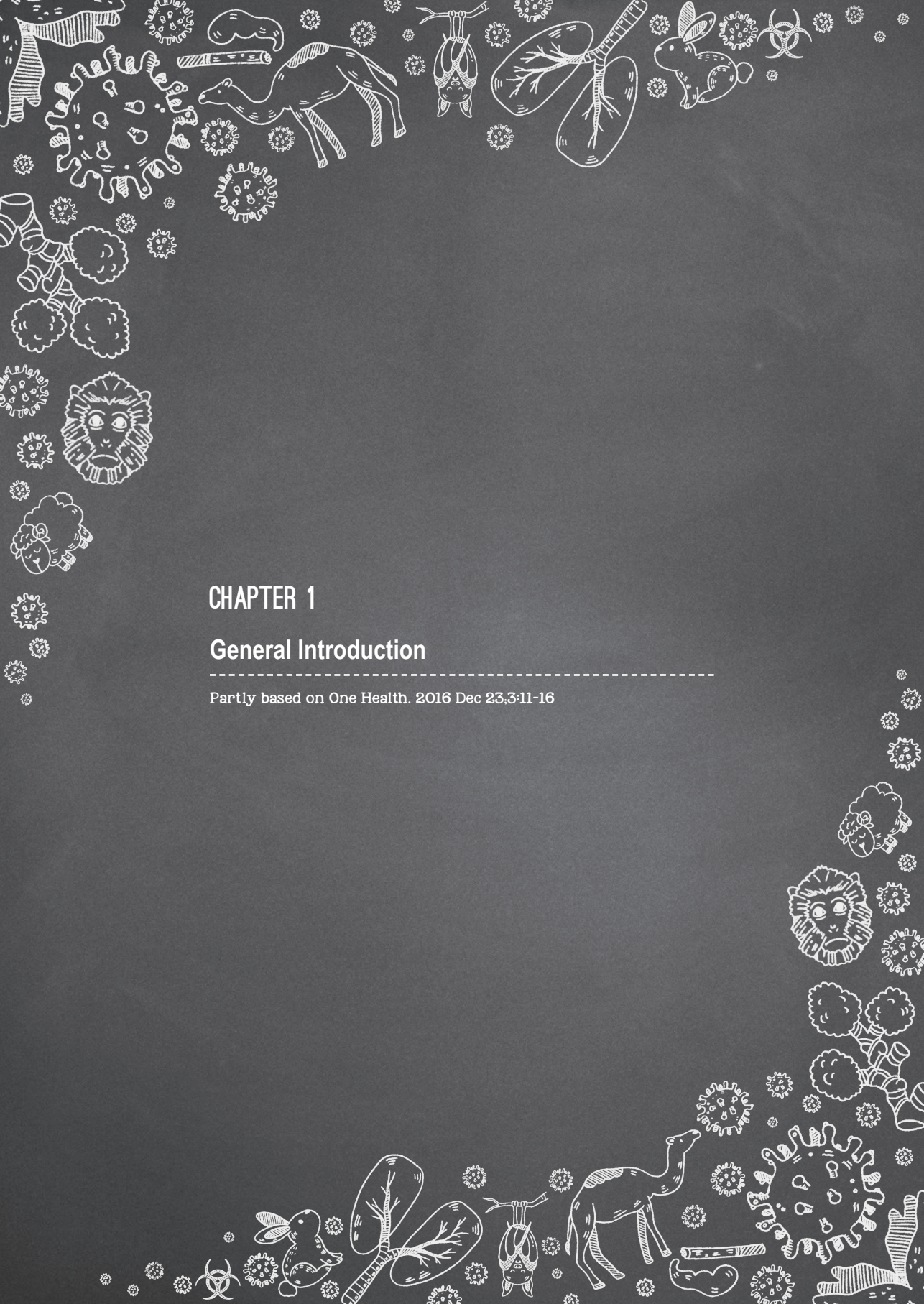

CHAPTER 1

General Introduction

Partly based on One Health. 2016 Dec 23;3:11-16

Emergence of MERS-CoV

Coronaviruses (CoVs) are known to cause mild upper respiratory tract infections in humans, as exemplified by OC43, NL63, 229E, and HKU1-CoV[1]. This paradigm was challenged when severe acute respiratory syndrome (SARS)-CoV emerged in 2002[2]. This virus mainly causes lower respiratory tract infections, such as bronchitis and pneumonia. Approximately 10% of SARS-CoV patients developed severe complications and succumbed to a fatal outcome. Further studies showed that this virus originated from bats and was transmitted to humans through civet cats, highlighting its zoonotic capacity. This virus managed to spread worldwide and infected ~8000 individuals within a year but was fortunately contained in 2003[3]. There is currently no evidence of SARS-CoV circulating in the human population, however, SARS-CoV-like-viruses that are able to directly infect human cells have been recently identified in horseshoe bats in China[4], hence continuous surveillance remains necessary.

At mid 2012s, a 60-year-old Saudi Arabian was admitted to a private hospital in Jeddah, Saudi Arabia with a 7-day history of fever, cough, expectoration, and shortness of breath. His chest radiography showed opacities in the middle and lower lung fields. His condition was quickly deteriorating thus he was transferred to an intensive care unit at day 2 post admission to receive mechanical ventilation. He developed acute renal failure at day 3 post admission. He died at day 11 due to progressive respiratory and renal failure[5]. Upon cell culture, PCR, and sequencing analysis on the sputum sample from this patient, a novel CoV belonged to genus betacoronavirus lineage C, later named Middle East respiratory syndrome (MERS)-CoV, was identified[5].

Since then MERS-CoV has been causing multiple outbreaks in the Arabian Peninsula, mainly in Saudi Arabia[6]. These outbreaks mostly occur in health-care settings[6,7], although have also been described to take place in dromedary camel farms and household settings[8,9]. Human MERS-CoV cases have also been reported in other countries, however they are associated with recent travel history to countries in the Arabian Peninsula[6,10,11], and mostly does not lead to an outbreak, except in South Korea[12]. Further studies suggest overcrowding of emergency room and lack of infection control measures in the health-care facilities in South Korea as the essential factors instigating this outbreak[12,13]. So far over 2000 individuals worldwide had been infected with MERS-CoV and ~35% of them succumbed to fatal outcome. New cases are still being reported[6], thus highlight the necessity to study this virus, especially its transmission and pathogenesis, as well as develop intervention measures.

Through molecular characterization, it has been shown that MERS-CoV is an enveloped single-stranded positive-sense RNA virus with crown-like structure, similar

to other CoVs[5,14]. Its genome consists of two large replicase open reading frames, ORF1a and ORF1b, that occupies three-fourth of the 5'-proximal part, and other genes that encode several nonstructural and structural proteins, i.e. spike (S), envelope (E), membrane (M), and nucleocapsid (N)[14]. The protruding trimeric S proteins of CoVs form the crown-like appearance and can further be divided into S1 and S2 protein (Figure 1A). S1 protein is known to initiate infection by attaching to host receptor at the surface of target cell[15,16]. Thus, S1 proteins can be used as a tool to assess specific immune response, develop vaccine candidate, as well as identify host receptor.

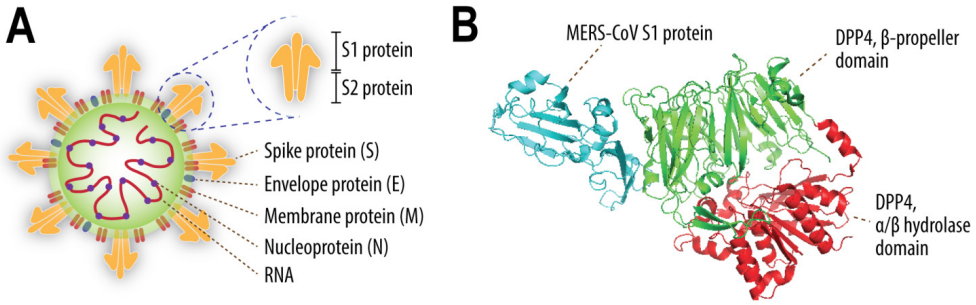

Figure 1. Schematic figure depicting four structural proteins of MERS-CoV, i.e. S, E, M, and N proteins (A); and a cartoon representation of MERS-CoV S1 protein binding to DPP4 (PDB code 4L72) (B). S protein can further be divided into S1 and S2 protein. α/β hydrolase domain of DPP4 is indicated in red, β-propeller domain in green, while part of MERS-CoV S1 protein in blue.

MERS-CoV Receptor, Dipeptidyl Peptidase-4

Our studies showed that MERS-CoV S1 protein recognizes two different host structures, a host exopeptidase called dipeptidyl peptidase-4 (DPP4) and several glycotopes of α2,3-sialic acids[17,18]. *In vitro*, polyclonal antibodies against DPP4 protect against MERS-CoV infection in susceptible cells, while transient expression of DPP4 in the non-susceptible Cos-7 cells renders these cells susceptible to MERS-CoV[18]. Meanwhile, elimination of α2,3-sialic acids in a susceptible cell line does not fully protect these cells against MERS-CoV, but still significantly reduce the number of infected cells[17]. Thus, we concluded that DPP4 is the functional receptor for MERS-CoV, while α2,3-sialic acids function as an attachment factor[17,18].

DPP4 is a serine exopeptidase known to cleave-off dipeptides out of polypeptides with either L-proline or L-alanine at the penultimate position. Accordingly, DPP4 is capable of cutting various substrates, such as hormones, cytokines, chemokines, neuropeptides, digestive enzymes, and etc. therefore involved in multiple physiological functions as well as pathophysiological conditions[19]. However, this enzymatic activity is not related to DPP4 function as MERS-CoV receptor, since DPP4 inhibitors are not able to abrogate this virus infection[18]. Crystal structure studies later confirmed that

MERS-CoV S1 protein binds to the β-propeller domain of DPP4 instead of the α/β hydrolase domain where its enzymatic reaction is taking place (Figure 1B). Besides that, these studies also reported eleven critical residues in DPP4 that binds to S1 protein[20-22]. Ferret and mice that merely have ~50% similarities in these residues to that of humans are not susceptible to MERS-CoV[22-24]. Meanwhile, DPP4 of camels, horses, llamas, pigs, sheep, rabbits, and bats, that have >80% similarities in these residues to that of humans, are able to recognize MERS-CoV S1 protein[22,23,25,26]. It is then important to investigate these animals' susceptibility to MERS-CoV, and more importantly their capability to transmit this virus.

MERS-CoV Transmission

The zoonotic capacity of MERS-CoV was already suspected at the beginning of its emergence since this virus is closely related to bat CoVs, i.e. HKU4 and HKU5, but not to the other human CoVs[5]. Subsequently, other MERS-like-CoVs have been sequenced from either fecal samples or rectal swabs of insectivorous bats[27-32]. This indicates insectivorous bats as the natural hosts for MERS-like-CoVs and further supports the animal origin of MERS-CoV. Regardless, there is currently limited evidence indicating transmission of these viruses from insectivorous bats to humans or other animal species (Figure 2).

Meanwhile, epidemiological studies reported that MERS-CoV seropositive dromedary camels are highly prevalent in the Arabian Peninsula and Africa[33-37]. These seropositive camels could even be detected as early as the 1980s[33,35,36], indicating that MERS-CoV did circulate in this animal long before being introduced to the human population. Upon experimental inoculation, dromedary camels developed mild upper respiratory infection[38,39]. Screening of nasal swabs obtained from dromedary camels subsequently led to identification and isolation of MERS-CoV from these animals, confirming its circulation in this animal species[9,40-42]. Two studies of human MERS cases post-contact with infected camels reported high similarity in virus sequences obtained from both the camels and humans[9,43]. These studies along with a case control study identifying direct exposure to camels as a risk factor for MERS-CoV infection[44,45], and serology studies showing higher seropositivity among camel contacts compared to non-camel contacts[46], support camel-to-human transmission of MERS-CoV (Figure 2). Wide geographical distribution of MERS-CoV seropositive dromedary camels, thus poses a risk of multiple zoonotic introductions to human population[37]. It remains to be determined whether there are other animals besides dromedary camels that could efficiently transmit MERS-CoV. Alpacas and llamas have been reported to be naturally seropositive and developing neutralizing antibodies against MERS-CoV[47,48]. However, MERS-CoV has not been isolated from these animals, and it is still unclear whether

they are capable of independently maintaining this virus in their population, as well as transmitting this virus to humans or other species. Meanwhile, sheep, horses, goats, and bovines have not been described to be naturally seropositive, but their DPP4 have been shown to facilitate MERS-CoV infection *in vitro*[25,49-52]. Experimental MERS-CoV infection and transmission experiment in these animals would then be necessary not only to confirm their susceptibility to MERS-CoV but also to assess their capacity to spread the virus.

Besides transmitted between camels and from camel to human, MERS-CoV has also been reported to be transmitted between humans[8]. This is in line with the fact that most of the outbreaks occur in healthcare settings[6,7]. However, unlike in camels, MERS-CoV mainly causes lower respiratory tract infection in humans[10,11,53]. Viral RNA levels in MERS-CoV patients are generally much higher in the lower respiratory tract compared to that in the upper respiratory tract[10,11,54]. MERS-CoV isolation from human samples was only successful when lower respiratory tract samples were used[5,10,55]. Autopsy result from fatal human MERS-CoV cases and *ex vivo* infection experiments using human lung explants showed that this virus does infect human lower respiratory tract epithelium[53,56,57]. This lower respiratory tract tropism might partly explain why contact tracing of symptomatic MERS-CoV patients showed that human-to-human transmission is rather limited, thus requires close contact and relatively high amount of virus being shed[7,8,13,58].

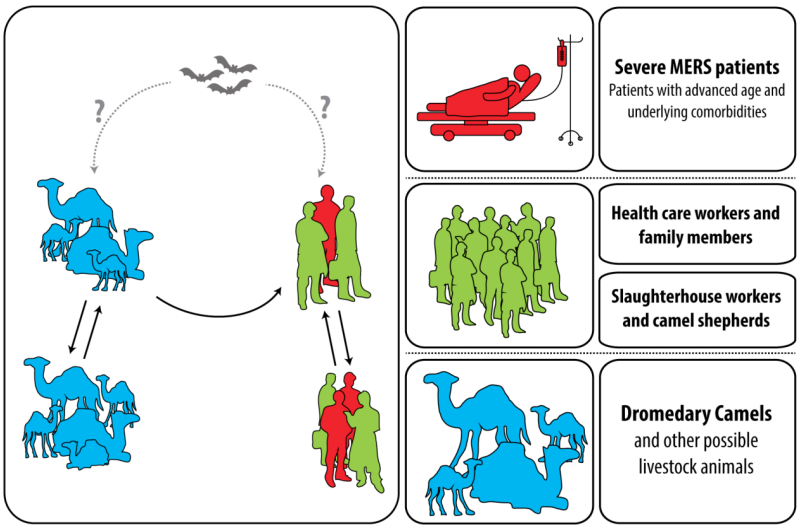

Figure 2. Schematic representation of MERS-CoV transmission. MERS-CoV has been circulating in dromedary camels for decades and could transmit to humans. Human-to-human transmission, besides camel-to-human transmission, has also been shown to occur. In humans, this virus causes respiratory infection ranging from asymptomatic to severe. Asymptomatic and mild cases consist of healthcare workers, family members, slaughterhouse workers, and camel shepherds. Severe MERS patients mainly consist of individuals with advance age and underlying comorbidities. Bats, on the other hand, have been suggested to be the natural hosts of MERS-CoV-like-viruses, however, the evidence supporting the transmission of these voruses from bats to other species is currently lacking.

MERS-CoV Pathogenesis

Besides offering insight in MERS-CoV transmission, contact tracing studies on MERS-CoV patients also reveal that MERS-CoV does not always cause severe pneumonia, acute respiratory distress syndrome, and fatal outcome. It can also cause mild and even subclinical manifestations[8,13,58,59]. These subclinical cases are easily missed and unrecorded in the field hence might lead to underestimation on the prevalence and overestimation on the fatality rate of MERS-CoV. These asymptomatic and mild cases mostly consist of slaughterhouse workers, camel shepherds, as well as health-care workers and family members having close contact with severe MERS-CoV patients[7,8,46,58]. While these individuals are mostly young-age and generally healthy, severe to fatal MERS-CoV cases are characterized by advance age and having multiple underlying chronic comorbidities, such as diabetes mellitus, chronic kidney diseases, heart diseases, chronic lung diseases, and etc.[6,60] (Figure 2). These differences between individuals having mild and severe MERS-CoV infection highlight the role of host factors in determining the outcome of MERS-CoV infection. It is currently unclear what these host factors are and how they influence MERS-CoV pathogenesis. DPP4, the MERS-CoV receptor, is one of the host factors that worth to look into. Since DPP4 is mainly studied in immunology and cancer field, there is not much known regarding the localization and function of DPP4 in the respiratory tract. Besides DPP4, MERS-CoV attachment factors, such as α2,3-sialic acids, carcinoembryonic antigen related cell adhesion molecule 5, and membrane-associated 78 kilodalton glucose-related protein[17,61,62]; as well as host innate and adaptive immune response might also influence MERS-CoV pathogenesis.

Outline of This Thesis

MERS-CoV is a novel zoonotic pathogen that uses DPP4 as its host receptor. The studies presented in this thesis aimed to gain insight into the role of DPP4 in MERS-CoV transmission and pathogenesis. The studies aimed to investigate the role of DPP4 in MERS-CoV transmission, i.e. **chapter 2-6**, are compiled in part 1 of this thesis. Meanwhile, **chapter 7 and 8** in part 2 of this thesis focused more on the role of DPP4 in MERS-CoV pathogenesis.

MERS-CoV is known to mainly causes lower respiratory tract infection in humans, while in dromedary camels, it merely causes upper respiratory tract infection. In **chapter 2**, we investigated whether such difference could be associated with DPP4 localization in the respiratory tract tissues of both species. We then extended our study by mapping DPP4 localization in various tissues of different bat species in **chapter 3** and different livestock animals, i.e. sheep, llamas, pigs, and horses in **chapter 4**,

and subsequently studied the association between DPP4 localization and MERS-CoV tropism in the different livestock animals. In **chapter 5**, we described our virus transmission experiment in rabbits, a susceptible small animal species, to gain insight on the host factors affecting MERS-CoV transmission. In **chapter 6**, we described α2,3-sialic acids glycotopes as an additional factor besides DPP4 that could influence MERS-CoV transmission and pathogenesis. To further study MERS-CoV pathogenesis in human, in **chapter 7** we reported our investigations on whether DPP4 expression in the lungs could be upregulated under certain chronic comorbidities. Later in **chapter 8** we investigated MERS-CoV tropism, DPP4 expression, and histopathological lesions in the lungs of a fatal MERS-CoV infection case; and also performed experimental MERS-CoV infection in cynomolgus macaques. Key findings of this thesis are then summarized and discussed in the context of current literature in **chapter 9**.

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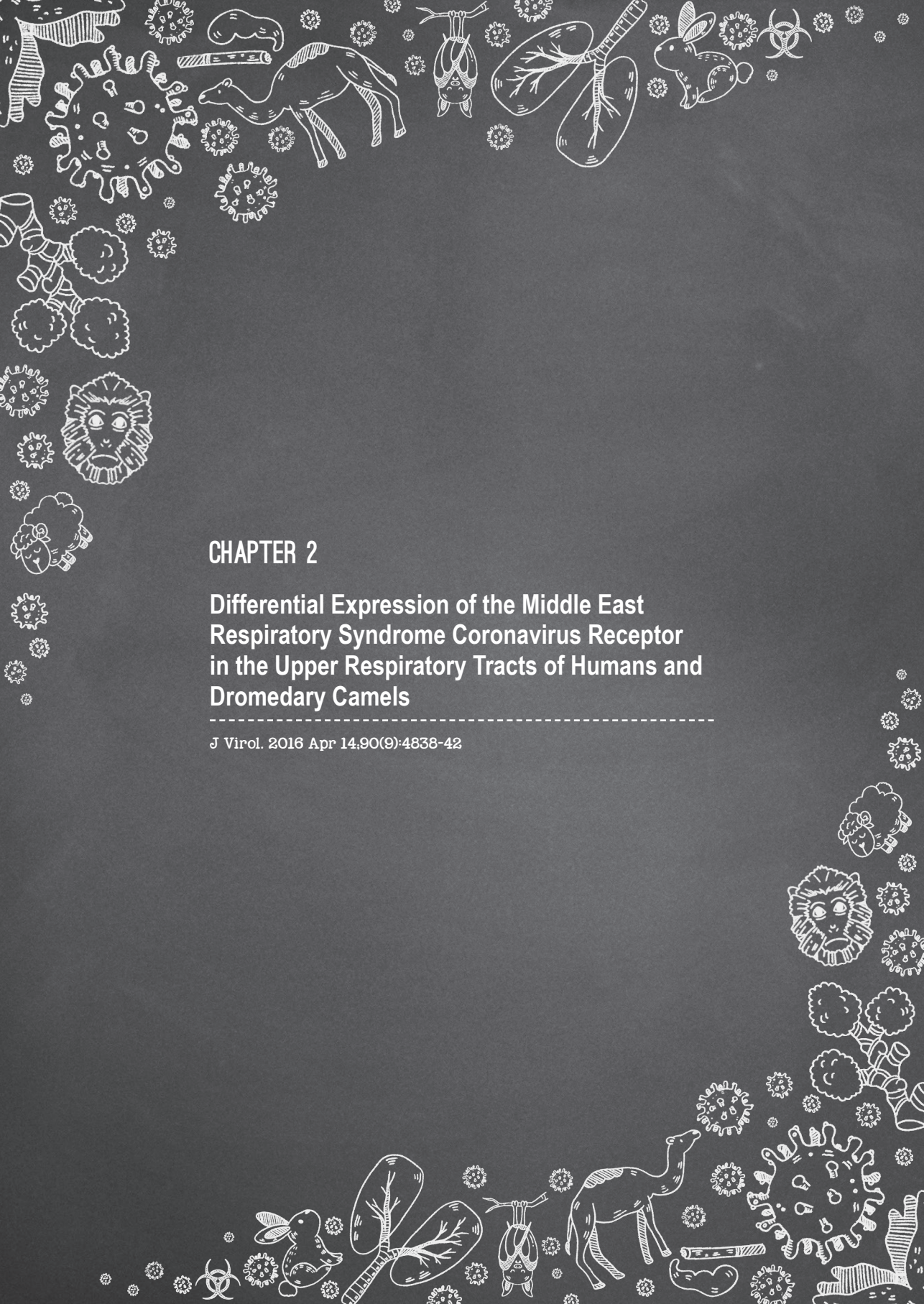

CHAPTER 2

Differential Expression of the Middle East Respiratory Syndrome Coronavirus Receptor in the Upper Respiratory Tracts of Humans and Dromedary Camels

J Virol. 2016 Apr 14;90(9):4838-42

Abstract

Middle East respiratory syndrome coronavirus (MERS-CoV) is not efficiently transmitted between humans, but it is highly prevalent in dromedary camels. Here we report that the MERS-CoV receptor – dipeptidyl peptidase 4 (DPP4) – is expressed in the upper respiratory tract epithelium of camels but not in that of humans. Lack of DPP4 expression may be the primary cause of limited MERS-CoV replication in the human upper respiratory tract and hence restrict transmission.

Brief Communication

Middle East respiratory syndrome coronavirus (MERS-CoV) is a novel coronavirus that causes pneumonia in humans, which may lead to acute respiratory distress syndrome[1]. Currently, more than 1,500 confirmed cases have been reported, with a relatively high case fatality rate. Although most MERS outbreaks have been reported in the Middle Eastern countries, travel-related cases may seed outbreaks in other regions, such as in South Korea[2]. In principle, they can be controlled through implementation of early viral diagnostics, strict hygiene measures, and isolation of patients. However, there is still a lack of understanding of how this virus is transmitted, both between humans and from camels to humans.

Dromedary camels are currently considered the only zoonotic source of MERS-CoV. This is largely based on the fact that closely related viruses have been isolated only from this species thus far[3,4]. Although studies in the Middle East and several northeastern African countries revealed a high percentage of serological positivity among dromedary camels[4-8], there seems to be limited MERS-CoV transmission from camels to humans. Recent studies have shown that only 2 to 3% of persons in Saudi Arabia and Qatar that come into close contact with dromedary camels have neutralizing antibodies to MERS-CoV[8,9]. Additionally, most of the notified MERS patients to date did not report any contact with camels or other livestock animals, consistent with the fact that most outbreaks took place in hospitals[10,11]. On the other hand, studies in hospital and household settings also reported a low percentage of confirmed MERS cases among patient contacts[10,11]. As a result, over a 3-year period, the number of MERS cases is relatively low, providing evidence that MERS-CoV transmission to humans and between humans is relatively inefficient.

One factor considered to be critical for the transmission of MERS-CoV is the ability of the virus to replicate in the upper respiratory tract. Differences in viral shedding in dromedary camels and humans have been observed. Relatively high levels of infectious virus can be detected in nasal swabs of dromedaries infected with MERS-CoV but

not in MERS patients[12,13]. We hypothesized that a critical determinant of MERS-CoV replication in the respiratory tracts of different hosts is the differential expression of the viral receptor. Dipeptidyl peptidase 4 (DPP4), a serine exopeptidase involved in various biological functions[14], has been shown to act as the functional MERS-CoV receptor[15]. Although there is ample evidence that it is expressed in different tissues and cell types, including kidney cells, small intestine cells, and T lymphocytes[14,16], its expression in the upper respiratory tract has not been investigated thus far. Here we addressed this knowledge gap by analyzing the tissue localization of DPP4 along the human and dromedary camel respiratory tracts.

We obtained 14 human respiratory tract and 3 human kidney formalin-fixed, paraffin-embedded (FFPE) tissue samples from the Erasmus MC Tissue Bank. These respiratory tract tissue samples were six nasal tissue samples (three superior and three inferior concha tissue samples) and two tracheal, three bronchial, and three lung tissue samples. These tissue samples were taken either from healthy donors or from patients with nonmalignant tumors. Kidney tissue was used as a positive control because of its abundant expression of DPP4[14]. These tissue samples were residual human biomaterials that were collected, stored, and issued by the Erasmus MC Tissue Bank under ISO 15189:2007 standard operating procedures. Use of these materials for research purposes is regulated according to reference[17]. Dromedary camel tissue samples were obtained from animals used in an experimental MERS-CoV infection[18]. DPP4 immunohistochemistry staining of these 3-μm-thick FFPE tissue sections was then performed. Antigen was retrieved by boiling these sections in 10.0 mM citric acid buffer, pH 6, for 15 min in a 600-W microwave. Endogenous peroxidase was blocked by incubating the slides with 3% hydrogen peroxidase for 10 min. DPP4 was detected with 5 μg/ml polyclonal goat IgG anti-human DPP4 antibody (R&D Systems, Abingdon, United Kingdom), while negative controls were stained with normal goat serum (MP Biomedicals, Santa Ana, CA, USA) in equal concentrations. This primary antibody staining was done overnight at 4°C. Secondary antibody staining was performed with peroxidase-labeled rabbit anti-goat IgG (Dako, Glostrup, Denmark) at a 1:200 dilution for 1 h at room temperature. The sections were then treated with 3-amino-9-ethylcarbazole (Sigma-Aldrich), counterstained with hematoxylin, and embedded in glycerol-gelatin (Merck, Darmstadt, Germany).

In the human respiratory tract tissue samples, DPP4 was detected in the lower part, i.e., alveolar epithelial cells and macrophages but mostly type II alveolar epithelial cells (Fig. 1). In addition, DPP4 expression was also detected to a limited extent on the apical surface of the terminal bronchioles and bronchial epithelium of two lung samples and one bronchus sample. In sharp contrast, DPP4 was not detected in any of our nasal respiratory and olfactory epithelium or trachea samples (Fig. 1). In the submucosal layer of these tissue samples, DPP4 was detected in the serous

glandular epithelium, inflammatory cells, and vascular endothelium. In contrast to humans, DPP4 was detected in the ciliated epithelial cells of the upper respiratory tract epithelium of dromedary camels (Fig. 1). Additionally, it was also present in the ciliated epithelial cells of the tracheal and bronchial epithelium of these animals. However, in the alveoli, it was detected mostly in the endothelial cells and barely in the alveolar epithelial cells. Therefore, we conclude that there is differential expression of DPP4 in the respiratory tracts of humans and dromedary camels. The absence of DPP4 in the upper respiratory tract epithelium of humans may keep MERS-CoV from replicating efficiently here. To confirm the localization of DPP4 expression, we performed in situ hybridization to detect mRNA transcripts. On the RNAscope platform[19] with commercially available probes for DPP4, mRNA was detected in human submucosal glands but not in the nasal epithelium (Fig. 2A and and B). Probes for ubiquitin C and DapB (Advanced Cell Diagnostics, Hayward, CA, USA) were used as positive and negative controls, respectively. Ubiquitin C is encoded by a housekeeping gene and is abundantly present in human tissue, while DapB is encoded by a bacterial gene and should not be present in healthy human tissue.

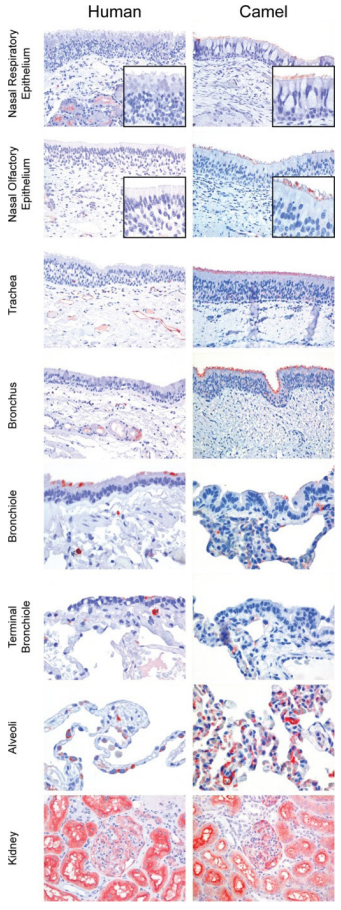

Figure 1. DPP4 expression in the upper respiratory tracts of camels and humans. DPP4 immunohistochemistry staining of human and dromedary camel respiratory tissue samples was performed; kidney tissue was used as the positive control. Nose, trachea, bronchus, and kidney samples, ×200 magnification; bronchiole, terminal bronchiole, and alveolar samples, ×400 magnification. Positive staining is red.

Alternatively, other as-yet-unidentified MERS-CoV receptors may localize in the upper respiratory tract. To investigate the presence of such receptors, we performed immunohistochemistry staining of frozen human tissue material with the spike S1 protein of MERS-CoV. The spike protein is one of the structural proteins that form the outer layer of the MERS-CoV particle and bind to DPP4[15]. By fusion of the MERS-CoV S1 protein to the mouse IgG2a Fc fragment (mFc-S1 MERS), binding of the S1 protein to cells or proteins in human tissue sections could be investigated. The S1 protein of coronavirus OC43 was used as a positive control, since this virus is commonly known to cause upper respiratory tract infection in human[20]. Meanwhile, as a negative control, we

used the S1 protein of porcine epidemic diarrhea virus (mFc-S1 PEDV) and mouse isotype antibodies (Dako, Glostrup, Denmark). Additionally, immunohistochemistry with mouse monoclonal antibody (MAb) against human DPP4 (anti-DPP4 MAb; Santa Cruz Biotechnology, Dallas, TX, USA) was performed to further confirm the absence of the MERS-CoV receptor in the same nasal epithelium. Frozen human nose and kidney tissue samples for this experiment were also obtained from the Erasmus MC Tissue Bank, and sections of 6 µm were cut. Kidney tissue was again used as a DPP4 positive control. These sections were fixed in acetone and incubated in room temperature for 1 h with mFc-S1 MERS-CoV, mFc-S1 OC43, mFc-S1 PEDV, anti-DPP4 MAb, or isotype mouse antibody at 1 µg/ml. They were subsequently incubated with peroxidase-labeled goat anti-mouse IgG (Dako, Glostrup, Denmark) at a 1:100 dilution for 1 h at room temperature and processed as described above. mFc-S1 OC43 bound to the nasal epithelium surface, while mFc-S1 MERS and anti-DPP4 MAb did not. Similar to our results depicted in Fig. 1 and 2, mFc-S1 MERS-CoV and anti-DPP4 MAb bound to the nasal submucosal glands and kidney proximal tubuli. Meanwhile, our negative control, mFc-S1 PEDV and mouse isotype antibodies, did not bind to either nasal or kidney tissue samples (Fig. 3). This result suggests that neither DPP4 nor any other alternative receptor is capable of binding spike protein of MERS-CoV in the upper respiratory tract epithelium of humans.

Here we report that the MERS-CoV receptor is expressed in the human lower respiratory tract but not in the upper respiratory tract epithelium. Similar results were recently reported by Meyerholz et al., who used a different MAb[21]. Our results with respect to the localization of DPP4 in the human lower respiratory tract are consistent with earlier studies showing MERS-CoV tropism in the alveolar and bronchial epithelial cells of *ex vivo* infected human lung tissue samples[22]. The presence of the receptor at this location is also in line with clinical observations showing that MERS is considered in essence a lower respiratory tract infection and the fact that MERS-CoV RNA is detected in larger amounts in the tracheal aspirate and sputum samples of MERS patients than in nasal or throat swabs[13,23]. The lack of DPP4 in the human upper respiratory tract epithelium may limit MERS-CoV infection and replication at this site and hence impede viral transmission. Expression of viral receptors in the upper respiratory tract epithelium has been shown to be critical in the transmission of viral infections, as exemplified by respiratory infections caused by influenza viruses. Efficient airborne transmission of influenza viruses between humans and ferrets requires binding to α2,6-sialic acid, which is strongly expressed in the upper respiratory tract. In contrast, influenza viruses that bind exclusively to α2,3-sialic acid, which is expressed mostly in the lower respiratory tract, are less likely to be transmitted[24].

Although there is limited DPP4 expression in the human upper respiratory tract

epithelium, we observed expression of the MERS-CoV receptor in glands located in the submucosa of the upper respiratory tract. These glands have been shown to be targeted by other coronaviruses, such as SARS-CoV and rat sialodacryoadenitis virus[25,26]. We therefore cannot exclude the possibility that MERS-CoV can replicate in submucosal glands that are connected to the respiratory epithelium by their secretory ducts. It remains to be investigated whether viral replication in patients who have been shown to shed MERS-CoV for a long time could be linked to the presence of virus at these locations. The susceptibility of these cells and their capacity to support MERS-CoV replication need to be investigated in future studies.

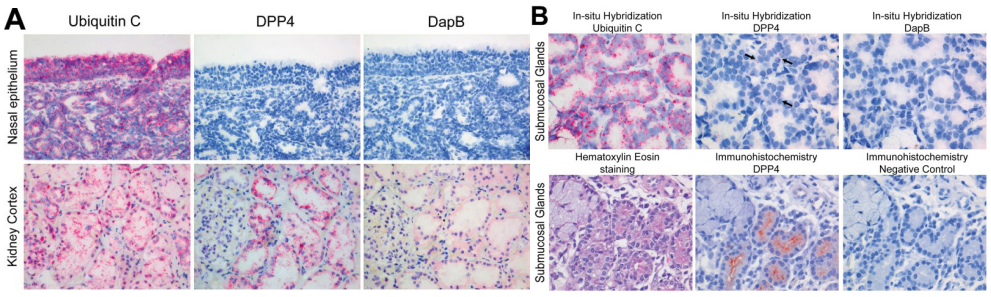

Figure 2. Presence of DPP4 mRNA and protein in the human nasal epithelium and submucosal glandular epithelial cells. (A) DPP4 mRNA was detected in the kidney but not in the nasal epithelium (×200 magnification). (B) DPP4 mRNA (arrows) and protein were detected in the submucosal gland cells by in situ hybridization and immunohistochemistry, respectively (×400 magnification). A positive in situ hybridization signal is marked by red dots. Kidney tissue was used as a positive control for both in situ hybridization and immunohistochemistry. For in situ hybridization, ubiquitin C and DapB mRNAs were used as positive and negative controls, respectively.

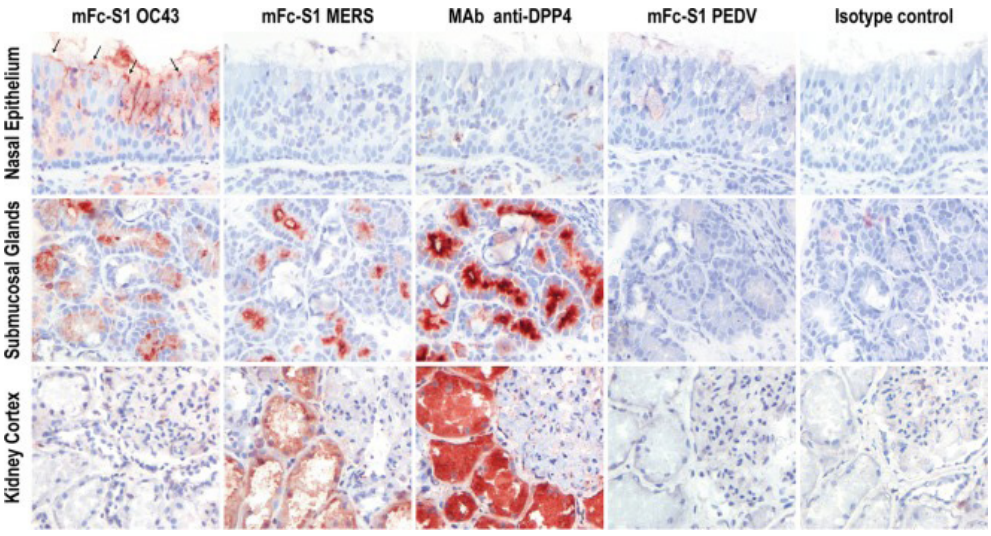

Figure 3. mFc-S1 MERS binds to human kidney proximal tubuli and nasal submucosal glands but not nasal epithelium. Mouse MAb against human DPP4 (MAb anti-DPP4) showed binding similar to that of mFc-S1 MERS-CoV. mFc-S1 OC43 binds to the nasal epithelium (indicated by arrows) and was used as a positive control. As a negative control, mFc-S1 PEDV and mouse IgG2a and IgG2b isotype antibodies were used. The panels showing the mouse isotype control antibody are representative of the two isotype control antibodies used in this experiment. Positive staining is red. All panels were made at magnification of ×200.

Although DPP4 is not expressed in the human upper respiratory tract epithelium tissue samples analyzed in this study, it remains possible that the expression pattern could depend on several factors. DPP4 expression in the lower respiratory tract seemed to vary between individuals and as shown by previous studies with T lymphocytes, DPP4 is not stably expressed on the cell surface but can be upregulated upon activation[16]. Interestingly, one study demonstrated that cultured primary human nasal epithelial cells expressed DPP4[27], which likely reflects upregulated expression as a result of cell division, as also observed in different cell lines[28]. Whether DPP4 expression in respiratory tract tissues is regulated by certain host or environmental factors remains to be studied. In general, our study highlights a critical difference between humans and camels in the distribution of DPP4 expression. Future studies should investigate this DPP4 distribution in other species, which would be relevant to further understand the transmission of MERS-CoV.

Acknowledgments

We thank Debby van Riel for the paraffin-embedded human respiratory tract tissue materials used in this study; Sarah Getu and Lonneke van Nes-Leijten for their technical suggestions on the in situ hybridization and immunohistochemistry staining method; David Solanes, Xavier Abad, Ivan Cordón, Mónica Pérez, and all of the animal caretakers at the CReSA biosecurity level 3 animal facilities for their technical assistance; and Alex Kleinjan and the Erasmus MC Tissue Bank for providing tissue samples for this study.

This study was supported by a TOP Project grant (91213066) funded by ZonMW and as part of the Zoonotic Anticipation and Preparedness Initiative (ZAPI project; IMI grant agreement no. 115760) with the assistance and financial support of IMI and the European Commission. The funders had no role in study design, data collection and interpretation, or the decision to submit the work for publication.

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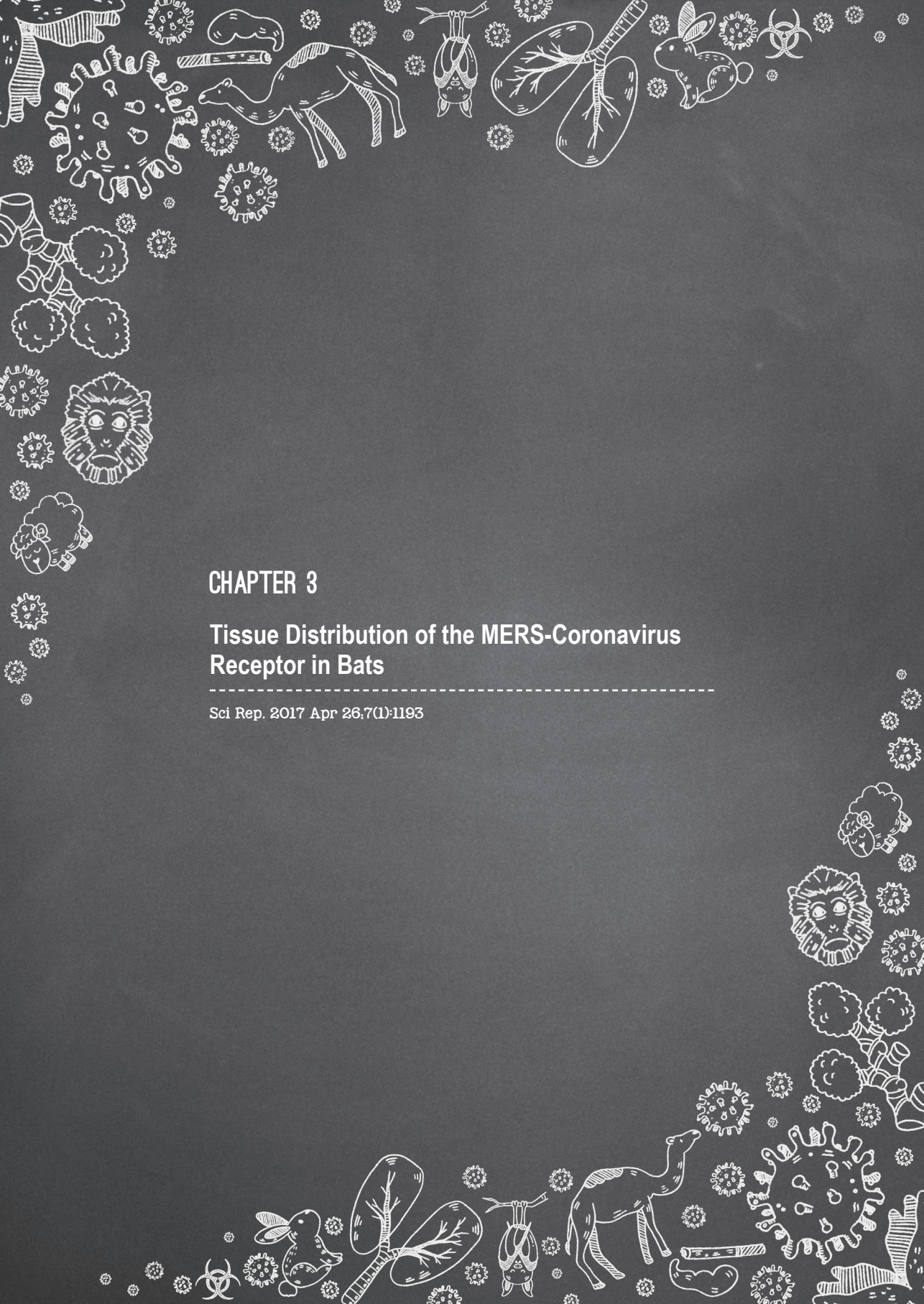

CHAPTER 3

Tissue Distribution of the MERS-Coronavirus Receptor in Bats

Sci Rep. 2017 Apr 26;7(1):1193

Abstract

Middle East respiratory syndrome coronavirus (MERS-CoV) has been shown to infect both humans and dromedary camels using dipeptidyl peptidase-4 (DPP4) as its receptor. The distribution of DPP4 in the respiratory tract tissues of humans and camels reflects MERS-CoV tropism. Apart from dromedary camels, insectivorous bats are suggested as another natural reservoir for MERS-like-CoVs. In order to gain insight on the tropism of these viruses in bats, we studied the DPP4 distribution in the respiratory and extra-respiratory tissues of two frugivorous bat species (*Epomophorus gambianus* and *Rousettus aegyptiacus*) and two insectivorous bat species (*Pipistrellus pipistrellus* and *Eptesicus serotinus*). In the frugivorous bats, DPP4 was present in epithelial cells of both the respiratory and the intestinal tract, similar to what has been reported for camels and humans. In the insectivorous bats, however, DPP4 expression in epithelial cells of the respiratory tract was almost absent. The preferential expression of DPP4 in the intestinal tract of insectivorous bats, suggests that transmission of MERS-like-CoVs mainly occurs via the fecal-oral route. Our results highlight differences in the distribution of DPP4 expression among MERS-CoV susceptible species, which might influence variability in virus tropism, pathogenesis and transmission route.

Introduction

Middle East respiratory syndrome coronavirus (MERS-CoV) emerged in the human population in 2012 and has been causing multiple outbreaks of human disease, mainly in the Arabian Peninsula[1]. The dromedary camel (*Camelus dromedarius*) has been shown to be the reservoir host for primary human infections[2-8], although other susceptible animals[9-11], including bats[12,13], are suspected also to be hosts for this virus. MERS-like-CoVs have been sequenced from bat samples, mainly from insectivorous bats, but they have not yet been successfully isolated[14-21]. Screening of over 5000 insectivorous bats from Ghana, Ukraine, Romania, Germany, and the Netherlands showed that MERS-CoV-like viruses were detected in 24.9% of Nycteris bats and 14.7% of Pipistrelle bats[14].

MERS-CoV uses dipeptidyl peptidase-4 (DPP4) as its receptor to infect its target cells, including bat cells[22]. Analysis of DPP4 sequences from different bat species and *in vitro* infection studies with various bat cell lines suggested that multiple bat species are susceptible to MERS-CoV[12,22,23]. MERS-like-CoVs probably also use DPP4 as indicated by studies on the Tylonycteris bat CoV HKU4, one of the MERS-like-CoVs[21]. HKU4 uses DPP4 to infect both bat and human cells *in vitro*[24,25]. It is known that DPP4 is differently distributed in the respiratory tract of humans and other

susceptible livestock animals, including dromedary camels[4,11]. DPP4 expression in the nasal epithelium of the camel, llama, and pig allows them to develop upper respiratory tract infection upon intranasal inoculation with MERS-CoV[4,11,26], while in humans, DPP4 is exclusively expressed in the lower respiratory tract epithelium, which is in line with acute pneumonia being the main clinical outcome of MERS-CoV infection[4,27]. Additionally, the absence of DPP4 expression in the upper respiratory tract epithelium of sheep renders this tissue to be non-susceptible *in vivo*[11]. These data indicate that the localization of DPP4 expression in tissues reflects MERS-CoV susceptibility and tropism *in vivo*. The localization of DPP4 expression in bat tissues, however, has not been studied, unlike that in other MERS-CoV susceptible species[4,11].

Our study aimed to understand the tropism of MERS-like-CoVs in bats by mapping the distribution of DPP4 expression in tissues from four bat species. DPP4 immunohistochemistry staining was performed on tissues collected from two widespread insectivorous bat species in Europe and Asia, the common pipistrelle bat (*Pipistrellus pipistrellus*) and the serotine bat (*Eptesicus serotinus*)[28,29]; and two common frugivorous bat species in Africa, i.e. the Gambian epauletted fruit bat (*Epomophorus gambianus*) and the Egyptian fruit bat (*Rousettus aegyptiacus*)[30,31]. These four bat species were chosen based on their interactions with humans as they roost and forage in the human habitat or serve as a human food source[28-31]. We show that DPP4 localization varies not only among MERS-CoV susceptible species[4,11], but also between bat species, which may imply variability in MERS-like-CoVs tropism, pathogenesis, and transmission route.

Results

Immunohistochemistry to detect DPP4 was performed on nose, lung, intestine, kidney, salivary gland, and liver tissues of different bat species: common pipistrelle bat, serotine bat, Gambian epauletted fruit bat (further referred as Gambian fruit bat), and Egyptian fruit bat. The assay was replicated two-three times for each tissue. We have used the same technique to map DPP4 localization in the respiratory tract tissues of human, dromedary camel, sheep, horse, pig, and llama[4,11]. The antibody used in this study recognizes bat DPP4 as was demonstrated in transfection experiments using cloned Pipistrelle bat DPP4[22]. Hematoxylin and eosin staining on subsequent slides of the same tissues from the bats did not show significant histological changes.

DPP4 was not found in the nasal olfactory epithelial cells of common pipistrelle bat, serotine bat, Gambian fruit bat, or Egyptian fruit bat (Fig. 1). In the nasal tissues of common pipistrelle bat, DPP4 was not detected in the respiratory epithelial cells lining the nasal cavity, but was detected in the epithelial cells lining the ducts of the

submucosal glands in this species. In the serotine bat and Gambian fruit bat, multifocal DPP4 expression was detected in a limited number of nasal respiratory epithelial cells. In contrast, in the nasal tissues of the Egyptian fruit bat, DPP4 was prominently detected at the apical surface of the respiratory epithelial cells lining the nasal cavity as well as in glandular and ductular epithelial cells of the submucosal glands. In the lungs of the common pipistrelle and serotine bat, DPP4 was found in the endothelial cells of the capillaries but not in the bronchial, bronchiolar or alveolar epithelial cells. In the Gambian and Egyptian fruit bat, DPP4 was detected in the bronchial, bronchiolar and alveolar epithelial cells as well as in endothelial cells of small blood vessels (Fig. 1).

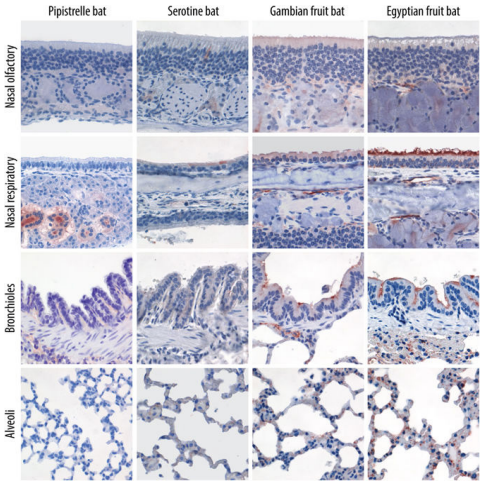

Figure 1. DPP4 expression in the respiratory tract tissues of common pipistrelle bat, serotine bat, Gambian epauletted fruit bat, and Egyptian fruit bat. DPP4 (indicated in red) is expressed in the nasal, bronchiolar and alveolar epithelium of the fruit bats, with limited expression in the epithelium lining the nasal cavity of serotine bats, and not detected in the epithelium lining the respiratory tract of the common pipistrelle bats. Original magnification x400 for all images.

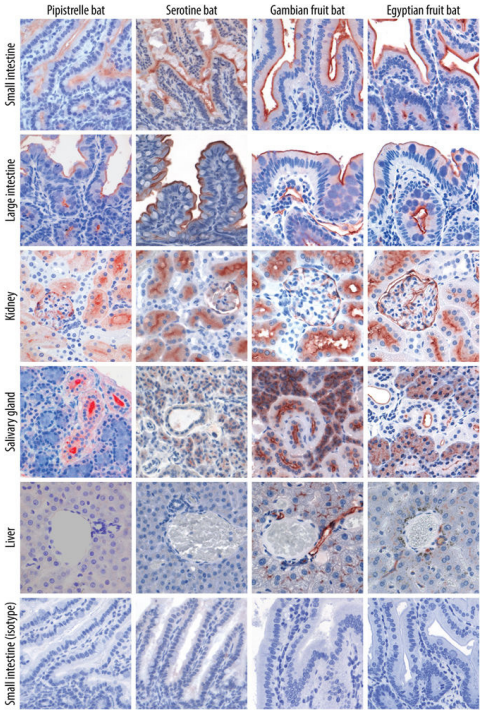

Figure 2. DPP4 expression in the intestine, kidney, salivary gland, and liver tissues of the common pipistrelle bat, serotine bat, Gambian epauletted fruit bat, and Egyptian fruit bat. In all four bat species, DPP4 (indicated in red) is detected on the apical surface of the intestinal epithelium, proximal tubular epithelium of the kidney, and in the salivary glands. Normal goat serum is used as isotype control for each tissue and showed no background signal. Only isotype control staining of the small intestines is shown. Original magnification x400 for all images.

In the intestinal tissues of all four bat species, DPP4 was prominently expressed on the apical surface of both small and large intestinal epithelial cells (Fig. 2). In the kidney of all four bat species, DPP4 was found in glomerular cells, parietal squamous epithelial cells of the Bowman's capsule, and in the proximal tubular epithelial cells. In the salivary gland of common pipistrelle bat, DPP4 was only detected in the ductular epithelial cells, while in the serotine bat, it was detected in a limited number of glandular epithelial

cells. In the Gambian and Egyptian fruit bat, it was detected in both the glandular and ductular epithelial cells of the salivary gland. In the liver of the common pipistrelle bat and serotine bat, DPP4 was present in a limited number of endothelial cells lining the sinusoids. In contrast, in the liver of the Gambian and Egyptian fruit bat, DPP4 was detected in the bile duct epithelial cells, in the endothelial cells of the hepatic arteries, and in the endothelial cells of the sinusoids (Fig. 2). Variation in DPP4 signal and localization were occasionally observed between animals within the same species. In one common pipistrelle bat, the paranasal sinus and pharynx were examined and showed a limited number of DPP4 positive epithelial cells. The results of the DPP4 immunohistochemistry staining were scored qualitatively and summarized in Table 1.

In general, our results showed that DP-P4 was prominently expressed in the intestine and the respiratory tract tissues of the frugivorous bats, i.e. the Gambian and the Egyptian fruit bat. However, it is limitedly expressed in the respiratory tract tissues of the insectivorous bats, i.e. the common pipistrelle bat and the serotine bat. In the common pipistrelle bat, DPP4 was not detected in the nasal respiratory, nasal olfactory, bronchiolar, or alveolar epithelium, but was abundant on the apical surface of the epithelium lining the small and large intestine. We compared these findings to our previous results on dromedary camel and human tissues[4]. In dromedary camels, DPP4 is strongly detected in the nasal respiratory, tracheal, and bronchial epithelium, while there is limited expression in the alveolar epithelium (Fig. 3). In humans, it is not found in the nasal epithelium and is present mainly in the alveolar epithelium. Additionally, we performed DPP4 staining on intestinal tissues of dromedary camels obtained from a previous study[26]. We found that DPP4 was expressed mainly on the apical surface of the small intestinal epithelium (data not shown), similar to what has been reported for humans[32-35](Fig. 3).

Discussion

The tissue distribution of the MERS-CoV receptor, DPP4, has previously been studied in humans, dromedary camels, and other livestock animals[4,11]. Here, we show that DPP4 is differentially expressed among bat species, especially between insectivorous and frugivorous bats. It is strongly detected in the intestine of the common pipistrelle bat, the serotine bat, the Gambian fruit bat and the Egyptian fruit bat. It is also prominent in the respiratory tract epithelium of the Gambian and Egyptian fruit bat, but expression is limited in that of the common pipistrelle and serotine bat. Given the essential role of DPP4 in the entry of MERS-CoV into cells, these results suggest that MERS-like-CoVs are not likely able to replicate in the respiratory tract in these two insectivorous bats. This is in line with our previous report on MERS-CoV infection experiment in sheep, showing that the lack of DPP4 in the respiratory tract of the sheep was associated

with restricted MERS-CoV replication in these animals upon intranasal inoculation[11]. Rather, in these two insectivorous bats, MERS-like-CoVs may preferentially replicate in the gastrointestinal tract. This is partly supported by the fact that viral genomes of MERS-like-CoVs were mainly obtained from faecal and intestinal tissue samples of insectivorous bats[14-20,36]. This intestinal tropism indicates that these viruses transmit mainly through the fecal-oral route. Therefore, future screening of MERS-like-CoVs from insectivorous bats, particularly the common pipistrelle bat, might focus on fecal material, rectal swabs, or intestinal tissues, rather than throat or nasal swabs.

Table 1. Overview of DPP4 expression in the tissues of the common pipistrelle bat, serotine bat, Gambian epauletted fruit bat and Egyptian fruit bat.

	Common pipistrelle bat	Serotine bat	Gambian fruit bat	Egyptian fruit bat
Nose				
✓ Nasal respiratory epithelial cells	-	+/-	+/-	+
✓ Nasal olfactory epithelial cells	-	-	-	-
✓ Submucosal glands	+	+/-	+/-	+
Lung				
✓ Bronchiolar epithelial cells	-	-	+/-	+
✓ Alveolar epithelial cells	-	-	+/-	+
✓ Endothelial cells of the capillaries and small blood vessels	+/-	+/-	+	+
Intestine				
✓ Small intestinal epithelial cells	+	+	+	+
✓ Large intestinal epithelial cells	+	+	+	+
Kidney				
✓ Glomerular cells	+	+	+	+
✓ Parietal squamous epithelial cells of the bowman capsule	+	+	+	+
✓ Proximal tubular epithelial cells	+	+	+	+
Salivary gland				
✓ Glandular epithelial cells	-	+/-	+/-	+
✓ Ductular epithelial cells	+	-	+	+
Liver				
✓ Hepatocytes	-	-	-	-
✓ Bile ductular epithelial cells	-	-	+	+
✓ Endothelial cells of the hepatic vein	-	-	-	-
✓ Endothelial cells of the hepatic artery	-	-	+	+
✓ Endothelial cells of the sinusoids	-	+/-	+	+

+, positive detection; +/-, only expressed in a limited number of cells; -, undetected.

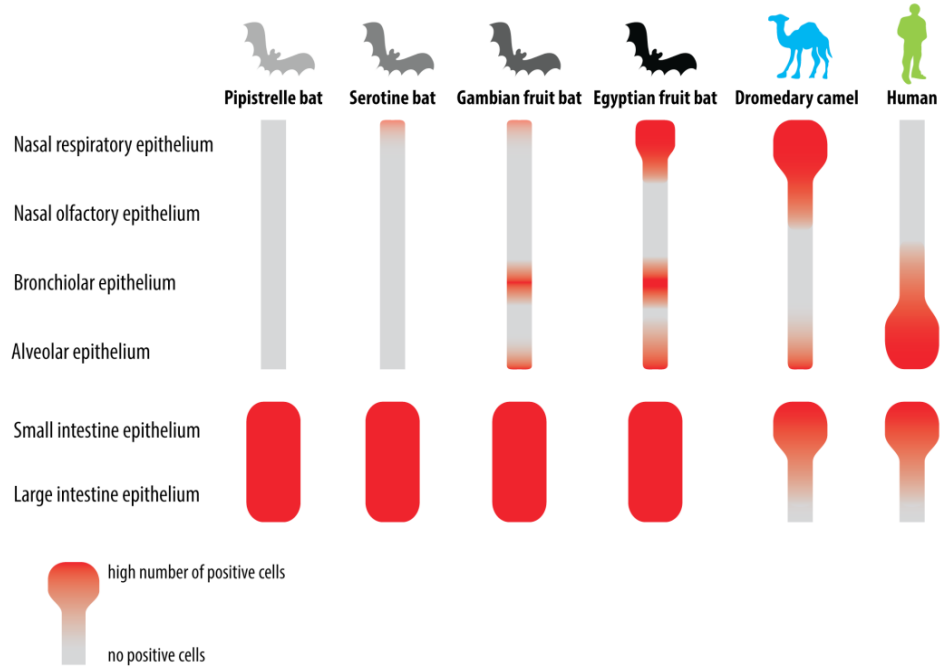

Figure 3. Different distribution of DPP4 expression in the lining respiratory tract and intestinal epithelium of the common pipistrelle bat, serotine bat, Gambian fruit bat, Egyptian fruit bat, dromedary camel, and human. In the common pipistrelle bat and serotine bat, DPP4 is limitedly detected in the respiratory tract and mainly expressed in the intestinal epithelium. In the Gambian and Egyptian fruit bat, DPP4 is found both in the respiratory tract and in the intestinal epithelium. In the dromedary camel, it is expressed in the upper respiratory tract and small intestine epithelium. In the human, it is predominantly expressed in the lower respiratory tract and small intestine epithelium.

Prominent DPP4 expression in both respiratory tract and intestinal epithelium of the Gambian fruit bat and the Egyptian fruit bat suggests that MERS-CoV is able to replicate in both the respiratory tract and intestine of the fruit bats. These results are in line with the fact that MERS-CoV was able to replicate in the lungs of Jamaican fruit bat (*Artibeus jamaicensis*) upon intranasal and intraperitoneal inoculation[37]. Interestingly, viral RNA could be detected in the rectal swabs of these animals up to day 9 p.i. and infectious virus was also isolated in the duodenum of one of the bats at day 28 p.i.[37]. These data suggest that MERS-CoV infects and replicates in the intestine of these bats, not only in the respiratory tract. MERS-CoV infection in these bats is likely mediated by DPP4, since hamster BHK cells, a non-susceptible cell line, could be infected by MERS-CoV when modified to express Jamaican fruit bat's DPP4[37]. DPP4 expression in the intestine and respiratory tract of these Jamaican fruit bats, however, was not investigated. Since the Jamaican fruit bat is a new world fruit bat, unlike the Gambian fruit bat and the Egyptian fruit bat, which are old world fruit bats, their genetic difference might influence the variation in DPP4 expression among these species. In contrast to the fruit bats, where DPP4 is expressed throughout the

respiratory tract, DPP4 is rarely detected in the respiratory tract tissues of insectivorous bats. This limited DPP4 expression in insectivorous bats might significantly restrict the replication of MERS-like-CoVs in these tissues and minimize the possibility of transmission of these viruses from the respiratory tract.

The limited DPP4 expression in the respiratory tract of the two insectivorous bat species, particularly the common pipistrelle bat, is different from what has been reported for dromedary camels and humans. In humans, DPP4 is merely expressed in the lower respiratory tract, while in the dromedary camels, it is detected in the upper respiratory tract epithelium[4]. This renders humans to develop pneumonia upon MERS-CoV infection, while camels develop upper respiratory tract infection[26,38,39]. In the intestine of both dromedary camels and humans, DPP4 is mainly present in the apical surface of the small intestine epithelium[32-35]. MERS-CoV has been isolated from faecal samples of a naturally infected dromedary camel, which suggests that this virus is able to replicate in the intestinal tract of this species[40]. However, in dromedary camels, the chance of detecting MERS-CoV RNA in faecal samples is much lower than from nasal swabs[40]. We also observed that low amounts of viral RNA are detectable in rectal swabs taken from MERS-CoV- inoculated dromedary camels[26]. While MERS-CoV has not yet been isolated from human faecal samples, low amounts of viral RNA could be detected in stool samples of MERS patients[41], and several MERS patients have also been reported to suffer from diarrhoea[42-44]. These observations suggest that MERS-CoV replicates in the intestine of both dromedary camels and humans although only to a limited extent. It is currently unclear what factors restrain MERS-CoV replication in the intestinal tract of dromedary camels and humans. The human intestinal tract is protected by a mucus layer, commensal microorganisms, multiple innate and adaptive immune cells[45]. Also, adenosine deaminase (ADA), a natural antagonist of DPP4 that can inhibit MERS-CoV infection *in vitro*[9], has also been found in the human intestine. The amount of ADA in the human intestine is four times higher compared to that in the lung[46]. The presence of DPP4 in the intestinal tract of bats suggests an intestinal tropism of MERS-like-CoVs. We also detected DPP4 in the salivary glands and kidneys in all of the bats. *In vitro*, MERS-CoV has also been shown to replicate in primary kidney cell culture derived from common pipistrelle bat[13]. However, there has been no further evidence supporting the susceptibility of these two tissues *in vivo*, nor have there been any reports of MERS-like-CoVs isolated from these two tissues or from bat urine samples. Whether these viruses are transmitted through bat saliva or urine, therefore, is currently unclear.

In general, our study describes the variation in DPP4 distribution among four bat species, with notable differences between insectivorous and frugivorous bats. More importantly, the tissue distribution of DPP4 in insectivorous bats, believed to be one

of the natural hosts for MERS-like-CoVs, is different to that in dromedary camels and humans. Our results indicate intestinal tropism of MERS-like-CoVs in the insectivorous bats we examined. The existence of a co-receptor that might influence MERS-like-CoVs tropism and replication in these bats, however, could not be disregarded. CEACAM5 is recently reported as an attachment factor that facilitates entry of MERS-CoV *in vitro*[47]. Whether CEACAM5 plays an important role *in vivo*, particularly in bats, remains to be investigated. *In vivo* infection experiments are necessary to confirm our findings, but such studies are ethically and technically challenging. Nevertheless, our data are relevant for future monitoring and surveillance of MERS-like-CoVs in insectivorous bats, particularly in the common pipistrelle bat[14,18], as well as for future efforts to better understand the pathogenesis and transmission of MERS-like-CoVs in their natural host.

Materials and Methods

Common pipistrelle and serotine bats were found stranded and severely wounded on different occasions, and admitted to an official local bat shelter in the Netherlands. The animals were euthanized by veterinarians due to ethical reasons using officially approved methods. The Gambian fruit bats and three of four Egyptian fruit bats used in this study originated from free-ranging populations in Ghana. The bats were sampled for an unrelated study and this study was approved by the Ethics Committee of the Zoological Society of London (ref. WLE715) and the council for scientific and industrial research in Accra, Ghana. One of the Egyptian fruit bats was obtained from the captive colony at the Friederich Loeffler Institute, Riems, Germany. It had been euthanized due to reasons not related to this study. All methods were performed in accordance with the relevant guidelines and regulations.

After euthanasia, the bats were necropsied and tissues were collected. Parts of the lung, intestine, salivary gland, liver, and kidney were obtained from nine common pipistrelle bats, seven serotine bats, three Gambian fruit bats, and four Egyptian fruit bats. Parts of the noses were obtained from five common pipistrelle bats, six serotine bats, three Gambian fruit bats, and three Egyptian fruit bats. These tissues were all fixed in 10% formalin and embedded in paraffin. The noses were decalcified in 10% EDTA for 9 days before being embedded in paraffin. The formalin fixed paraffin embedded tissues were sectioned (4 μm), deparaffinized, and subsequently hydrated. Citric acid buffer 10 mM pH 6 was used to retrieve antigens. Blocking with normal rabbit serum 5% was performed prior to staining with polyclonal goat IgG anti-DPP4 (R&D systems, Abingdon, UK) in 5 μg/ml concentration. Normal goat serum (MP Biomedicals, Santa Ana, CA, USA) in equal concentration was used as negative control. DPP4 staining was performed at 4 °C overnight. Secondary antibody rabbit

anti-goat IgG labeled with peroxidase were applied subsequently in 1:200 dilution for 1 hour at room temperature (Dako, Glostrup, Denmark). The red signal was revealed with 3-amino-9-ethyl-carbazole (Sigma-Aldrich, St. Louis, Missouri, USA) before counterstaining with hematoxylin.

Dromedary camel intestinal tissues were obtained from three different animals sacrificed at day 14 post infection with MERS-CoV in a previous MERS-CoV vaccination experiment[26]. Two of these animals were vaccinated beforehand, while one was not. MERS-CoV was not detected in the intestinal tissues of these animals using PCR, virus titration or immunohistochemistry detecting nucleoprotein of MERS-CoV. Information on DPP4 expression in human respiratory and intestinal tissues was derived from the previous studies[4,32-34].

Acknowledgements

This study is supported by a TOP project Grant (91213066) and by the Zoonoses in the night project (5O-52200-98-308) both funded by ZonMW. The *E. gambianus* and three of four *R. aegyptiacus* used in this study originated from a study in Ghana in collaboration with Richard Suu-Ire, from the Forestry Commission, Accra. We would like to thank Anne Buschmann-Balkema for her assistance in preparing the *R. aegyptiacus* tissues that come from the Friederich Loeffler Institute, Riems, Germany. We would like to thank Stichting Vleermuisopvang Oss for providing the tissues of *P. pipistrellus*. We thank Brigitta M Laksono for her advice on the schematic figure.

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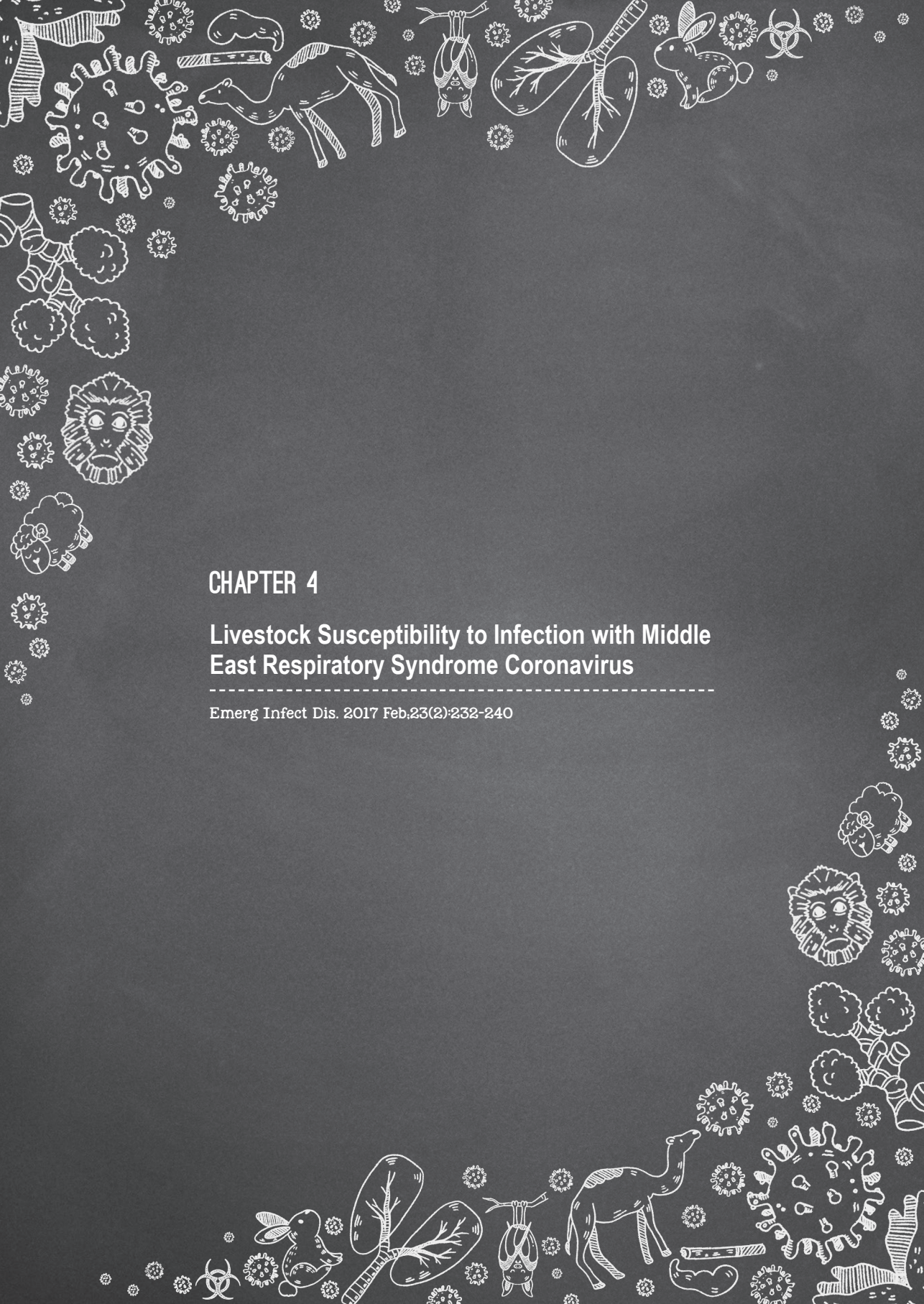

CHAPTER 4

Livestock Susceptibility to Infection with Middle East Respiratory Syndrome Coronavirus

Emerg Infect Dis. 2017 Feb;23(2):232-240

Abstract

Middle East respiratory syndrome (MERS) cases continue to be reported, predominantly in Saudi Arabia and occasionally other countries. Although dromedaries are the main reservoir, other animal species might be susceptible to MERS coronavirus (MERS-CoV) infection and potentially serve as reservoirs. To determine whether other animals are potential reservoirs, we inoculated MERS-CoV into llamas, pigs, sheep, and horses and collected nasal and rectal swab samples at various times. The presence of MERS-CoV in the nose of pigs and llamas was confirmed by PCR, titration of infectious virus, immunohistochemistry, and in situ hybridization; seroconversion was detected in animals of both species. Conversely, in sheep and horses, virus-specific antibodies did not develop and no evidence of viral replication in the upper respiratory tract was found. These results prove the susceptibility of llamas and pigs to MERS-CoV infection. Thus, the possibility of MERS-CoV circulation in animals other than dromedaries, such as llamas and pigs, is not negligible.

Introduction

Middle East respiratory syndrome (MERS) coronavirus (MERS-CoV) first emerged in 2012 in Saudi Arabia and is currently a worldwide concern[1]. As of September 21, 2016, the World Health Organization had confirmed ~1,800 MERS cases and 643 associated deaths[2]. Although during most reported outbreaks the virus is mainly transmitted by human-to-human contact, infection through contact with dromedary camels (*Camelus dromedarius*) plays a major role in the primary cases. In the Middle East and some countries from East Africa where MERS is endemic, high prevalence of MERS-CoV–specific antibodies in dromedaries has been reported[3-7]. Moreover, a recent surveillance study in Saudi Arabia demonstrated that MERS-CoV strains isolated from humans were also detected in the upper respiratory tract of dromedaries of several geographic origins, indicating that the virus did not require mutations to jump between species[8]. However, not all index cases can be explained by direct contact with dromedaries, and transmission from other domestic livestock or animal species to humans cannot yet be ruled out. Recently, evidence that alpacas (*Vicugna pacos*) were also susceptible to MERS-CoV infection was provided and confirmed by field studies performed in Qatar[9-11]. In contrast, despite the ability of the virus to infect a plethora of cell lines and tissues from mammals of multiple species *in vitro*[12], serologic surveys of ruminants and horses did not conclusively determine circulation of MERS-CoV among these domestic animals[3,13,14]. Sampling design could explain negative results, and experimental infections provide, in many instances, a more straightforward answer to virus host susceptibility. This knowledge is crucial for determining risk factors with regard to possible globalization of the disease.

Our aim with this study was to address these critical research gaps and to understand the potential role that other animals (besides dromedaries and alpacas) could play in MERS-CoV dissemination. We experimentally inoculated MERS-CoV into llamas (*Lama glama*), pigs (*Sus scrofa*), horses (*Equus ferus caballus*), and sheep (*Ovis aries*). We based our selection of species on epidemiologic interest and on sequence similarities in the MERS-CoV receptor binding domain of dipeptidyl peptidase-4 (DPP4).

Materials and Methods

Ethics

All experiments with MERS-CoV were performed at Biosafety Level 3 (BSL-3) facilities of the Biocontainment Unit of the Institut de Recerca i Tecnologia Agroalimentàries (IRTA) Centre de Recerca en Sanitat Animal (CReSA) in Barcelona, Spain. The study was approved by the Ethical and Animal Welfare Committee of IRTA and the Ethical Commission of Animal Experimentation of the Autonomous Government of Catalonia.

Cells and MERS-CoV

Vero cells were cultured in Dulbecco modified Eagle medium (DMEM; Lonza, Basel, Switzerland) supplemented with 1% fetal calf serum (EuroClone, Pero, Italy), 100 U/mL penicillin, 100 mg/mL streptomycin, and 2 mmol/L glutamine. Passage 7 human isolate MERS-CoV stock (HCoV-EMC/2012) was propagated in Vero cells at 37°C in a CO_2 incubator for 3 days. The infectious virus titer was determined in Vero cells and calculated by determining the dilution that caused cytopathic effect in 50% of the inoculated cell cultures (50% tissue culture infectious dose endpoint [$TCID_{50}$]).

Animal Studies

All animals used in this study were obtained from France and Spain by private sale and housed at BSL-3 animal facilities (IRTA-CReSA, Barcelona, Spain). We obtained 8 llamas (6–8 months of age), 8 horses (6–8 months), 14 sheep (2–3 months), and 14 pigs (2 months). We intranasally inoculated a 10^7 $TCID_{50}$ dose in 3 mL saline solution into each animal (1.5 mL in each nostril) by using a mucosal atomization device (LMA; North-America, Inc., San Diego, CA, USA) and monitored the animals daily for clinical signs (sneezing, coughing, nasal discharge, dyspnea). Rectal temperatures were recorded with a fast display digital thermometer (Digi-Temp; Kruuse Veterinary Products, Langeskov, Denmark) until postinoculation day 10. Nasal and rectal swabs were obtained on postinoculation days 1, 2, 3, 4, 7, 10, 14, and 24 in phosphate-

buffered saline (PBS) (for PCR analysis) and DMEM (for virus isolation and titration) containing antimicrobial drugs (100 U/mL penicillin and 0.1 mg/mL streptomycin). All samples were stored at −80°C until tested. Serum samples were obtained before challenge and at postinoculation days 14 and 24 and were subsequently used to detect MERS-CoV–specific antibodies.

Virus Titration

Nasal swabs collected at different times after inoculation were evaluated for infectious virus by titration in Vero cells. We prepared 10-fold dilutions, starting with a dilution of 1:10, and transferred the dilutions to Vero cells. Plates were monitored daily under a light microscope, and wells were evaluated for cytopathic effect. The final determination was conducted on postinoculation day 5. The amount of infectious virus in swab samples was calculated by determining the $TCID_{50}$.

Pathology Studies

On postinoculation day 2, we euthanized 4 pigs and 4 sheep with an overdose of pentobarbital followed by exsanguination; using the same procedure, on postinoculation day 4, we euthanized 4 animals of each species (including llamas and horses) and on postinoculation day 24, the remaining animals. We performed complete necropsies and collected respiratory tissues (frontal, medial, and caudal turbinates; proximal, medial and distal trachea; large and small bronchi; and right cranial, mediodorsal, and caudal lung parenchyma) for virologic, histopathologic, immunohistochemical (IHC), and in situ hybridization (ISH) examination.

Tissues for pathology studies were fixed by immersion in 10% neutral-buffered formalin, embedded in paraffin, and sectioned at 3 µm for slides. Sequential slides were either stained with hematoxylin and eosin or used to detect the DPP4 receptor and MERS-CoV antigen by IHC and viral genome by ISH[15,16]. In brief, we performed DPP4 IHC staining by using 5 µg/mL of polyclonal goat IgG anti-human DPP4 antibody (R&D Systems, Abingdon, UK) and peroxidase-labeled rabbit anti-goat IgG (1:200; DAKO; Agilent Technologies Company, Santa Clara, CA, USA) as a secondary antibody. To detect MERS-CoV antigen, we used a monoclonal antibody to the nucleocapsid protein (SinoBiological Inc., Beijing, China) as described[15]. We performed ISH according to the manufacturer's instructions by using probes targeting the nucleocapsid gene of MERS-CoV (Advanced Cell Diagnostics, Hayward, CA, USA). ISH staining was detected by using the Fast Red substrate as previously reported[17]. For detection of mucous substances, we stained selected slides (from animals euthanized on postinoculation day 24) with periodic acid–Schiff (PAS)

according to standard methods.

Viral RNA Detection by Reverse Transcription PCR

We collected tissues (0.2–0.5 g) for viral RNA quantification and placed them in cryotubes containing beads and 500 µL DMEM supplemented with 100 U/mL penicillin, 100 mg/mL streptomycin, and 2 mmol/L glutamine. In brief, samples were homogenized at 30 Hz for 2 min by using a TissueLyser II (QIAGEN, Hilden, Germany) and stored at −70°C until use. We extracted viral RNA from nasal swabs, rectal swabs, and tissue homogenates by using a NucleoSpin RNA virus kit (Macherey-Nagel, Düren, Germany) according to the manufacturer's instructions. The RNA extracts were tested by UpE PCR[18]. We conducted reverse transcription quantitative PCR (RT-qPCR) by using AgPath-ID One-Step RT-PCR reagents (Applied Biosystems, Foster City, CA, USA) and performed amplification by using a 7500 Fast Real-Time PCR System (Applied Biosystems) programmed as follows: 5 min at 50°C, 20 s at 95°C, and 45 cycles of 3 s at 95°C and 30 s at 60°C. We considered samples with a cycle threshold <40 positive for MERS-CoV RNA.

MERS-CoV S1 ELISA

We determined specific S1 antibodies in serum samples from postinoculation days 0, 14, and 24 by a MERS-CoV S1 ELISA as previously described[15], with some modifications. In brief, 96-well high-binding plates (Sigma-Aldrich, St. Louis, MO, USA) were coated with 100 µL of S1 protein at 1 µg/mL in PBS overnight at 4°C. After blocking with 1% bovine serum albumin/PBS/0.5% Tween20 for 1 h at 37°C, individual serum samples were added at 1:100, followed by 1 h incubation at 37°C. Plates were washed 4 times with PBS, and the species-specific secondary antibody was added. We used the following conjugates: anti-llama IgG:horseradish peroxidase (HRP) (diluted 1:60,000; no. A160–100P, Bethyl Laboratories, Montgomery, TX, USA); anti-pig IgG:HRP (diluted 1:20,000; no. A5670, Sigma-Aldrich); anti-horse IgG:HRP (diluted 1: 10,000; no. AAI38P, Bio-Rad, Hercules, CA, USA); and anti-sheep IgG:HRP (diluted 1:10,000; no. 5184–2504, Bio-Rad). After 1 h of incubation at 37°C, wells were washed 4 times with PBS, and TMB (3,3',5,5'-tetramethylbenzidine) substrate solution was added and allowed to develop for 8–10 min at room temperature, protected from light. We measured optical density at 450 nm.

MERS-CoV Neutralization Assay

We also tested serum samples collected on postinoculation days 0, 14, and 24 by a specific virus neutralization assay. First, the samples were inactivated at 56°C for

30 min. Following a previous protocol[15], we mixed 400 PFU of MERS-CoV (HCoV-EMC/2012) with serial 2-fold dilutions of heat-inactivated serum, incubated the mixture 1 h at 37°C, and inoculated it onto Huh7 cells. The presence of viral antigen was assessed 8 h after inoculation. Cells were fixed with formaldehyde and stained by using rabbit anti–MERS-CoV antibodies and fluorescein isothiocyanate–conjugated swine anti-rabbit immunoglobulins as secondary antibodies. We calculated 90% plaque-reduction neutralization test values for the MERS-CoV neutralization assay.

Results

Clinical Signs

After challenge, 3 of 8 llamas showed clinical signs (moderate mucus secretion in 1 nostril) at postinoculation days 4–18 (Technical Appendix, Figure 1, panel A). Soon after inoculation, mild excretion of white mucus from the nose was noted for 3 pigs. Minimal mucus excretion in the nose was noted at variable times (postinoculation days 2–16) for 3 horses. We did not detect nasal discharge in any of the sheep during the experiment. No animal of any species showed a significant increase in body temperature after MERS-CoV inoculation (Technical Appendix, Figure 1, panel B).

MERS-CoV RNA and Infectious Virus in Nasal Swab Samples and Viral RNA in Respiratory Tissues

Pigs and llamas excreted virus in the nose, as evidenced by RT-qPCR of nasal swab samples from postinoculation days 1–10; in 1 llama, viral RNA was detected until postinoculation day 15 (Figure 1, panel A). At postinoculation day 7, the amount of MERS-CoV RNA was still high in all llamas, but a decrease in RNA level was noted at postinoculation day 10. In pigs, high levels of MERS-CoV RNA were demonstrated until postinoculation day 4 and started decreasing at postinoculation day 7; only 1 animal remained positive at postinoculation day 10 (Figure 1, panel A). We subsequently tested positive nasal swab samples for the presence of infectious virus. During the experiment, 7 of 8 llamas and 7 of 14 pigs excreted infectious virus during at least 1 day from postinoculation day 2 on. We detected infectious virus until postinoculation day 4 in pigs and postinoculation day 7 in llamas (Figure 1, panel B). Relatively low levels of viral RNA were detected only until postinoculation day 2 in 5 of 8 inoculated horses and only at postinoculation day 1 in 7 of 14 sheep, suggesting the presence of residual inoculum in these animals (data not shown). We did not detect virus in rectal swab samples from any animal.

To determine the presence of viral RNA in tissues, we euthanized representative

numbers of animals on postinoculation days 2 (pigs), 4 (llamas and pigs), and 24 (llamas and pigs) and tested tissue homogenates by RT-qPCR. Early after infection, virus RNA transcripts were detected mainly in the nose, trachea, and small and large bronchi of llamas and pigs (Figure 2). At postinoculation day 24, viral RNA was detected only in some tissues (caudal nose and trachea) in 2 llamas (Figure 2).

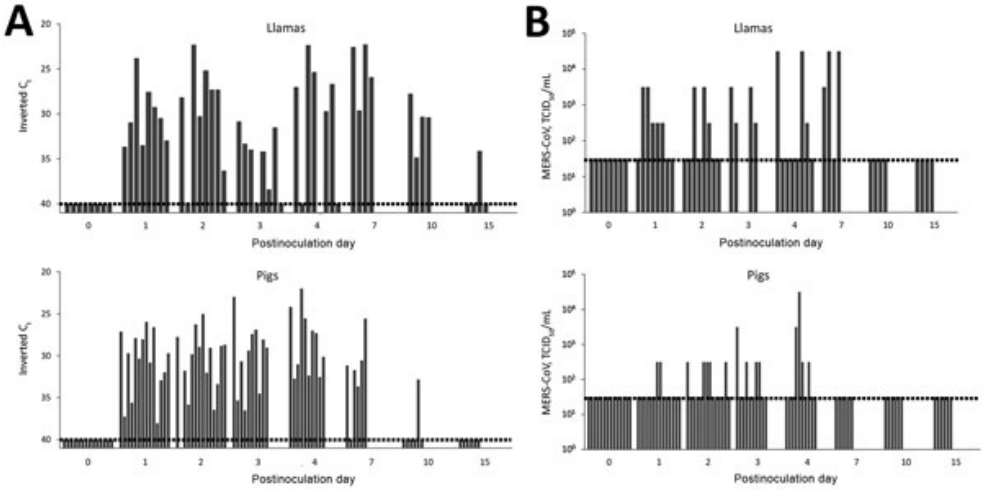

Figure 1. Viral shedding of llamas and pigs after experimental inoculation with MERS-CoV. A) Viral RNA and B) infectious MERS-CoV from nasal swab samples collected from llamas (top) and pigs (bottom) at different times after challenge. Each bar represents an individual animal. Dashed lines depict the detection limit of the assays. Ct, cycle threshold; MERS-CoV, Middle East respiratory syndrome coronavirus; TCID50, 50% tissue culture infective dose.

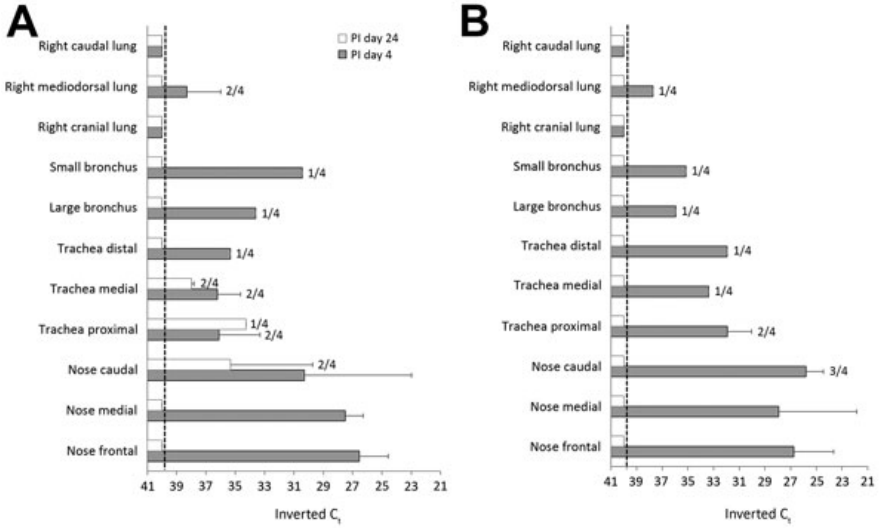

Figure 2. MERS-CoV viral RNA in respiratory tissues of llamas (A) and pigs (B). Viral RNA was determined in tissue homogenates at postinoculation days 4 and 24. Error bars indicate SDs when results were positive in >1 animal. Dashed lines depict the detection limit of the assays (Ct ≤40). Ct, cycle threshold; MERS-CoV, Middle East respiratory syndrome coronavirus; PI, postinoculation.

Pathology, IHC, and ISH

We observed no substantial macroscopic changes attributable to MERS-CoV infection in any animals. All horses exhibited purulent inflammation of the guttural pouch (empyema), which was most likely of bacterial origin.

At postinoculation day 4, IHC and ISH demonstrated virus antigen and nucleic acid in a few nasal respiratory epithelial cells in 3 of 4 llamas and in 2 of 4 pigs (Figure 3). At postinoculation day 2, occasional alveolar epithelial cells were positive for antigen and nucleic acid in 1 of 4 pigs. Also at postinoculation day 2, occasional bronchiolar cells from 1 of 4 sheep were positive for MERS-CoV (Technical Appendix, Figure 2). MERS-CoV antigen was absent in the rest of the respiratory tissues of animals of all species collected on any of the days. Associated with the presence of virus antigen and nucleic acid, llamas and pigs demonstrated a mild to moderate rhinitis characterized by mild to moderate epithelial necrosis with infiltration of variable numbers of neutrophils in the epithelium (exocytosis) and in the lumen and mild to severe infiltration of the lamina propria with variable numbers of macrophages, lymphocytes, neutrophils, and plasma cells and multifocal mild edema. We also observed mild to moderate multifocal epithelial hypertrophy consistent with regeneration (Figure 3). We observed no other relevant microscopic lesions in horses (besides the evidence of purulent empyema) and sheep.

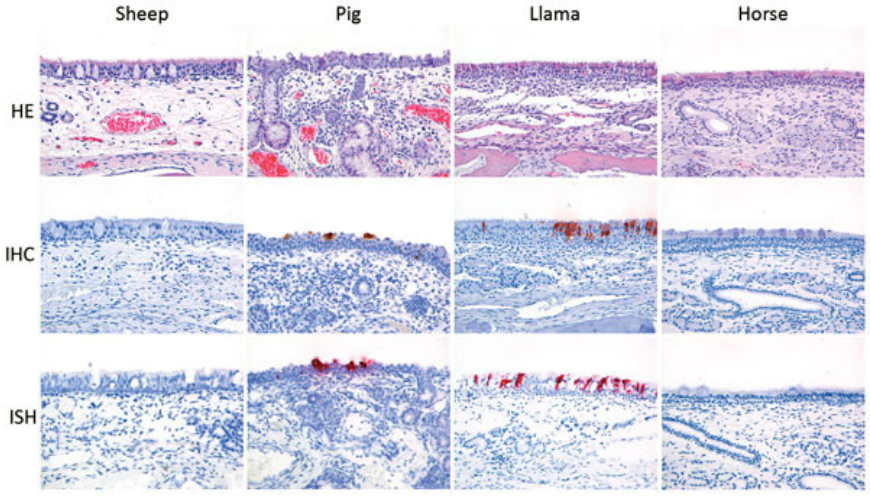

Figure 3. Histology and expression of viral antigen (IHC) and viral RNA (ISH) at postinoculation day 4 in the nasal respiratory epithelium of sheep, pigs, llamas, and horses inoculated with MERS-CoV. A mild to severe rhinitis with epithelial necrosis and hypertrophy and inflammation of the epithelium and lamina propria was observed in the nasal respiratory tissue of pigs and llamas. Associated with these was presence of virus antigen (IHC) and RNA (ISH). No substantial lesions, virus antigen, or virus RNA were detected in the nasal respiratory tissues of sheep and horses (HE, IHC, ISH). Original magnification ×200 for all images. HE, hematoxylin and eosin; IHC, immunohistochemistry; ISH, in situ hybridization; MERS-CoV, Middle East respiratory syndrome coronavirus.

DPP4 Receptor Distribution and Presence of Mucosubstances in Respiratory Tissues

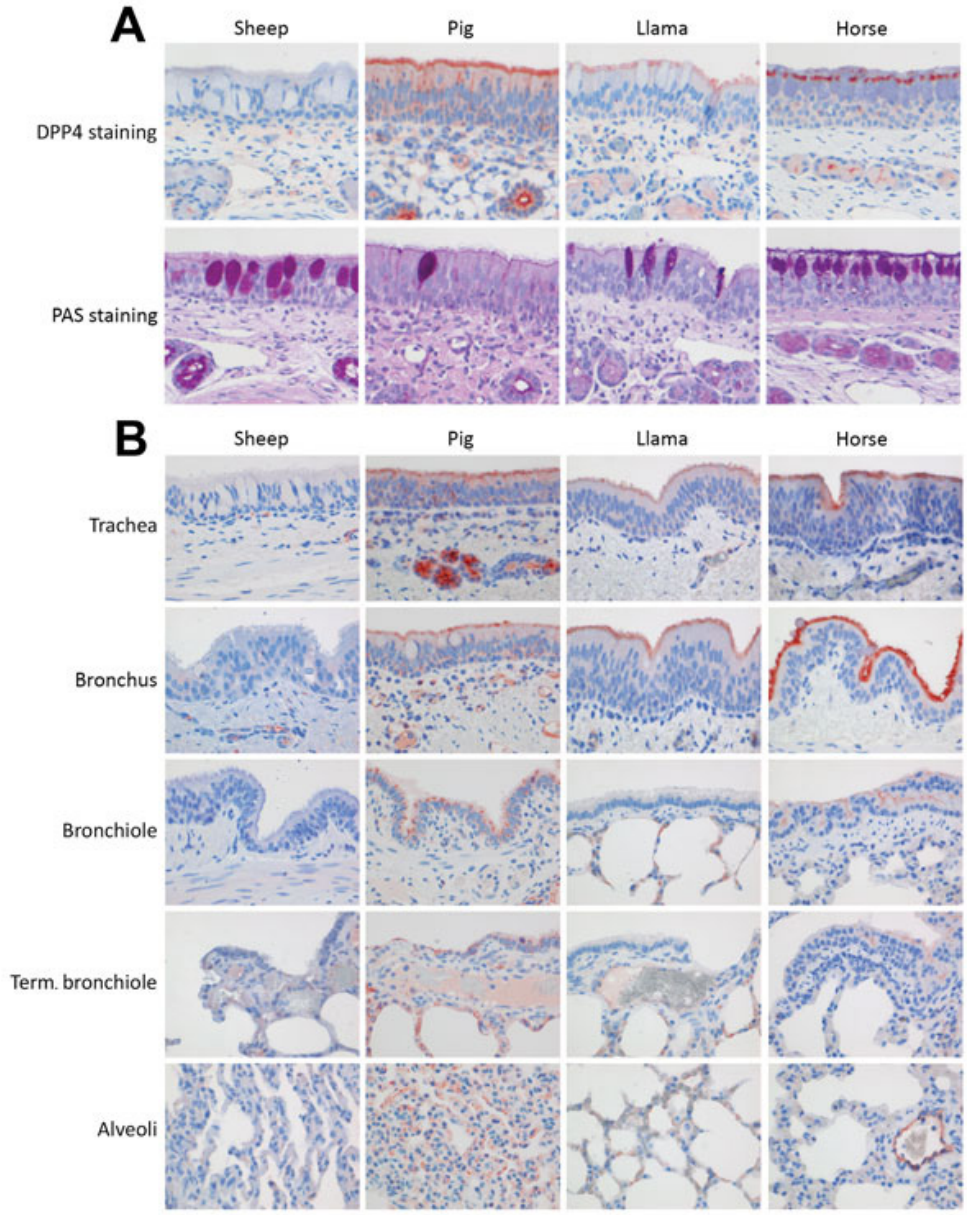

Figure 4. Presence of MERS-CoV receptor DPP4 (IHC) and of mucosubstances (PAS) in upper and lower respiratory tract tissues from sheep, pigs, llamas, and horses. A) In the nose, DPP4 (red cytoplasmic or membrane staining) was present on the lining epithelium of pigs, llamas, and horses but not sheep. PAS staining (magenta) demonstrated more mucous cells in the lining epithelium of sheep and horses and a layer of mucus on the lining epithelium of the horses. B) DPP4 (red cytoplasmic or membrane staining) was present on the lining epithelium of the trachea, bronchus/bronchioles, and alveoli in the pigs, llamas and horses but not in the sheep. Original magnification ×400 for all images. DPP4, dipeptidyl peptidase-4; IHC, immunohistochemistry; MERS-CoV, Middle East respiratory syndrome coronavirus; PAS, periodic acid–Schiff; term., terminal.

DPP4 IHC staining of upper and lower respiratory tract tissues collected on postinoculation day 24 found DPP4 expression on the respiratory epithelium of the nose of llamas, pigs, and horses but only to a very limited extent on that of sheep (Figure 4, panel A). We also detected DPP4 expression in the lower respiratory tract (but restricted to tracheal and bronchial epithelia) of horses, llamas, and pigs (Figure 4, panel B). PAS staining demonstrated the presence of mucosubstances in the nose of llamas, pigs, horses, and sheep, with a relatively higher number of mucous (goblet) cells in the lining epithelium of sheep and horses. In the horses only, there was also a layer of mucus covering lining epithelium with a multifocal distribution.

Specific Humoral Immune Response

ELISA results showed antibodies against the S1 protein in all llamas and pigs from postinoculation day 14 on, although the response in pigs was weaker than that in llamas (Figure 5, panel A). We confirmed the specificity of the response by virus neutralization assay (Figure 5, panel B). In all llamas, serum neutralizing MERS-CoV-specific antibody titers (1:80 to 1:320) were detected at postinoculation days 14 and 24. In addition, 14 days after challenge, in 5 of 6 pigs, MERS-CoV neutralizing antibodies were detected (1:80 to 1:160). However, 10 days later, these virus neutralizing antibodies decreased (1:20 to 1:40) (Figure 5, panel B). We detected no MERS-CoV–specific antibodies in serum of sheep and horses.

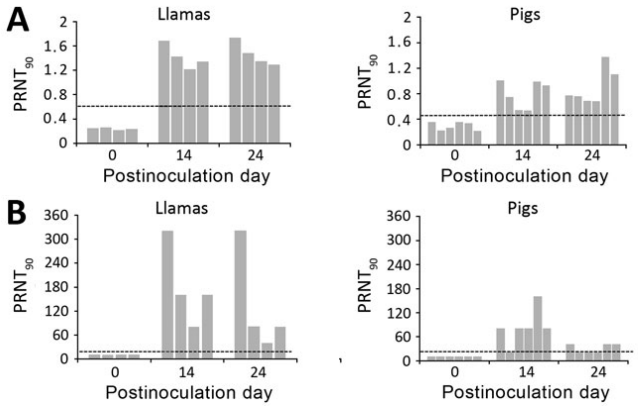

Figure 5. Antibody responses after experimental inoculation of MERS-CoV into llamas and pigs. A) MERS-CoV S1 antibody responses were analyzed in serum from all animals at postinoculation days 0, 14, and 24. An ELISA with recombinant MERS-CoV S1 protein was used, and results are represented individually. B) Individual MERS-CoV neutralization titers from llamas and pigs as determined from serum. Dashed lines depict the detection limit of the assays. MERS-CoV, Middle East respiratory syndrome coronavirus; OD, optical density; PRNT90, 90% plaque reduction neutralization test.

Discussion

Our study results indicate that pigs and llamas are susceptible to MERS-CoV infection. These animals shed infectious virus until postinoculation days 4 (pigs) and 7 (llamas), although titers were lower among pigs. In pigs and llamas, as well as dromedary camels[15] and alpacas[11], we detected virus predominantly in the nasal respiratory

epithelium by IHC, ISH, or both. Accordingly, we mainly detected viral RNA, as assessed by RT-qPCR, in the nose, trachea, and bronchi of those animals. We also detected viral RNA in lung tissue from 2 of 4 pigs euthanized at postinoculation day 2. Virus shedding in dromedary camels and alpacas for longer periods, up to 14 days after experimental inoculation, has been reported[11,15,19]. Of note, the level of MERS-CoV excreted in the nose of dromedaries seems to be much higher[15,19] than that of other animal species described so far, suggesting a more prominent role of dromedaries in transmission of MERS-CoV to humans.

Differences in virus susceptibility and pathogenicity between animals of different species could be explained by a distinct tissue distribution of DPP4, the MERS-CoV receptor. In our study, DPP4 distribution in the respiratory tract was similar among llamas and pigs but differed from that of dromedary camels[16]. In contrast, DPP4 was barely detected in the respiratory tract of sheep, probably accounting for the lack of infection reported here. These results are in concordance with those reported by Adney et al., that MERS-CoV experimentally inoculated sheep showed no clinical disease and that only small amounts of virus were detected in nasal swab samples[20]. Differences in susceptibility to MERS-CoV infection and level of virus excretion might also result from host factors associated with innate immunity. Surprisingly, horses were not susceptible to MERS-CoV despite high expression and wide distribution of the virus receptor along the respiratory tract. Moreover, the receptor binding domain, and in particular key amino acids on the docking site, are identical in horses and humans[21]. Although human, camel, and horse DPP4 served as potent and nearly equally effective MERS virus receptors[22], horses were not productively infected by the strain of MERS-CoV used in this study. Detection of low levels of viral RNA in nasal swab samples until postinoculation day 2 can be attributed to residual inoculum. Similarly, a recent study with horses also showed low levels of MERS-CoV excretion and no virus neutralizing antibodies[20].

These results highlight that other mechanisms, such as epithelial cell permissibility or strong innate immune responses, may influence the establishment of infection. In that respect, PAS staining revealed differences in the number of goblet cells in the lining epithelium and mucus covering epithelial surfaces, which may have impeded the binding of the virus to the respiratory epithelium of horses. Also, virus tends to bind more to ciliated or nonciliated non–mucus-producing cells and, in proportion, these cells may be fewer in horses than in llamas and pigs. However, it is possible that the guttural pouch empyema, which most likely was of bacterial origin (probably *Streptococcus spp.*), may have influenced mucus production in the horses. Although these observations are in line with those from studies in the field indicating the absence of antibodies to MERS-CoV in equids[14], this aspect should be studied further.

Epidemiologic studies have provided evidence of endemic MERS-CoV infection among dromedaries in the Greater Horn of Africa as far back as 1983[5,23] and in Saudi Arabia as far back as 1992–1993[24]. To implement optimal serologic surveillance in countries where MERS is and is not endemic, identifying which animal species might be potential reservoirs for MERS-CoV, besides dromedaries, is crucial. The finding that pigs can be infected with MERS-CoV suggests that other members of the family Suidae could be susceptible to the virus, such as common warthogs (*Phacochoerus africanus*), bushpigs (*Potamochoerus larvatus*), and wild boars (*Sus scrofa scrofa*). Indeed, these animals are commonly found in the Greater Horn of Africa or the Middle East, sharing territories and water sources with dromedaries. Thus, members of the family Suidae might merit inclusion in MERS surveillance programs. Further studies need to be done to investigate MERS-CoV transmission within and among species to provide a better understanding of the role of potential reservoirs during an outbreak. Moreover, studies comparing the innate immunity of horses with susceptibility of other animal species (i.e., dromedary camels, alpacas, llamas, or pigs) are needed.

Acknowledgments

We thank Xavier Abad, Enric Vidal, Mónica Pérez, and all animal caretakers from the IRTA-CReSA BSL-3 laboratories and animal facilities for technical assistance. We also express our gratitude to Sebastian Napp for his critical reading and discussion insights. This study was performed as part of the Zoonotic Anticipation and Preparedness Initiative (Innovative Medicines Initiative grant 115760), with assistance and financial support from Innovative Medicines Initiative and the European Commission and contributions from European Federation of Pharmaceutical Industries and Associations partners. This study was also partly funded by Nederlandse Organisatie voor Wetenschappelijk Onderzoek (grant 91213066).

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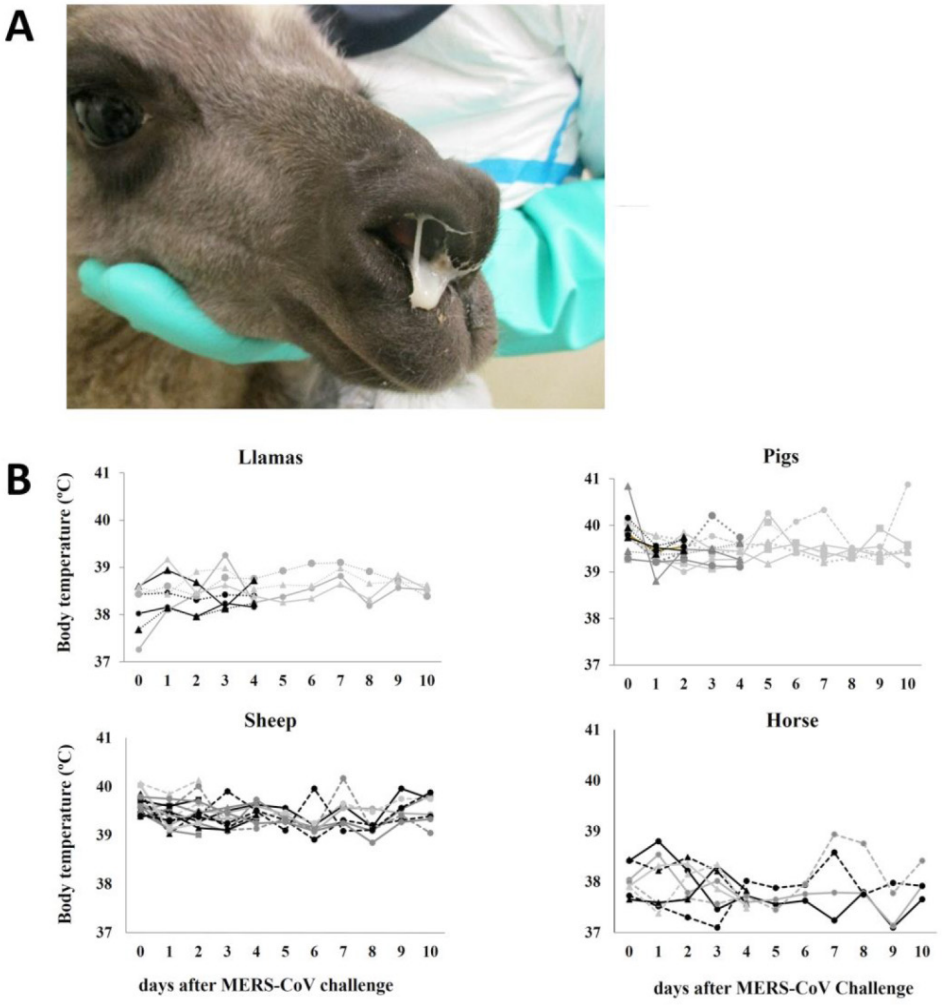
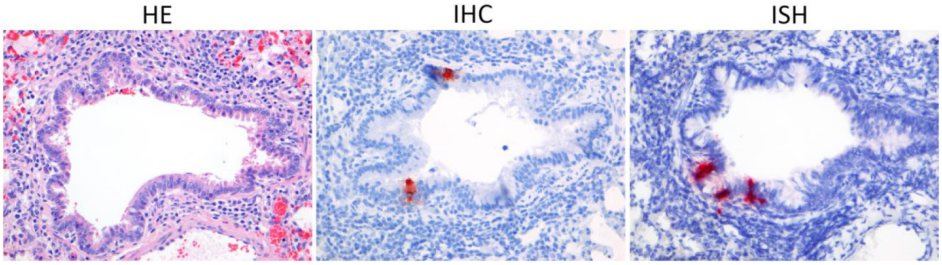
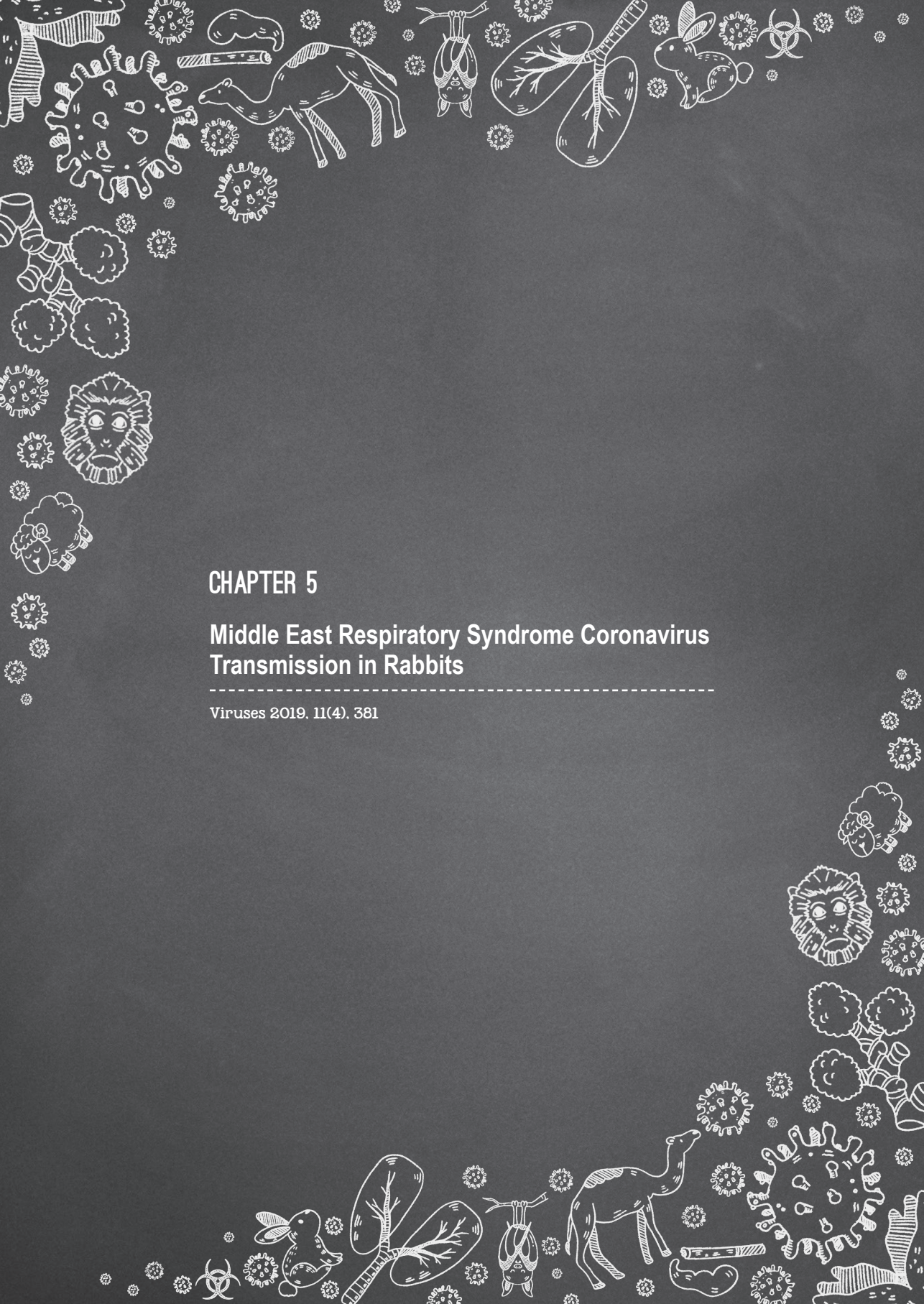
Technical Appendix



Technical Appendix Figure 1. Clinical signs after MERS-CoV inoculation in llamas, pigs, horses, and sheep. A) Presence of mucus excretion in a llama at 4 days postinoculation. B) Rectal body temperatures in llamas, pigs, sheep, and horses at different times postinoculation.



Technical Appendix Figure 2. Histology, IHC, and ISH in the lung of a sheep 2 days postinoculation with MERS-CoV. Few bronchiolar cells stained positive for MERS-CoV antigen in 1 out of 4 sheep (HE, IHC, ISH x200 magnification).



CHAPTER 5

Middle East Respiratory Syndrome Coronavirus Transmission in Rabbits

Viruses 2019, 11(4), 381

Abstract

Middle East respiratory syndrome coronavirus (MERS-CoV) transmission from dromedaries to humans has resulted in major outbreaks in the Middle East. Other livestock such as sheep, horses, rabbits and goats, potentially could contribute to zoonotic transmission - evidenced by the susceptibility of their cells *in vitro* - but it is not fully understood why this has not been observed in the field. We used rabbits to further characterize the transmission potential of MERS-CoV. In line with the presence of MERS-CoV receptor in the nasal epithelium, high levels of viral RNA were shed following virus inoculation. However, no transmission by contact or airborne routes was observed in rabbits, consistent with limited shedding of infectious virus from the nose. Our data reveal that the relatively low levels of infectious virus compared to viral RNA produced - possibly through host-mediated restriction - limit viral transmission in rabbits and possibly other host species.

Background

Middle East respiratory syndrome coronavirus (MERS-CoV) is a novel pathogen isolated late 2012 [1]. Seroepidemiological studies indicate that this virus has been circulating in dromedary camels in the Arabian Peninsula and Africa for decades [2-4]. MERS-CoV sequences obtained from these camels are largely similar to those obtained from human MERS cases in corresponding regions, thus providing evidence that dromedary camels act as the zoonotic source of this virus [5,6]. However, many primary human MERS cases do not have a history of direct contact with these animals [7]. This suggests the presence of unidentified routes of human-to-human transmission or the involvement of other animal species in spreading the virus to humans. Besides dromedary camels, other animals, i.e. llamas, alpacas, and pigs have been shown to be susceptible and develop upper respiratory tract infection upon experimental intranasal MERS-CoV inoculation [8-10]. This is in line with the expression of the MERS-CoV receptor, dipeptidyl peptidase-4 (DPP4), in their nasal epithelium [8]. MERS-CoV-seropositive llamas and alpacas have been reported in the field, and MERS-CoV-experimentally-inoculated alpacas have also been shown to transmit the virus via contact [11-13].

To further understand the zoonotic potential of MERS-CoV, it is crucial to delineate the factors involved in the spread of the virus among dromedaries as well as other animal species. In order to gain insight on these factors, we performed MERS-CoV transmission experiments in rabbits. We have previously shown that rabbits are susceptible to MERS-CoV and develop both upper and lower respiratory tract infection upon virus inoculation [14,15]. Naïve recipient rabbits were housed with MERS-

CoV-inoculated donor rabbits either in the same or in adjacent cages to determine whether MERS-CoV can be transmitted via contact or airborne routes, respectively [16]. Donor rabbits were found to shed high levels of viral RNA but a limited amount of infectious virus thus potentially restricting MERS-CoV transmission in these animals.

Methods

Virus stocks

In vivo experiments in this study were performed using Passage 7 human isolate MERS-CoV (HCoV-EMC/2012) and passage 3 isolate MERS-CoV (Qatar15/2015; GenBank Acc. No. MK280984) that were propagated in Vero cells as described earlier [8]. Qatar15 was isolated from a 69 years old Qatari man that developed severe pneumonia and was PCR confirmed to have a MERS-CoV infection [17].

Animal experiments

Animal experiments were approved and performed according to the guidelines from the Institutional Animal Welfare Committee (no. 201300121, 122-17-01, and AVD277002015283-WP01). The studies were performed under biosafety level 3 (BSL3) conditions. To compare whether different routes of MERS-CoV inoculation generate similar clinical outcomes, twelve 6-month-old New Zealand rabbits (*Oryctolagus cuniculus*), specific pathogen free, and seronegative for MERS-CoV were divided into four groups. Animals were inoculated under ketamine-medetomidine anesthesia either (a) intranasally with 200 µl of $1x10^6$ $TCID_{50}$/ml MERS-CoV; (b) intranasally with 1 ml $1x10^6$ $TCID_{50}$/ml MERS-CoV; (c) intranasally with 200 µl of $1x10^6$ $TCID_{50}$/ml MERS-CoV and intratracheally with 3 ml $4x10^6$ $TCID_{50}$/ml MERS-CoV; or (d) intranasally with 1ml PBS. The intranasal inoculums were divided equally over both nostrils. Nasal and throat swabs were obtained daily from day 1 up to day 4 post inoculation. These animals were then sacrificed on day 4 and respiratory tract tissues were collected. To compare the clinical outcomes of the MERS-CoV EMC strain and the Qatar15 strain, ten New Zealand rabbits were divided into two groups and inoculated with 1 ml of $1x10^6$ $TCID_{50}$/ml of each MERS-CoV strain intranasally. Nasal and throat swabs were obtained from day 1 up to day 4 post inoculation and these animals were sacrificed on day 4.

To study MERS-CoV transmission, a modified version of the previously described influenza A virus ferret transmission set-up was used. This set-up consists of two clear polymethyl methacrylate cages of different sizes. Donor rabbits and direct contact recipients were housed in a cage of 35 cm x 30 cm x 65 cm (W x H x L), whereas

airborne recipients were housed in a cage of 30 cm x 30 cm x 55 cm (W x H x L). These cages are separated by two stainless steel grids 10 cm apart to prevent direct contact, but still allow airflow from the donor rabbit to the airborne recipient rabbit. These transmission cages allow the experiment to be conducted in negatively pressured isolators in the BSL3 facility, with HEPA-filtered airflow <0.1 m/sec [18]. Since these cages were too small for New Zealand rabbits, we chose a smaller-sized breed, the Netherland dwarf rabbits (*Oryctolagus cuniculus*), for the MERS-CoV transmission experiment. We used both male and female rabbits with an age range of 6 months – 3 years in these experiments. First, three MERS-CoV seronegative Netherland dwarf rabbits were inoculated intranasally with 1 ml of $1x10^6$ $TCID_{50}$/ml MERS-CoV (500 µl per nostril) to show that they are equally susceptible to MERS-CoV as the New Zealand rabbits. Nasal and throat swabs were obtained daily up to 4 days post inoculation. These animals were then sacrificed on day 4 and their respiratory tract tissues were collected. For the virus transmission experiment, twelve Netherland dwarf rabbits were randomly distributed into four individually housed groups. One naïve rabbit from each group was inoculated intranasally with 1 ml of $1x10^6$ $TCID_{50}$/ml MERS-CoV (500 µl per nostril). Six hours post inoculation, naïve direct contact recipients were co-housed in the same cage with the donor rabbits. Donor and direct contact animals were of the same sex. The next day, naïve airborne recipients were placed in the adjacent cage. Nasal and throat swabs were obtained every other day from day 1 until day 7 or 9 post inoculation from the donor and direct contact rabbits, respectively, and from day 1 until day 9 post exposure from the airborne recipients. All animals were sacrificed at 14 days post exposure and blood was collected to assess seroconversion.

Virological analysis

Nasal swabs, throat swabs, and respiratory tract tissue samples were evaluated for the presence of infectious virus by virus titration, and for viral RNA by RT-qPCR against the UpE gene as previously described [8]. Samples with a cycle threshold less than forty were considered as positive for MERS-CoV RNA. Virus titration was performed in serial 10-fold dilutions on Vero cells. Cells were monitored under a light microscope at day 6 for cytopathic effect. The amount of infectious virus in swab samples was calculated by determining the $TCID_{50}$. Statistical analysis was performed using the GraphPad Prism program (La Jolla, CA, USA). Kruskal wallis test was applied due to the non-normal distribution of our data as priorly determined by Saphiro-Wilk test. The significant difference between groups was determined at a P-value <0.05.

Histopathology and histochemistry analysis

Respiratory tract tissue samples were collected in formalin and embedded in

paraffin for pathological analysis. Hematoxylin-eosin staining was performed for histopathological analysis. The presence of MERS-CoV nucleoprotein and MERS-CoV RNA was detected by immunohistochemistry and in-situ hybridization, respectively, using previously published protocols [15]. The localization of DPP4 in the respiratory tract of non-infected New Zealand rabbits was analyzed using an optimized immunohistochemical assay [15,19].

Serological analysis

Collected serum samples were tested for MERS-CoV neutralizing antibodies using a virus neutralization assay and for MERS-CoV S1-specific antibodies using MERS-CoV S1 ELISA according to the previously published protocols [8]. Goat anti-rabbit IgG conjugated with HRP (1:2000, DAKO, Glostrup, Denmark) was used as a secondary antibody in the ELISA.

Results

DPP4 is expressed in the upper and lower respiratory tract of rabbits

Rabbits are the smallest animal species that can be infected by MERS-CoV. We previously reported that they develop both upper and lower respiratory tract infection upon MERS-CoV inoculation [15], suggesting the expression of the viral receptor at these locations. Using immunohistochemistry, we analyzed the DPP4 expression in rabbit respiratory tract tissues. In the upper respiratory tract, DPP4 is strongly expressed at the apical surface of both nasal respiratory and olfactory epithelium (Figure 1). In the lower respiratory tract, DPP4 is present in both bronchiolar and alveolar epithelial cells, although some variation in DPP4 expression was observed throughout the lungs. DPP4 is either absent, limitedly expressed on alveolar type II cells, or expressed on both alveolar type I and II cells (Figure 1). Thus, these data highlight a broad DPP4 expression in the respiratory tract tissues of rabbits. Our results show that rabbits express DPP4 in both the upper and lower respiratory tract epithelium, in line with MERS-CoV tropism in this species [8,19].

MERS-CoV infects both upper and lower respiratory tract of rabbits upon intranasal inoculation

In our previous study, we inoculated rabbits both intranasally and intratracheally [15]. Intratracheal inoculation is quite invasive thus requires a skillful operator to minimize procedure-related damage in the respiratory tract. We then investigated whether intranasal MERS-CoV inoculation is sufficient to induce both upper and lower respiratory tract infection in rabbits. Three New Zealand rabbits (*Oryctolagus*

cuniculus) were inoculated with MERS-CoV EMC strain either intranasally with 200 µl of 1 x 10^6 $TCID_{50}$/ml (group a); intranasally with 1 ml of 1 x 10^6 $TCID_{50}$/ml (group b); intranasally with 200 µl of 1 x 10^6 $TCID_{50}$/ml and intratracheally with 3 ml of 4 x 10^6 $TCID_{50}$/ml (group c); or intranasally with 1ml of PBS as a negative control (group d). All three groups of MERS-CoV inoculated rabbits developed minimal clinical mani-festations and histopathological lesions. The amount of viral RNA shed in the nasal and throat swabs did not vary among the inoculated groups (Figure 2A, B). However, in the lungs of the rabbits, the amount of viral RNA was significantly lower in group a than in groups b and c (Figure 2C). In line with these observations, fewer MERS-CoV-infected cells were observed in the lungs of group a animals compared to groups b and c (Figure 2D). Based on these results, we decided to use intranasal inoculation with 1 ml of 1 x 10^6 $TCID_{50}$/ml MERS-CoV for our subsequent experiments.

Different human MERS-CoV strains have been isolated since the EMC/2012 strain was first characterized [20,21]. However, studies that evaluate phenotypic differences between these strains in animals are currently lacking. We investigated whether a more recent MERS-CoV strain (Qatar15/2015) rep-licates differently in rabbits in comparison to the EMC strain. We found that rabbits inoculated with the MERS-CoV EMC strain and those with the Qatar15 strain developed an equally mild infection and shed similar levels of viral RNA in their nasal and throat swabs (Figure 3). Following this result the MERS-CoV transmission experiment was performed using the Qatar15 strain, the more recent strain of these two.

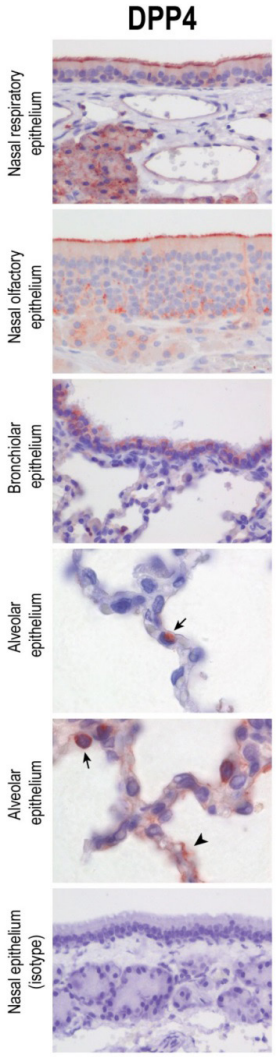


Figure 1. DPP4 expression in the respiratory tract tissues of rabbits. DPP4, indicated in red, is detected in the epithelial cells lining the respiratory tract of rabbits and the nasal olfactory ensheating cells. The DPP4 expression is varied throughout the lungs, either limited in the type II cells (arrow) or expressed in both type II and type I cells (arrowhead). Nasal epithelium and bronchiolar epithelium pictures were taken in 400x magnification, while alveolar epithelium in 1000x. The isotype control showed no background signal in our assay.

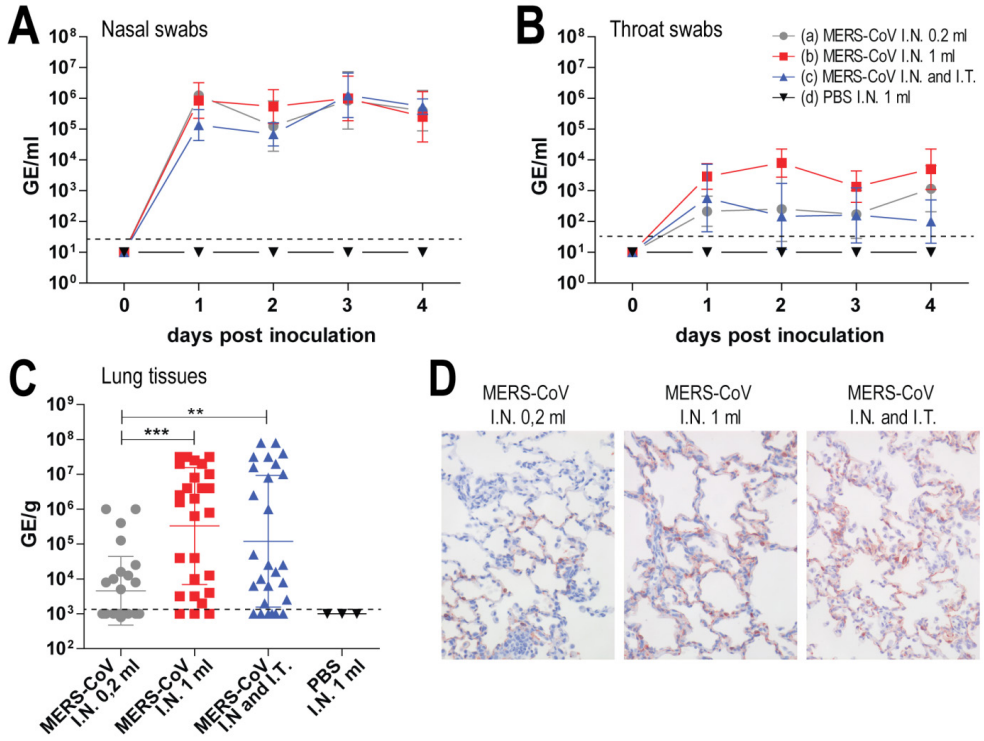


Figure 2. MERS-CoV inoculation in rabbits with different routes and volumes. Three New Zealand rabbits were each infected either with (a) 200 µl of 1x10^6 TCID$_{50}$/ml MERS-CoV intranasal (I.N.); (b) 1 ml of 1x10^6 TCID$_{50}$/ml MERS-CoV I.N.; (c) 200 µl of 1x10^6 TCID$_{50}$/ml MERS-CoV I.N. combined with 3 ml of 4x10^6 TCID$_{50}$/ml MERS-CoV intratracheal (I.T.); or (d) 1ml of PBS I.N as negative control. These animals were sacrificed at day 4 post inoculation. Viral RNA shed by the MERS-CoV-inoculated rabbits in the nasal swabs (A) and throat swabs (B) are reported in genome equivalents per ml (GE/ml). Viral RNA detected in the lungs of these rabbits are reported in genome equivalents per gram tissues (GE/g). Dashed lines depict the detection limit of the assays. All error bars represent standard deviations. Statistical analysis is performed using Kruskal wallis test (**, p value <0.01; ***, p value <0.001). Representative figures of immunohistochemistry detecting MERS-CoV nucleoprotein (displayed in red) in the lungs of these rabbits were taken at a 200x magnification (D).

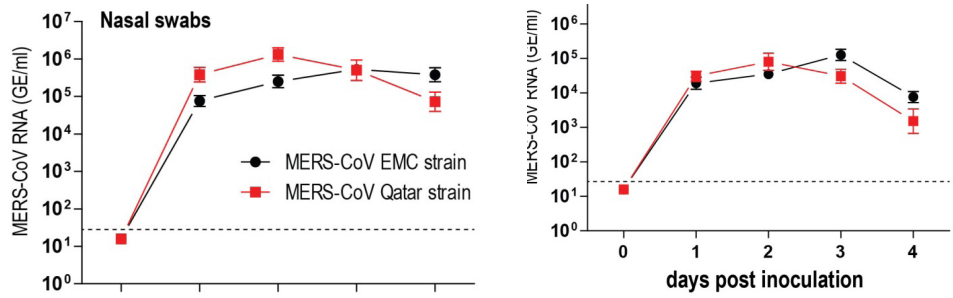


Figure 3. MERS-CoV EMC strain and Qatar strain replicate equally in the upper respiratory tract of rabbits. Five New Zealand rabbits were each intranasally inoculated either with 1 ml of 1x10^6 TCID$_{50}$/ml MERS-CoV EMC strain or Qatar strain. Nasal and throat swabs were obtained from day 0 (before inoculation) until day 4 post inoculation. There is no difference in the viral RNA being shed between the rabbits inoculated with EMC strain and Qatar strain. The amount of viral RNA is displayed in genome equivalent per ml (GE/ml). Dashed lines depict the detection limit of the assay. All errorbars represent standard deviations.

Unlike MERS-CoV-inoculated rabbits, both contact and airborne-exposed rabbits did not develop an infection

To study MERS-CoV transmis-sion, an experimental set up previously used to investigate influenza A virus transmission between ferrets was used. This set up consists of two polymethyl methacrylate cages and separated with two steel grids, 10 cm apart [16]. Because this set-up was too small to house New Zealand rabbits; we used a smaller-sized breed, the Netherland dwarf rabbits. Prior to the virus transmission experiment, Netherland dwarf rabbits were inoculated with MERS-CoV to determine their susceptibility to the virus. Similar to the New Zealand rabbits [15], Netherland dwarf rabbits shed viral RNA in the nasal and throat swabs as well as in the respiratory tract tissues upon intranasal inoculation (Figure 4A, B). Identical to the New Zealand rabbits [15], the MERS-CoV-inoculated Netherland dwarf rabbits did not develop any clinical signs and showed minimal histopathological lesions and immune cell infiltration in the respiratory tract.

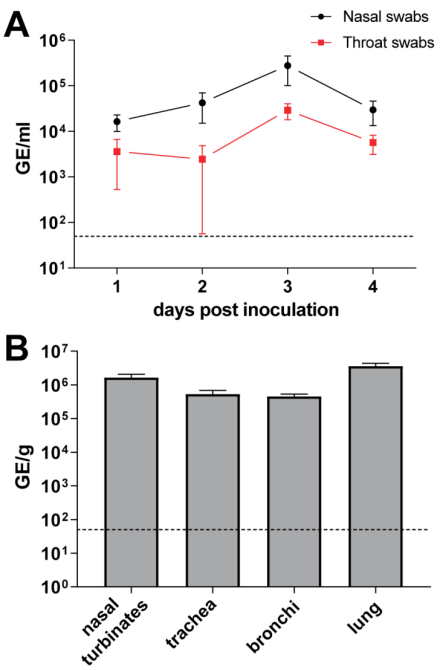


Figure 4. MERS-CoV infects the upper and lower respiratory tract of Netherland dwarf rabbits. Upon intranasal inoculation, viral RNA can be detected in the nasal and throat swabs (A) as well as in the respiratory tract tissues obtained at day 4 post inoculation (B). The amount of viral RNA in the nasal and throat swabs as well as in the respiratory tract tissues are displayed in genome equivalent per ml (GE/ml) and genome equivalent per gram tissues (GE/g), respectively. Dashed lines depict the detection limit of the assays. All error bars represent standard error of means.

To study MERS-CoV transmission, four Netherland dwarf rabbits were intranasally inoculated with 1 ml of 1 x 10^6 $TCID_{50}$/ml MERS-CoV. Six-hours later, each of them was co-housed with one naïve rabbit in the same cage, and 24 hours later another one was co-housed in an adjacent cage to determine whether MERS-CoV could be transmitted through contact and/or airborne routes. Nasal and throat swabs were collected every other day up to day 7 or 9 post inoculation/exposure for the donor and direct contact rabbits, respectively, and day 9 post exposure for the airborne recipient ones. Both viral RNA and infectious virus were quantified in these samples. We found that all donor rabbits shed relatively high loads of viral RNA in both the nasal swabs (~10^5 – 10^6 $TCID_{50}$ GE/ml) and the throat swabs (~10^3 – 10^4 $TCID_{50}$ GE/ml). The amount of viral RNA shed by the inoculated rabbits remained high until day 7 post inoculation. On the other hand, recipient rabbits housed in the same cage (direct contact

recipients), or adjacent cage (airborne recipients), shed limited amounts of viral RNA (~10 $TCID_{50}$ GE/ml) in both nasal and throat swabs. Among four animals in each group, only two direct contact recipient and two airborne recipient rabbits had detectable viral RNA up to day 5 post inoculation in the nasal swabs, while in the throat swabs, viral RNA was only detected in one direct contact recipient and one airborne recipient rabbit at day 1 post inoculation (Figure 5A, B). Infectious virus was detected at low level (~10^2 $TCID_{50}$/ml) both in the nasal and throat swabs of the donor rabbits. Infectious virus was detected in the nasal swabs of all donor rabbits at day 1 post inoculation, and in one of the donors up to day 7. In the throat swabs, infectious virus was only detected in two donors on day 1, but up to day 5 in one of them. In contrast, none of the swabs from recipient rabbits was positive for virus titration (Figure 5C, D). Serological analysis of samples collected 14 days after exposure showed that only the donor rabbits seroconverted and developed neutralizing antibodies (Figure 6A, B). This indicates that these rabbits developed MERS-CoV infection while the contact and airborne-exposed rabbits did not, supporting the results of the virus titration.

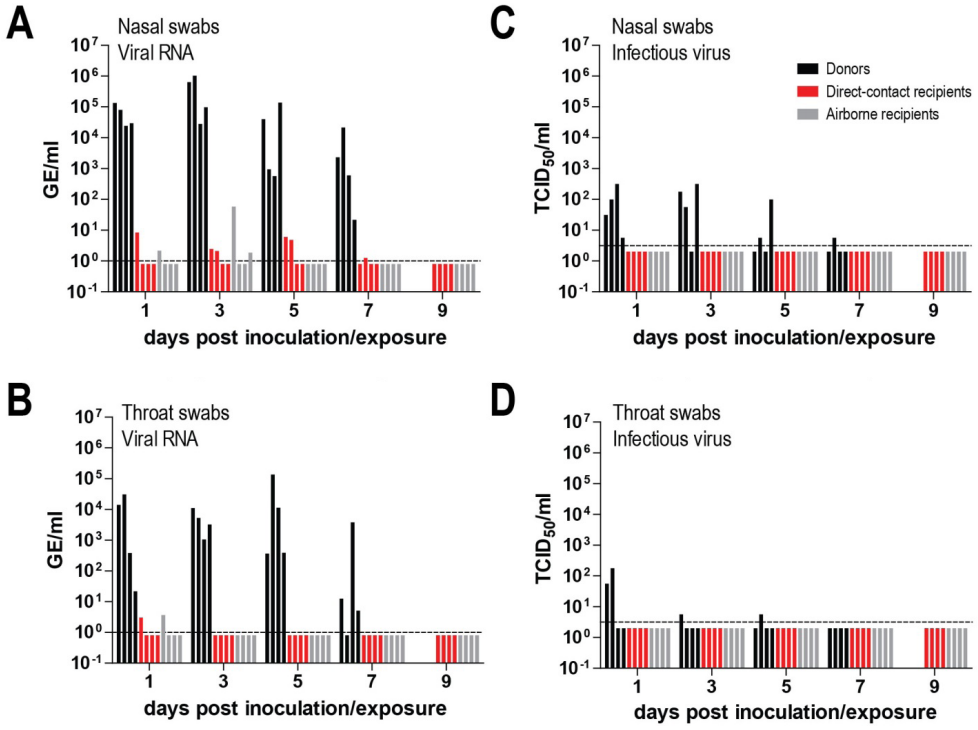


Figure 5. Shedding of MERS-CoV RNA and infectious virus in inoculated, contact-exposed and airborne-exposed rabbits. MERS-CoV RNA and infectious virus were measured in the nasal (A, B) and throat swabs (C, D). In the inoculated rabbits, both MERS-CoV RNA and infectious virus were detected. Meanwhile, in the contact and airborne-exposed rabbits, only MERS-CoV RNA was detected. The amount of viral RNA is displayed in genome equivalent per ml (GE/ml), while infectious virus in 50% tissue culture infective dose per ml ($TCID_{50}$/ml). Dashed lines depict the detection limit of each assay.

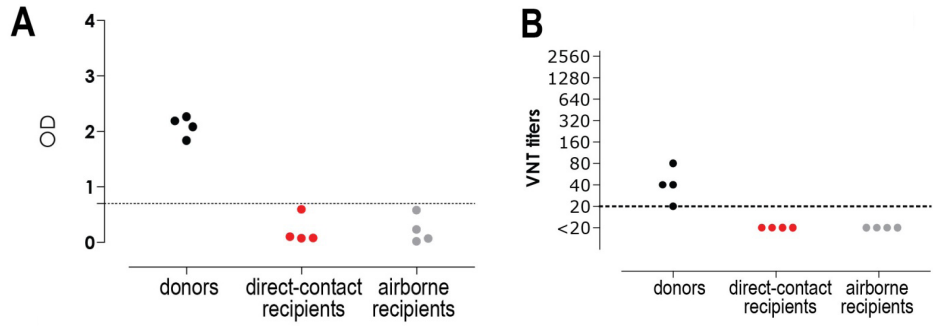


Figure 6. MERS-CoV-specific antibody response in rabbits. Inoculated rabbits developed S1-specific MERS-CoV antibody (A) and MERS-CoV neutralizing antibodies (B), while contact and airborne-exposed developed none. S1-specific MERS-CoV antibody was measured with ELISA and displayed in optical density (OD) value, while MERS-CoV neutralizing antibodies was measured with the virus neutralization test (VNT) and displayed in titers. Dashed lines depict the detection limit of the assay.

Discussion

Current data indicate that MERS-CoV is highly endemic in dromedary camels in the Arabian Peninsula and Africa and has been circulating in these animals for decades [2-4,22]. This suggests that this virus is easily transmitted between dromedary camels. From an epidemiological point of view, it is important to know whether other animal species in the region may also spread the virus to humans or other animal species. *In vitro*, MERS-CoV has been found to infect cells from a broad range of animal species including Old and New World camelids as well as primates, bats, cows, sheep, goats, pigs, horses, and rabbits [15,23,24]. The DPP4 of these species, especially rabbits, has high similarity to that of humans and dromedary camels, especially in the region that interacts with the spike protein, thus can facilitate MERS-CoV infection [23-25]. The New World camelids, i.e. llamas and alpacas, have been shown to seroconvert to MERS-CoV when present in regions where MERS-CoV is circulating and may transmit the virus [11-13]. It is currently unclear why, besides camelids, other livestock animals do not seem to transmit the virus to humans [22,26-28]. To further understand the transmission potential of MERS-CoV, we performed virus transmission experiments using rabbits as animal model.

To perform the virus transmission experiments, we housed MERS-CoV-inoculated rabbits together with naïve contact rabbits either in the same or adjacent cages. Rabbits developed both upper and lower respiratory tract infection upon MERS-CoV inoculation [15], either via intranasal or combined intranasal and intratracheal routes, in line with the localization of DPP4 in their respiratory tract epithelium. The amount of viral RNA being shed by the inoculated rabbits during the first three days post inoculation is almost as high as that of the MERS-CoV-inoculated dromedary camels [29,30]. However, none of the direct contact and airborne-exposed rabbits developed infection. They hardly shed viral RNA, did not shed infectious virus nor did they seroconvert. One

possible reason for this lack of transmission is the limited amount of infectious virus being shed by the inoculated rabbits. Similar to rabbits, MERS-CoV-infected pigs and goats shed a limited amount of infectious virus, and hardly spread the virus to naïve animals [26,31]. In humans, levels of infectious virus shed by MERS-CoV patients have rarely been reported. However, MERS-CoV patients that transmit the virus have been shown to shed a higher amount of viral RNA in their swabs compared to those that do not, supporting the quantity of virus shed as an important factor in the transmission of MERS-CoV between humans [32]. For influenza A viruses, infectious virus shedding has been documented as one of the main determinants of airborne virus transmission. Using ferrets as an animal model, it has been reported that a reduction in infectious virus shedding in the nasal swabs can subsequently limit virus transmission [33-35].

Our results show that despite the presence of DPP4 in the upper respiratory tract, accompanied by MERS-CoV replication at this site, a limited amount of infectious virus was shed. Similarly, titration of rabbit lung homogenates, that show high levels of viral RNA and presence of nucleoprotein (Fig. 2 C and D), resulted in only low levels of infectious virus (not shown), in line with earlier observations [15]. At this stage it is not clear which host mediated mechanisms limit the production of infectious virus while allowing viral RNA to still be shed at high levels. Since restriction of infectious virus shedding in the rabbits already occurred one day post inoculation, activation of host innate immune responses, including type I interferon induction, may be relevant. *In vitro* studies have shown that MERS-CoV is relatively sensitive to type I interferon-mediated antiviral activities [36,37]. In human plasmacytoid dendritic cells, MERS-CoV inoculation leads to secretion of large amount of type I and III interferons and production of viral RNA, but hardly any infectious virus is being produced [38]. Further studies are needed to elucidate the mechanisms that restrict MERS-CoV replication in rabbits compared to dromedary camels. Potentially, some of the MERS-CoV accessory proteins shown to antagonize immune responses including production of interferon, may not work efficiently in some MERS-CoV susceptible species, including rabbits. It is intriguing to investigate whether a similar phenomenon occurs in some human MERS-CoV infections and whether this is linked to the development of asymptomatic to mild clinical manifestations [39]. This might partly explain why MERS-CoV transmission in humans is rather inefficient in comparison to dromedary camels [40,41], and why camelids that secrete high levels of infectious virus are the only known zoonotic source of MERS-CoV [6,13,29,42]. Deciphering these mechanisms could potentially offer insight into understanding MERS-CoV transmission as well as developing novel treatments to tackle the ongoing outbreaks.

Notes

Financial support. This work was supported by Zoonotic Anticipation and Preparedness Initiative (Innovative Medicines Initiative grant 115760), with assistance and financial support from Innovative Medicines Initiative

and the European Commission and contributions from European Federation of Pharmaceutical Industries and Associations partners. W.W. was supported by Nederlandse Organisatie voor Wetenschappelijk Onderzoek (grant 91213066). S.H is funded by an NWO VIDI grant (contract number 91715372). The research of M.R., T.M.B. and S.H. is supported by NIAID/NIH contract HHSN272201400008C.

Potential conflicts of interest. K.S., L.d.W., and G.J.A. are full time employees at Viroclinics Biosciences BV. The other authors have no conflict of interest to disclose. All authors have submitted the ICMJE Form for Disclosure of Potential Conflicts of Interest. Conflicts that the editors consider relevant to the content of the manuscript have been disclosed.

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CHAPTER 6

Species-specific Co-localization of MERS-CoV Entry and Attachment Receptors

Submitted manuscript

Abstract

MERS-CoV uses the $S1^B$ domain of its spike protein to bind to dipeptidyl peptidase-4 (DPP4), its functional receptor, and its $S1^A$ domain to bind to sialic acids. The tissue localization of DPP4 in humans, bats, camelids, pigs, and rabbits generally correlates with MERS-CoV tropism, highlighting the role of DPP4 in virus pathogenesis and transmission. However, MERS-CoV $S1^A$ does not indiscriminately bind to all α2,3-sialic acids and the species-specific binding and tissue distribution of these sialic acids in different MERS-CoV susceptible species has not been investigated. We established a novel method to detect these sialic acids on tissue sections of various organs of different susceptible species by using nanoparticles displaying multivalent MERS-CoV $S1^A$. We found that the nanoparticles specifically bound to the nasal epithelial cells of dromedary camels, type II pneumocytes in human lungs, and the intestinal epithelial cells of common pipistrelle bats. Desialylation by neuraminidase abolished nanoparticle binding and significantly reduced MERS-CoV infection in primary susceptible cells. In contrast, $S1^A$ nanoparticles did not bind to the intestinal epithelium of serotine bats and frugivorous bat species, nor to the nasal epithelium of pigs and rabbits. Both pigs and rabbits have been shown to shed less infectious virus than dromedary camels and do not transmit the virus, neither via contact nor airborne routes. Our results depict species specific co-localization of MERS-CoV entry and attachment receptors which may be relevant in the transmission and pathogenesis of MERS-CoV.

Introduction

Coronaviruses use their spike (S) protein to attach to host cell surface molecules and enter target cells. The N-terminal part of this S protein, known as S1, is responsible for attachment to host cells, while the C-terminal part mediates virus fusion to host cells post-attachment [1]. For Middle East respiratory syndrome coronavirus (MERS-CoV), the S1 protein comprises four individually folded domains, designated $S1^A$ through $S1^D$ [2,3]. Two of these domains, $S1^A$ and $S1^B$, are involved in binding to host cell surface molecules during the attachment phase. The $S1^A$ domain preferentially binds to several glycotopes of α2,3-sialic acids, while the $S1^B$ recognizes a host exopeptidase named dipeptidyl peptidase-4 (DPP4), the viral receptor [4,5]. The absence of DPP4 renders cells insusceptible to MERS-CoV[4]. Meanwhile, elimination of sialic acids in susceptible cell lines significantly reduces MERS-CoV infection [5]. These findings indicated that besides DPP4, the functional entry receptor of MERS-CoV, α2,3-sialic acids acts as attachment receptor [4,5].

DPP4 expression has been mapped in tissues of different susceptible species. It is

expressed in the nasal epithelium of camelids, pigs, and rabbits, in which MERS-CoV causes upper respiratory tract infection [6-12]. In the human respiratory tract, it is mainly expressed in the type II pneumocytes in the lungs [8,13], in line with clinical data showing that in humans MERS-CoV mainly replicates in the lower respiratory tract [14-16]. In insectivorous bats, a potential reservoir for MERS-CoV-like viruses, DPP4 is scarcely detected in the respiratory tract but abundantly present in the intestinal tract [17]. Accordingly, MERS-CoV-like viruses are detected mostly in fecal samples of these species of bats [18-20]. Sheep, on the other hand, do not seem to express DPP4 in their respiratory tract, thus hardly shed infectious virus and did not seroconvert upon intranasal MERS-CoV inoculation [7,21]. Altogether, these data support the role of DPP4 in determining the host range and tissue tropism of MERS-CoV.

The localization of α2,3-sialic acids in the respiratory tract of both humans and dromedary camels has been mapped using lectin histochemistry (5). These molecules are mainly present in the lower respiratory tract epithelium of humans and the upper respiratory tract epithelium of dromedary camels, in line with the localization of DPP4 [5,22]. However, it is important to note that MERS-CoV $S1^A$ does not indiscriminately bind to all α2,3-sialic acids. It does not recognize those with 5-N-glycosylation nor 9-O-acetylation, but preferentially binds 5-N-acetyl modified sialic acids [5]. Among these α2,3-linked, N-acetyl modified sialic acids, MERS-CoV $S1^A$ predominantly binds to short, sulfated, α2,3-linked mono-sialosaccharides; and to long, branched, di- and triantennary α2,3-linked sialic acids with a minimum extension of 3 N-Acetyl-D-Lactosamine tandem repeats [5]. These glycotopes were previously identified by glycan array analysis using nanoparticles displaying multivalent MERS-CoV $S1^A$ [5]. Here, using these nanoparticles, we developed a histochemistry-based technique to map the MERS-CoV-recognized glycotopes in the tissues of different susceptible species. The results of our study offer further insight into the importance of these glycotopes in MERS-CoV host range, tropism, and transmission.

Results

MERS-CoV $S1^A$ binds specifically to the nasal epithelium of dromedary camels

The nasal epithelia of dromedary camels express DPP4 and are susceptible to MERS-CoV upon experimental inoculation [8,23]. Recent studies revealed that these tissues also express α2,3-sialic acids based on Maackia Amurensis Lectin II binding [5]. However, this lectin binds to a broad range of modified α2,3-sialic acids, and thus may not represent a specific marker for glycotopes recognized by the MERS-CoV $S1^A$ [24]. In order to map these glycotopes, we displayed MERS-CoV $S1^A$ in a multivalent manner using 60-meric self-assembled nanoparticles generated from the lumazine

synthase protein of the Aquifex aeolicus bacterium (np-S1^{A}) [5] and subsequently used these nanoparticles to set up a histochemistry assay. We previously showed that both np-S1^{A} and MERS-CoV virions can agglutinate human erythrocytes while dimeric MERS-CoV S1^{A} cannot, indicating that multivalent presentation is necessary for the hemagglutination phenotype of the MERS-CoV S1^{A}. Similarly, the np-S1^{A}, but not the dimeric MERS-CoV S1^{A}, can bind to tissues in our assay. As shown in Figure 1, the np-S1^{A} binds specifically to the nasal epithelium of dromedary camels. We found that these glycotopes are expressed in clusters of ciliated and goblet cells in the nasal epithelium in a random multifocal pattern (Fig. 1A). Goblet cells, however, are DPP4 negative thus are not susceptible to MERS-CoV despite expressing these glycotopes (Fig. S1). We determined the binding specificity of the np-S1^{A} by using blank nanoparticles and tissues pre-treated with neuraminidase as negative controls (Fig. 1A).

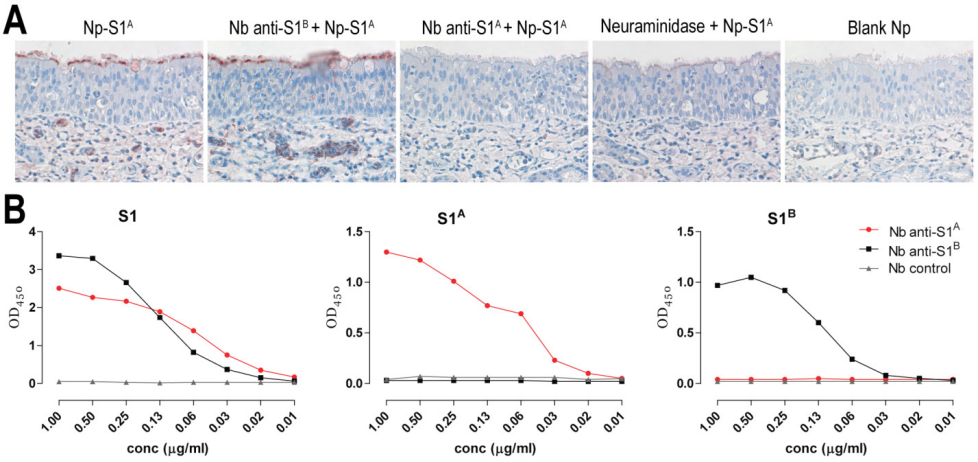

Figure 1. MERS-CoV S1^{A} binds specifically to the nasal epithelium of dromedary camels. The binding of nanoparticles displaying multivalent MERS-CoV S1^{A} protein (np-S1^{A}) to camel nasal ciliated epithelial cells (red) is abrogated by prior neuraminidase treatment and nanobodies against S1^{A} protein (Nb anti-S1^{A}), but not by those against S1^{B} protein (Nb anti-S1^{B}) (A). The tissues used in this experiment were sequentially cut. All pictures are taken in 400x magnification. Nb anti-S1^{A} and anti-S1^{B} bind to both S1^{A} and S1^{B} proteins respectively and to S1 protein as revealed by ELISA. The control Nb does not bind to S1, S1^{A}, and S1^{B} proteins (B). Nb binding is measured in OD values with ELISA.

In a previous study, we generated a nanobody library from the bone marrow of dromedary camels vaccinated with Modified Vaccinia virus Ankara (MVA) expressing the MERS-CoV spike protein and identified S1^{B}-reactive nanobodies [25]. We rescreened this library using S1^{A} protein and identified an S1^{A}-reactive nanobody. We confirmed its specific binding to S1^{A} domain through S1, S1^{B}, and S1^{A} ELISAs. While the control nanobody [25] was negative in all three ELISAs, each of the anti-S1^{A} and anti-S1^{B} nanobodies reacted specifically to its corresponding domain and to S1 in a dose-dependent manner (Fig. 1B). The identified S1^{A} nanobody inhibited np-S1^{A} binding to nasal epithelial cells, whereas the S1^{B} reactive nanobody did not,

further confirming the np-S1^A binding specificity and the potential role of S1^A specific antibodies in blocking MERS-CoV attachment (Fig. 1A).

MERS-CoV S1^A does not bind to the nasal epithelium of pigs and rabbits

Similar to dromedary camels, pigs and rabbits also develop upper respiratory tract infection upon MERS-CoV inoculation. However, both pigs and rabbits shed less infectious virus than dromedary camels and do not transmit the virus, neither via contact nor airborne routes [6,7,9-11,23,26]. In line with these findings, there were fewer numbers of MERS-CoV-infected cells in the nasal epithelium of pigs and rabbits compared to that of dromedary camels at day 4 post-inoculation (Fig. 2), while DPP4 was highly expressed in the nasal epithelium of these three species (Fig. 2). Subsequent screening for the presence of α2,3-sialic acids in these tissues using lectin histochemistry revealed that the nasal epithelium of pigs does not express these sialic acids, in accordance with the results of previous studies [27,28]. In contrast, these sialic acids were detected in the nasal epithelium of dromedary camels and rabbits (Fig. 2). We then used the np-S1^A to test for the presence of MERS-CoV-recognized glycotopes in the nasal epithelium of pigs and rabbits and found that unlike dromedary camels, both species do not express these glycotopes (Fig. 2). The absence of these glycotopes in the nasal epithelium of rabbits, despite the abundant presence of α2,3-sialic acids further supports the fine specificity of the A domain binding.

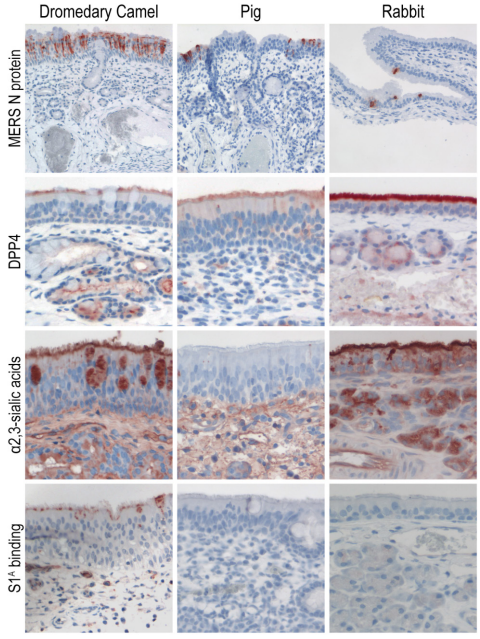

Figure 2. Detection of MERS-CoV N protein, DPP4, α2,3-sialic acids, and MERS-CoV S1^A binding in the nasal epithelium of dromedary camels, pigs, and rabbits. MERS-CoV N protein, DPP4, α2,3-sialic acids, and MERS-CoV S1^A binding are all indicated in red. MERS-CoV N protein is detected on the nasal epithelium tissues of MERS-CoV-infected dromedary camels, pigs, and rabbits. DPP4, α2,3-sialic acids, and MERS-CoV S1^A binding are evaluated on the tissues of non-infected animals. MERS-CoV N protein and DPP4 are detected in the nasal epithelium of dromedary camel, pig, and rabbit. α2,3-sialic acids are detected in the nasal epithelium of dromedary camel and rabbit but not in that of pig. Meanwhile, MERS-CoV S1^A merely binds to the nasal epithelium of dromedary camel. All pictures are taken in 400x magnification.

MERS-CoV S1^A binds specifically to the intestinal epithelium of Pipistrelle bats

DPP4 has been reported to mediate MERS-CoV infection in various bat cell lines derived from different bat species [29,30]. It has also been shown to mediate entry of

pseudotyped viruses expressing spike proteins of two MERS-CoV-like-viruses, i.e. HKU4 and Hp-BatCoV HKU25, in target cells [31-33]. DPP4 has also been shown to be abundantly expressed in the intestinal epithelium of both insectivorous and frugivorous bats [17]. Altogether, these studies suggest the susceptibility of various bat species to MERS-CoV-like-viruses. However, not all susceptible bat species may act as hosts for these viruses, as they were preferentially detected in insectivorous bats [18-20]. One study conducted a large screening of over 5000 insectivorous bats, from Ghana, Ukraine, Romania, Germany, and the Netherlands, showing that these viruses were mainly detected in Nycteris bats and Pipistrelle bats [20]. Using the np-S1^A, we investigated whether MERS-CoV-recognized glycotopes were differentially expressed in the intestinal epithelium of insectivorous bats, i.e. common pipistrelle (*Pipistrellus pipistrellus*) and serotine bats (*Eptesicus serotinus*); and frugivorous bats, i.e. Gambian epauletted (*Epomophorus gambianus*) and Egyptian fruit bats (*Rousettus aegyptiacus*). We observed that np-S1^A bind to the apical surface of the intestinal epithelium of common pipistrelle bat both in villi and crypts, while in others, np-S1^A only bind to the intestinal crypts (Fig. 3).

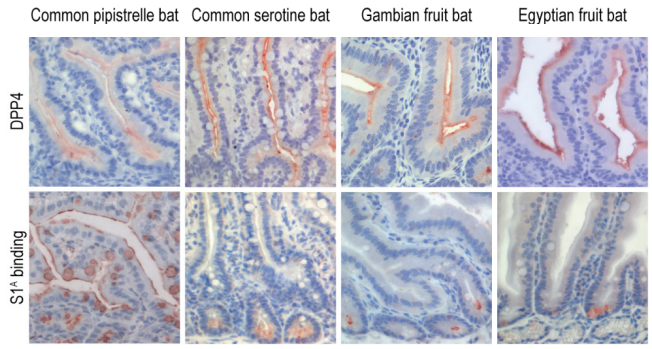

Figure 3. DPP4 expression and MERS-CoV S1^A binding in the intestinal tissues of common pipistrelle bat, serotine bat, Gambian epauletted fruit bat, and Egyptian fruit bat. DPP4 expression and MERS-CoV S1^A binding are indicated in red. DPP4 is expressed at the apical surface of the intestinal epithelial cells of these four bat species. MERS-CoV S1^A binds to the apical surface of the intestinal epithelial cells of common pipistrelle bats both in villi and crypts, while in other bat species, it binding is mostly to intestinal epithelial cells within the crypts. All pictures are taken in 400x magnification.

MERS-CoV S1^A binds specifically to cells in the human lower respiratory tract

Since lower respiratory tract samples of MERS human cases have higher levels of virus and the virus is detected by immunohistochemistry in the lungs of some cases, it is concluded that MERS-CoV mainly replicates in the human lower respiratory tract [14-16]. In line with these observations, DPP4 is expressed in the lower airway epithelium but not in the upper airway epithelium. It is detected in bronchiolar and alveolar epithelial cells, but primarily in the type II pneumocytes (Fig. 4A), consistent with previous studies [8,13]. Our lectin histochemistry staining showed that both bronchiolar epithelial cells and type II pneumocytes express α2,3-sialic acids [22]. Both cell types also express MERS-CoV-recognized glycotopes as indicated by the binding of np-S1^A (Fig. 4B). The expression of these glycotopes in bronchial epithelial cells allowed us to

confirm their function as an attachment factor using primary well-differentiated normal human bronchiolar epithelial (wd-NHBE) cell cultures. These cells were obtained from healthy human bronchial epithelial cells and cultured at air-liquid interface to mimic the human airway environment. These cells have been shown to express DPP4 and are susceptible to MERS-CoV [34-36]. Thus far, the function of MERS-CoV-recognized glycotopes as an attachment factor has only been observed in Calu3 cells, which is a cell line originating from a human lung adenocarcinoma. These cells express both DPP4 and the glycotopes of α2,3-sialic acids recognized by MERS-CoV. Removal of sialic acids on these cells using neuraminidase prior to MERS-CoV infection significantly reduced the number of infected cells [5]. In contrast, neuraminidase treatment on Vero cells, that do not express MERS-CoV-recognized glycotopes, had no effect on the number of infected cells [5]. Neuraminidase treatment of wd-NHBE cells prior to MERS-CoV infection significantly reduced the number of infected cells (Fig. 4C), similar to our previous findings in Calu3 cells [5]. Thus, our results concur with the importance of MERS-CoV-recognized glycotopes as an attachment factor during infection of human airway epithelial cells.

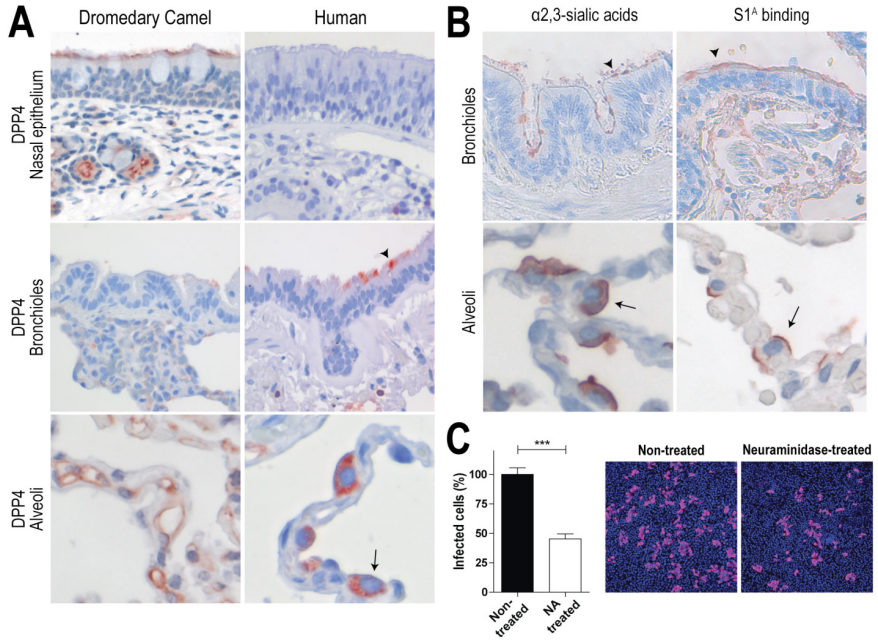

Figure 4. MERS-CoV receptor and attachment factor in the human lower respiratory tract epithelium. The MERS-CoV receptor, DPP4, is expressed in the nasal epithelium of dromedary camels, while in the lungs it is mainly expressed in the endothelial cells. In the human respiratory tract, DPP4 is expressed in the bronchiolar epithelial cells (arrowhead) and type II pneumocytes (arrow) in the lungs, but not in the nasal epithelium (A). α2,3-sialic acids expression and MERS-CoV $S1^A$ binding are also detected in human bronchiolar epithelial cells (arrowhead) and type II pneumocytes (arrow) (B). DPP4 expression, α2,3-sialic acids presence, and MERS-CoV $S1^A$ binding are indicated in red. Pictures of the nasal epithelium are taken in 400x magnification, while the alveoli in 1000x magnification. MERS-CoV infection in the primary normal human bronchial epithelial cells is inhibited up to 50% upon prior removal of sialic acids using neuraminidase treatment (B). MERS-CoV N protein is indicated in red, while nuclei are in blue.

Discussion

The S1 domain of the spike protein is an important determinant for the host range and tissue tropism of coronaviruses. This domain initiates infection by binding to host cell surface molecules, either proteinaceous, sialoglycans-based, or both [37]. The S1 protein of feline coronavirus, transmissible gastroenterovirus, and MERS-CoV has been demonstrated to have dual-binding specificity, allowing them to engage both sialoglycans and proteinaceous molecules [5,34,37-41]. We have previously reported that for MERS-CoV, this dual-binding is facilitated by distinct domains of its S1 protein, i.e. $S1^A$ and $S1^B$ [4,5]. The $S1^B$ domain binds DPP4, the functional receptor of MERS-CoV [34]. DPP4 has been demonstrated to have a major influence on the viral host range and tropism since its tissue localization varies between species [7,8,13,17,29,34,42-44]. It is detected in the nasal epithelium of camelids, pigs, and rabbits [7,8]. In bats, it is mainly expressed in the small intestinal epithelium of common pipistrelle and serotine bats – and in both the respiratory and intestinal epithelium of Gambian epauletted and Egyptian fruit bats [17]. Meanwhile, in the human airways, it is merely present in the lower respiratory tract epithelium, particularly in the type II pneumocytes [8,13]. Besides DPP4, MERS-CoV preferentially binds α2,3-linked sialic acids via its $S1^A$ domain and uses these sialic acids as an attachment factor [5]. Using nanoparticles displaying multivalent $S1^A$ domain, we show that the tissue localization of these glycotopes varies between various tissues in susceptible species. The $S1^A$ protein bound to the nasal epithelium of dromedary camels and the type II pneumocytes in human lungs, but not the nasal epithelium of pigs and rabbits.

Previous studies have shown that MERS-CoV-inoculated pigs and rabbits shed less infectious virus in comparison to dromedary camels [6,7,10,11,23]. In addition, these animals did not transmit the virus, neither via contact nor airborne routes [45] (W. Widagdo, N.M.A. Okba, et al, submitted for publication). In contrast, MERS-CoV is easily transmitted among dromedary camels [12,46-49]. The absence of MERS-CoV-recognized glycotopes in the nasal epithelium of pigs and rabbits might render them less permissive to MERS-CoV, thus shed less infectious virus which subsequently limited virus transmission. This is supported by our finding that the loss of these glycotopes, e.g. through desialylation, could significantly reduce MERS-CoV infection [5]. However, it is important to note that expression of other factors such as proteases and interferons may also influence MERS-CoV replication and transmission. MERS-CoV has been reported to use host cell proteases, such as TMPRSS2, furin, and cathepsins to mediate fusion to host cells [50-52]. The virus has been shown to be sensitive towards type I interferon, an essential host innate immune cytokine [5,50-54]. The importance of glycotopes in MERS-CoV transmission, either solely or in combination with other factors, remains to be further elucidated. These studies would likely require

experimental infection of camelids, particularly dromedary camels [46,47,55,56].

Our data also show that besides its presence in the respiratory tract of dromedary camels and humans, MERS-CoV-recognized glycotopes are expressed at the apical surface of the villi and crypts of intestinal epithelium of common pipistrelle bats. Interestingly, in serotine bats and both Gambian epaulleted and Egyptian fruit bats, these glycotopes are merely detected in the intestinal crypts. This finding further supports the belief that insectivorous bats are one of the natural hosts of MERS-CoV-like-viruses [18-20] and also suggests that not all insectivorous bats express the α2,3-sialic acid glycotopes recognized by MERS-CoV in their intestines. Whether these glycotopes influence the permissiveness of different bat species towards MERS-CoV, still remains to be elucidated and would need the availability of primary bat intestinal cell culture or bat intestinal organoids. However, experiments in insectivorous bats may be difficult to perform due to legal restrictions.

In general, our results showed that the tissue localization of α2,3-sialic acid glycotopes recognized by MERS-CoV $S1^A$ varies between susceptible species. These glycotopes and DPP4 are both expressed in the nasal ciliated epithelial cells of dromedary camels, type II pneumocytes of humans, and intestinal epithelial cells of common pipistrelle bats, providing further evidence that these tissues are the main replication site of MERS-CoV in the respective species. This study corroborates α2,3-sialic acids glycotopes as an important attachment factor for MERS-CoV [5], and highlights the necessity to further understand their role in MERS-CoV pathogenesis and transmission. Importantly, our results also imply that the MERS-CoV $S1^A$ domain should be considered a target for vaccines [57,58].

Materials and Methods

Tissue samples

Human formalin fixed paraffin embedded (FFPE) lung tissues were obtained from the Erasmus MC Tissue Bank and had been used in a previous study [8]. These tissue samples were residual human biomaterials taken either from healthy donors or from patients with nonmalignant lung tumors that were collected, stored, and issued by the Erasmus MC Tissue Bank under ISO 15189:2007 standard operating procedures. Use of these materials for research purposes is regulated according to human tissue and medical research: code of conduct for responsible use (2011). Dromedary camel, pig, and rabbit FFPE nasal tissues, both MERS-CoV-infected and mock-infected were obtained from previous studies. Infected animals were all sacrificed at day 4 post-inoculation [6,7,23]. Bat FFPE intestinal tissues were also obtained from a previous study

[17]. All of these samples were obtained in accordance with the relevant guidelines and regulations.

Histochemistry and immunofluorescence analysis

MERS-CoV nucleoprotein was detected with 5 µg/ml mouse anti-MERS nucleoprotein (Sino-Biological, Beijing, China), while DPP4 expression with 5 µg/ml goat anti-human DPP4 (R&D, Minneapolis, Minnesota, USA) using the protocol described in previous studies [7,8,17]. Periodic acid-Schiff staining was also performed according to the protocol described in a previous study [7]. Expression of α2,3-sialic acids was detected using biotinylated Maackia Amurensis Lectin II (Vector Labs, Burlingame, California, USA) in 1:800 dilution and streptavidin-HRP in 1:300 dilution, both diluted in 1x Tris buffered saline containing 0.1M $MnCl_2$, 1M $MgCl_2$, and 0.1M $CaCl_2$, then subsequently visualized using 3-Amino-9-ethylcarbazole and counterstained with Hematoxylin. $S1^A$ protein binding on the tissues was performed using nanoparticles displaying multivalent $S1^A$ protein (np-$S1^A$) and a strep-tag generated in a previous study [5]. The tissues were boiled in citric acid buffer 10 mM pH 6 for 15 minutes and blocked with 5% normal goat serum (DAKO, Glostrup, Denmark) before staining with 3 µg/ml np-$S1^A$ overnight at 4°C. Tissues that were stained with an equal concentration (3 µg/ml) of blank nanoparticles and those that were pre-treated with 800 mU/ml neuraminidase from Vibrio cholerae (Sigma-Aldrich, St. Louis, Missouri, USA) for 4 hours at 37°C were used as negative controls. Additional controls were performed by staining tissues with np-$S1^A$ priorly incubated for 1 hour at 37°C with either anti-$S1^A$, anti-$S1^B$ or control nanobodies in 15 µg/ml concentration. These nanobodies were obtained from a nanobody library generated in a previous study [25]. These tissues were subsequently stained with rabbit anti-strep-tag sera generated in-house and goat anti rabbit-IgG-HRP (DAKO, Glostrup, Denmark), each in 1:100 dilution for 1 hour at room temperature, and then visualized with 3-amino-9-ethylcarbazole and counterstained with Hematoxylin. For immunofluorescence staining, fluorescence conjugated secondary antibody was applied in the experiment, i.e. goat anti-rabbit IgG conjugated with Alexa Fluor 488 and goat anti-mouse IgG conjugated with Alexa Fluor 594 (Life technologies, Carlsbad, California, USA), both in 1:250 dilution and 1 hour incubation at room temperature.

MERS-CoV S1, $S1^B$, and $S1^A$ ELISAs

The specificity of anti-$S1^A$, anti-$S1^B$ and control nanobodies to MERS-CoV S1, $S1^A$, and $S1^B$ proteins was determined using ELISA as previously described [25]. In brief, 96-well ELISA plates were coated with 1 µg/ml MERS-CoV S1 (amino acid 1-751), $S1^A$ (amino acids 1-357), or $S1^B$ (amino acids 358-588) proteins in PBS (pH 7.4) and

incubated overnight at 4°C. Wells were then washed with PBS, and blocked with 1% bovine serum albumin in PBS/0.5% Tween-20 for 1 hour at 37°C. Nanobodies were two-fold serially diluted in blocking buffer starting at a 1 µg/ml concentration, 100 µl of each dilution was added per well, and plates were incubated at 37°C for 1 hour. Next, plates were washed three times in PBS/0.05% Tween-20 (PBST), after which they were incubated with mouse anti-his tag antibodies (1:2000, Thermo Fisher Scientific) at 37°C for 1 hour. Following incubation, the plates were washed and further incubated with goat anti-mouse HRP (1:2000, Dako) at 37°C for 1 hour. After this incubation, plates were washed three times in PBST and 3,3',5,5'-tetramethylbenzidine substrate (eBioscience) was added and incubated for 10 min. The reaction was stopped with 0.5N H_2SO_4 (Sigma). The absorbance of each sample was read at 450 nm with an ELISA reader (Tecan Infinite F200).

MERS-CoV infection in well-differentiated primary normal human bronchial epithelial (wd-NHBE) cells

Primary NHBE cells (Lonza, Basel, Switzerland) were cultured on Transwell® permeable support (Costar) according to the protocol suggested by the manufacturer (Clonetics™ Airway Epithelial Cell Systems, Lonza, Basel, Switzerland). The cells were differentiated at the air-liquid interface for 6 weeks to promote mucociliary differentiation, resulting in the presence of multilayered epithelium, ciliated cells, and goblet cells [59]. These cells were subsequently either mock-treated or pre-treated with a mixture of 40U of Arthrobacter urefaciens neuraminidase (Sigma-Aldrich, St. Louis, Missouri, USA) and 50U of Clostridium perfringens neuraminidase (NEB, Ipswich, Massachusetts, USA) for 1 hour before infection. Each well was inoculated with MERS-CoV EMC/2012 strain [6,7] 10^6 TCID50/ml (MOI 5), incubated for 36 hours and fixed in formalin 10%. MERS-CoV infected cells were visualized with 5 µg/ml mouse anti-MERS nucleoprotein (Sino-Biological, Beijing, China) and 1:250 goat anti-mouse IgG conjugated with Alexa Fluor 594 (Life technologies, Carlsbad, California, USA). Experiments were performed in triplicates. The number of infected cells was counted for each well and the percentage of infected cells was determined, as performed in our previous study [5]. Statistical analysis was performed using student's t-test and then presented in mean ± standard deviation for each group.

Acknowledgements

The E. gambianus and R. aegyptiacus tissues used in this study originated from Ghana in collaboration with Dr. Richard Suu-Ire, Forestry Commission, Accra, Ghana, Prof. Andrew A Cunningham, Institute of Zoology, Zoological Society of London, United Kingdom (ZSL) and Prof. James Wood, University of Cambridge, United Kingdom. We thank Louise Gibson, ZSL for assistance with sampling the African fruit bats. We would like to thank Stichting Vleermuisopvang Oss for providing the tissues of common pipistrelle and serotine bats.

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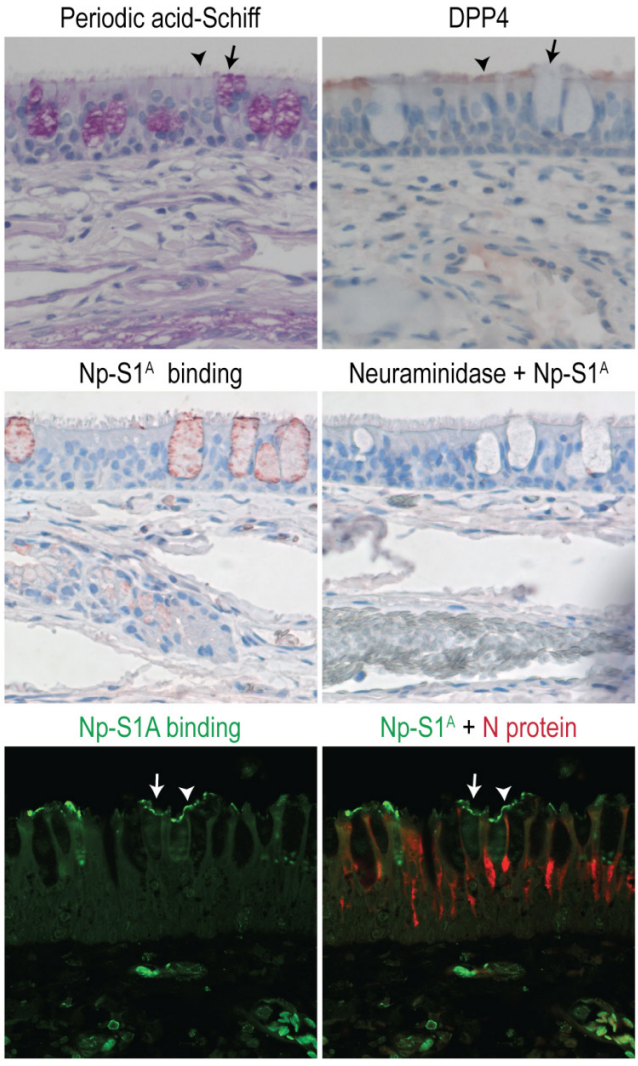
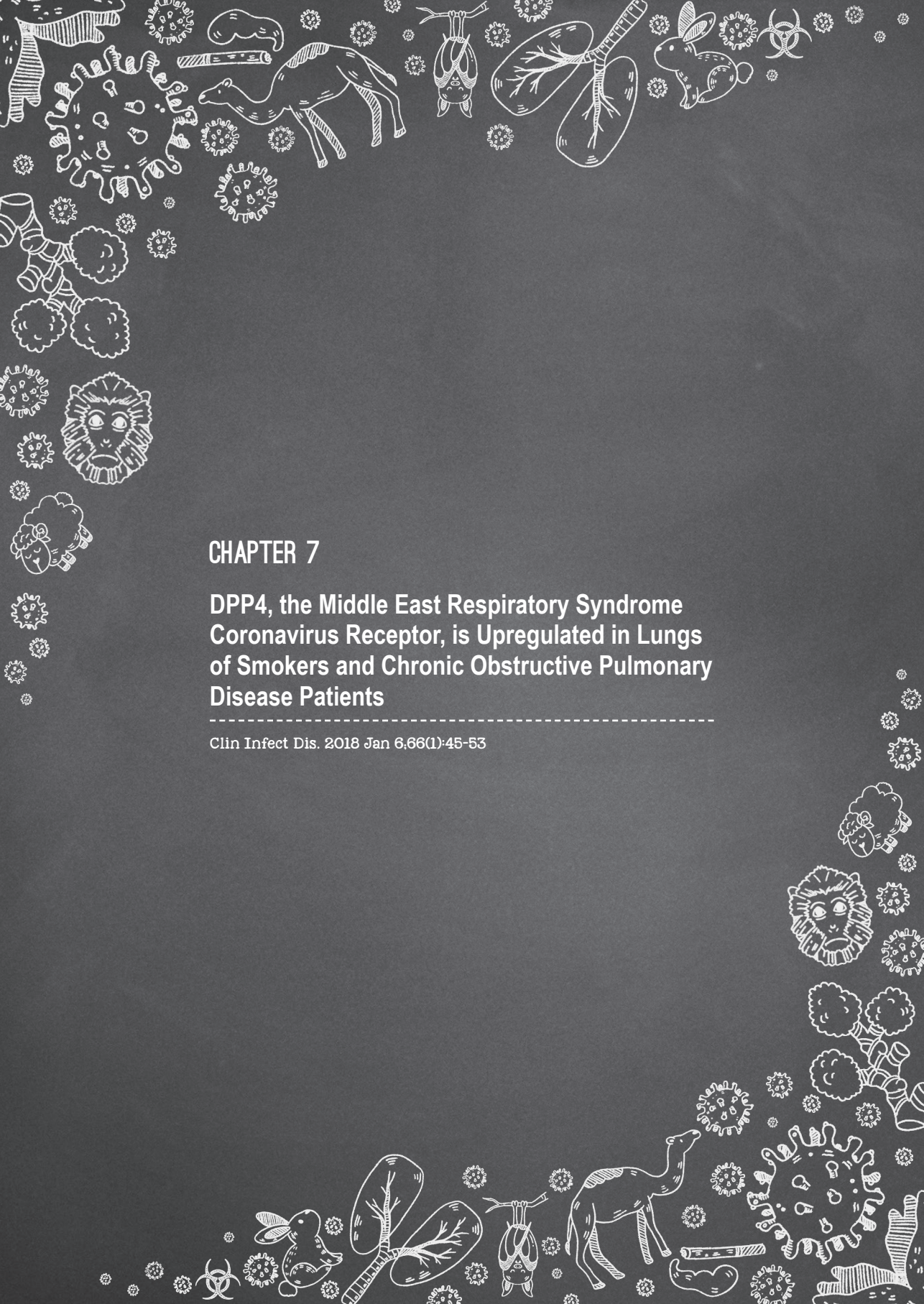


Supplementary figure 1. Periodic acid-Schiff staining, DPP4 expression, MERS-CoV $S1^A$ binding, and MERS-CoV N protein detection in the nasal epithelium of dromedary camel. Goblet cells (arrows), visualized in purple by periodic acid-Schiff stain, are DPP4 negative unlike the nasal ciliated columnar epithelial cells (arrowheads). The DPP4 expression is indicated in red. MERS-CoV $S1^A$ binding to these goblet cells (red) can be abrogated by neuraminidase treatment. In MERS-CoV-infected camels, MERS-CoV $S1^A$ (green) binds to both nasal ciliated columnar epithelial cells and goblet cells, while MERS-CoV N protein (red) is only detected in the nasal ciliated columnar epithelial cells. All pictures are taken in 400x magnification.



CHAPTER 7

DPP4, the Middle East Respiratory Syndrome Coronavirus Receptor, is Upregulated in Lungs of Smokers and Chronic Obstructive Pulmonary Disease Patients

Clin Infect Dis. 2018 Jan 6;66(1):45-53

Abstract

Background: Middle East respiratory syndrome coronavirus (MERS-CoV) causes pneumonia with a relatively high case fatality rate in humans. Smokers and chronic obstructive pulmonary disease (COPD) patients have been reported to be more susceptible to MERS-CoV infection. Here, we determined the expression of MERS-CoV receptor, dipeptidyl peptidase IV (DPP4), in lung tissues of smokers without airflow limitation and COPD patients in comparison to nonsmoking individuals (never-smokers). **Methods:** DPP4 expression was measured in lung tissue of lung resection specimens of never-smokers, smokers without airflow limitation, COPD GOLD stage II patients and in lung explants of end-stage COPD patients. Both control subjects and COPD patients were well phenotyped and age-matched. The mRNA expression was determined using qRT-PCR and protein expression was quantified using immunohistochemistry. **Results:** In smokers and subjects with COPD, both DPP4 mRNA and protein expression were significantly higher compared to never-smokers. Additionally, we found that both DPP4 mRNA and protein expression were inversely correlated with lung function and diffusing capacity parameters. **Conclusions:** We provide evidence that DPP4 is upregulated in the lungs of smokers and COPD patients, which could partially explain why these individuals are more susceptible to MERS-CoV infection. These data also highlight a possible role of DPP4 in COPD pathogenesis.

Introduction

Middle East Respiratory Syndrome coronavirus (MERS-CoV) is a newly emerging pathogen that mainly causes pneumonia with a relatively high case-fatality rate[1,2]. Since 2012, ~2000 laboratory-confirmed cases have been reported to the World Health Organization (WHO)[2]. The majority of cases occurred in familial or hospital-related clusters through human-to-human transmission[3-5]. The clinical course of MERS-CoV infection ranges from asymptomatic to acute respiratory distress syndrome with need for ventilatory support[4-7]. To infect its host, MERS-CoV attaches to its receptor, an exopeptidase called dipeptidyl peptidase 4 (DPP4), also known as CD26[8].

DPP4 is a type II transmembrane glycoprotein that is expressed in many cell types and organs in the body. It serves multiple functions among which post-translational cleavage of hormones and chemokines, T-cell activation, cell adhesion, and apoptosis[9-11]. In lungs, however, DPP4 is expressed at a minimum level[12], mainly in alveolar epithelial cells and endothelial cells, and to a lesser extent in bronchiolar epithelial cells, airway submucosal glands, alveolar macrophages, lymphocytes, and plasmacytoid dendritic cells[13-16]. Importantly, the alveolar epithelial cells are the main

target for MERS-CoV[13,17].

Several underlying comorbidities, including chronic lung diseases, have been reported to increase the risk of acquiring MERS-CoV infection[18]. Chronic obstructive pulmonary disease (COPD) is a highly prevalent chronic lung disease in older subjects and is currently the leading cause of death worldwide[19,20]. The most common cause of COPD is chronic cigarette smoking. The inflammatory response to cigarette smoke results in an excessive release of chemokines and cytokines with a subsequent high influx of immune cells[19]. Because smoking has also been reported to increase susceptibility to MERS-CoV infection[18], we aimed to investigate the expression of the MERS-CoV receptor, DPP4, in a large well-phenotyped cohort of smokers, with and without airflow limitation, in comparison to age-matched individuals that never smoked (never-smokers).

Methods

Human Lung Tissue Samples

Table 1. Characteristics of study population (n=117)

	Never-smokers	Smokers[a]	COPD II[b]	COPD III–-IV[c]
Number	21	32	37	27
Sex (M/F)	6/15[d]	23/9[d]	34/3[d]	12/14[d]
Age (years)	65 (58–71)	64.5 (55–71)	65 (58–69)	56.5 (54–60)[e,f,g]
Current- / ex-smoker	-	19/13[d]	24/13[d]	0/27[d]
Smoking history (PY)	0 (0–0)	33 (14–51)[e]	45 (40–60)[e,f]	30 (25–36)[e,g]
FEV_1 post (L)	2,4 (2,1–3)	2,7 (2,3–3,3)	2,1 (1,8–2,4)[e,f]	0,7 (0,5–1)[e,f,g]
FEV_1 post (% predicted)	103 (92–117)	95 (92–112)	69 (61–75)[e,f]	27 (21–33)[e,f,g]
FEV_1 / FVC post (%)	78 (74–83)	76 (72–79)	56 (51–61)[e,f]	30 (27–35)[e,f,g]
DLCO (% predicted)	88 (81–103)	83 (65–104)	67 (51–86)[e,f]	34 (32–37)[e,f,g]
KCO (% predicted)	95 (86–121)	93 (78–106)	85 (65–107)[e]	52 (46–59)[e,f,g]
ICS (yes/no)	1/19[d]	2/30[d]	16/21[d]	25/1[d]

Abbreviations: COPD, chronic obstructive pulmonary disease; DLCO, diffusing capacity of the lung for carbon monoxide; FEV1 forced expiratory volume in 1 second; FVC, forced vital capacity; IQR, interquartile range; KCO, transfer of carbon monoxide coefficient, ICS, inhaled corticosteroids; PY, pack years.

[a]Smokers without airflow limitation; [b]subjects with COPD stage II as defined by the Global initiative for Obstructive Lung Disease (GOLD). Data are presented as median (IQR), Mann-Whitney U test:cSubjects with COPD stage III–IV as defined by GOLD. Fisher's exact test:dP < .001. [e]P < .05 versus never smokers; [f]P < .05 versus smokers without COPD; [g]P < .05 versus COPD GOLD I–-II.

Lung resection specimens were obtained from patients diagnosed with solitary pulmonary tumors at Ghent University Hospital (Ghent, Belgium) or from explant lungs from end-stage COPD patients (UZ Gasthuisberg, Leuven, Belgium). Based on preoperative spirometry, diffusion capacity tests and questionnaires, patients were categorized as never-smokers with normal lung function, smokers without airflow limitation or patients with COPD. COPD severity was defined according to the Global

Initiative for Chronic Obstructive Lung Disease (GOLD) classification[20]. None of the patients were treated with neo-adjuvants chemotherapy. Lung tissue of patients diagnosed with solitary pulmonary tumor was obtained at a maximum distance from the pulmonary lesions and without signs of retro-obstructive pneumonia or tumor invasion and collected by a pathologist. Lung tissue of patients with COPD GOLD III-IV was obtained from lung explants of end-stage COPD patients undergoing lung transplantation. Written informed consent was obtained from all subjects. This study was approved by the medical ethical committees of the Ghent University Hospital (2011/114) and the University Hospital Gasthuisberg Leuven (S51577). Patient characteristics are listed in Table 1. Detailed patient characteristics per read-out are provided in supplementary Tables S1 and S2.

Human Proximal Bronchi Samples

Table 2. Characteristics of the Patients in Which Proximal Bronchi Biopsy Samples Were Obtained

	Mean ± SD	Median	Range
Age, years	56.3 ± 8.9	60	42–46
Actual smoking, cigarettes/day	15.4 ± 7.4	13	6–30
Pack-years	25.3 ± 11.2	21	5–50
FEV1, % predicted	62.5 ± 12.9	65	34–93
Reversibility, % predicted	5.3 ± 3.1	5	0–9.0
PC20, mg/ml			
For histamine	1.7 ± 2.1	0.87	0.11–8
For methacholine	4.6 ± 5.5	1.72	0.6–17.4

Abbreviations: FEV1, forced expiratory volume in 1 second; SD, standard deviation; PC20, provocative concentration causing a 20% fall in FEV1.

Table 3. Overview of the Cohorts and Samples Used in This Study

90 patients (21 never-smokers, 32 smokers without airflow limitation and 37 patients with COPD GOLD stage II) who underwent lobectomia or pneumectomia due to lung cancer.	
	73/90 patients: samples for both qRT-PCR and IHC analyses. 5/90 patients: samples only for qRT-PCR analysis. 12/90 patients: samples only for IHC analysis.
27 patients with COPD GOLD stage III–IV who underwent lung transplantation due to end-stage COPD.	
	14/27 patients: samples for qRT-PCR analysis. 13/27 patients: samples for IHC analysis.
37 patients who underwent bronchial biopsies.	
	21/37 patients with moderate-to-severe COPD (ref): samples used for IHC staining. 16/37 control patients with airflow limitation (ref): samples used for IHC staining.

In this study we used resection lung tissue of 90 patients who underwent lobectomia/pneumectomia due to lung cancer, explant lung tissue of 27 end-stage COPD patients and bronchial biopsy tissue of 37 patients.

Abbreviations: COPD, chronic obstructive pulmonary disease; GOLD, global initiative for chronic obstructive lung disease; IHC, immunohistochemistry; qRT-PCR, quantitative reverse-transcription polymerase chain reaction.

Biopsy samples of proximal bronchi were obtained from 21 patients (17 male and 4 female) with moderate-to-severe COPD previously recruited for a separate study[21]. Inclusion criteria were the following: chronic productive cough, age between 40 and 70 years, current smokers, negative skin tests for inhalation allergens, FEV1 < 70% of predicted normal value or FEV1/VC < 0.70, reversibility of FEV1 < 10% pred after 750 µg terbutaline inhalation, and suffering from moderate-to-severe bronchial hyper-responsiveness, as determined by PC20 value upon challenge with histamine and methacholine. Exclusion criteria were a history of asthma, complaints of wheezing, recent respiratory tract infection, and recent or concurrent usage of anti-inflammatory drugs. Oral anti-inflammatory medication was discontinued for at least 3 months and inhaled glucocorticoids at least 6 weeks before the start of the study. Bronchoscopy was performed with an Olympus BF 1T10. At least 6 biopsies were taken from the bronchi of the right and the left upper and lower lobes using a fenestrated forceps (FB-18C or FB-20C). All was according to published guidelines[22]. The study was approved by the Medical Ethics Committee of the Erasmus University Medical Center Rotterdam, and written informed consent was obtained from all participants. Patient characteristics are listed in Table 2. Proximal bronchi biopsy samples of 16 healthy individuals (8 male and 8 female), previously described in the earlier study[23], were used as negative control.

Purification of Human Lung Dendritic Cell-subsets

Lung dendritic cells (DC) were isolated from single cell suspensions of lung tissue of 3 patients, as described previously[24]. Lung tissues were rinsed, cut into small fragments, and incubated in digestion medium. Next, the samples were resuspended in Ca^{2+} and Mg^{2+}–free PBS containing 10 mM EDTA and passed through a 40 µm filter. Subsequently, pulmonary mononuclear cells were separated on a Ficoll density gradient. The cells were labeled with anti-CD3-FITC, anti-CD19-FITC, anti-CD207-PE, anti-CD209-PerCp-Cy5 and anti-BDCA2-APC and sorted on a FACSAria (BD Biosciences).

RNA Extraction and Real-Time Polymerase Chain Reaction Analysis

RNA extraction and polymerase chain reaction (PCR) analysis of lung tissue were performed as described previously[25]. RNA extraction from lung tissue blocks of 92 subjects (18 never-smokers, 26 smokers without airflow limitation, 34 patients with COPD GOLD II, 14 patients with COPD GOLD IV) was performed with the miRNeasy Mini kit (Qiagen, Hilden, Germany), following manufacturer's instructions. Next, complementary DNA (cDNA) was prepared with the iScript™ Advanced cDNA Synthesis Kit for RT-qPCR (Bio-Rad, Hercules, California). Taqman Gene Expression

Assays (Applied Biosystems, Forster City, California) were used to measure the expression of DPP4 and the reference genes Glyceraldehyde-3-phosphate dehydrogenase (GAPDH), Hypoxanthine phosphoribosyltransferase-1 (HPRT-1) and Succinate Dehydrogenase complex flavoprotein subunit A (SDHA). Data were analyzed using the standard curve method, and expression of DPP4 was calculated relative to the expression of the 3 reference genes, using the geNorm applet according to the guidelines and theoretical framework previously described[25,26].
For human lung DC subsets, RNA extraction was performed with miRNeasy Mini kit (Qiagen, Hilden, Germany), whereas RNA amplification was with the Qiagen QuantiTect Whole Transcriptome kit, both following manufacturer's instructions. DPP4 expression in the DC subsets was calculated relative to the expression of GAPDH, HPRT1 and peptidylprolyl isomerase A (PPIA) as described previously[25].

DPP4 Immunohistochemistry and Analyses

Sections obtained from formalin-fixed paraffin-embedded lung tissue blocks of 98 subjects (19 never-smokers, 30 smokers without COPD, 36 subjects with COPD GOLD II, and 13 subjects with COPD GOLD III-IV) were incubated with anti-DPP4 antibody (polyclonal goat-anti-human, R&D Systems, AF1180)[16] after antigen retrieval with citrate buffer (Klinipath, Olen, Belgium). Next, slides were colored with diaminobenzidine (Dako, Carpinteria, California) and counterstained with Mayer's hematoxylin (Sigma-Aldrich, St-Louis, Missouri). The isotype control was goat immunoglobulin G (IgG) from R&D Systems (Abingdon, UK) (AB-108-C). To co-stain DPP4 with alveolar epithelial cells, anti-aquaporin 5 (Abcam, Cambridge, UK) (ab92320) and pro-surfactant C (Abcam) (ab90716) were used to detect, respectively, type I and type II alveolar cells and subsequently colored with Vector Blue (Vector, Peterborough, UK).

Quantitative scoring of the amount of DPP4-positive scoring in alveolar tissue and airway epithelium was performed using the Axiovision software (Zeiss, Oberkochen, Germany). In order to measure the area of DPP4-positive signal in alveolar tissue, 15 images of alveolar tissue were recorded from an average of 3 tissue blocks per patient. The intensity of brown staining we wished to score was selected by means of selecting specific hue, lightness, and saturation values. The hue, saturation, and lightness values were identical for all images, therefore restricting our scoring to a specific signal. In every image the alveolar tissue was selected and the DPP4-positive signal was calculated only within the alveolar tissue and normalized to the area of alveolar tissue present in each image. The final score of each patient was the average ratio of DPP4-positive signal of the 15 images. In the airway epithelium, the amount of DPP4 signal was normalized to the length of the basement membrane (Pbm). The

final score of each patient was the average DPP4 staining in all airways present in all tissue blocks available of that patient. The number of airways per patient was between 3 and 20.

DPP4 detection in the frozen samples of proximal bronchi was performed with 1 µg/mL mouse anti-DPP4 monoclonal antibody (Santa Cruz Biotechnology, Dallas, Texas)[16], after previously fixed with acetone and incubated with 10% normal goat serum (Dako, Glostrup, Denmark) for 1 hour at room temperature. These slides were subsequently stained with biotinylated goat antimouse Ig serum (1:50 in PBS/BSA plus 10% human serum) and with streptavidin alkaline phosphatase (1:50 in PBS/BSA plus 10% human serum; Biogenex, Klinipath, Duiven, The Netherlands) for 30 minutes each. A positive signal was revealed with New Fuchsin substrate (Chroma, Kongen, Germany). Counterstaining was performed with Gill's hematoxylin. Negative control staining was performed by the substitution of the primary monoclonal antibody with an isotype antibody.

Statistical Analysis

Statistical analysis was per-formed with Sigma Stat software (SPSS 23.0, Chicago, Illinois), using Kruskal-Wallis, Mann-Whitney U, Fisher exact test, and Spearman correlation analysis. In addition, one-way analysis of variance (ANOVA) and T-tests were used for statistical analyses of the DC subsets. Characteristics of the study population are presented as a median and interquartile range. Differences at P-values < .05 were considered to be significant (*P < .05, **P < .01, and *** P < .001).

Results

DPP4 mRNA Expression is Upregulated in Lungs of Smokers and COPD Patients

Messenger RNA (mRNA) expression of DPP4 was analyzed in lung tissue of 92 subjects. Lung tissue was derived from either resection tissue of lobectomy (never smokers, smokers without airflow limitation and patients with COPD GOLD stage II) or explant lungs of lung transplantation (patients with COPD GOLD stage III–IV). Patient characteristics are described in supplementary Table 1.

Compared to never-smokers, mRNA expression of DPP4 in lung tissue of smokers without airflow limitation and patients with COPD was significantly increased (Figure 1A). Moreover, DPP4 mRNA expression in lung tissue of patients with COPD GOLD stage III–IV was significantly higher than in lung tissue of smokers without airflow limitation and patients with COPD GOLD stage II (Figure 1A). Quantification according

to smoking status (ex- vs. current smokers) is shown in Supplementary Figure S1. Furthermore, DPP4 mRNA expression was inversely correlated with the severity of airflow limitation: FEV1 ($R = -0.376$, $P < .001$) and FEV1/FVC ratio ($R = -0.527$, $P < .001$) (Figure 1B–C). In addition, the mRNA expression of DPP4 was also correlated inversely with the diffusing capacity of the lung, DLCO ($R = -0.402$, $P < .001$) and KCO ($R = -0.408$, $P < .001$) (Figure 1D–E). Linear regression analysis revealed that the association of DPP4 mRNA expression with the presence of COPD was significant even when corrected for age, sex, pack-years, and use of inhaled corticosteroids (Supplementary Table S3).

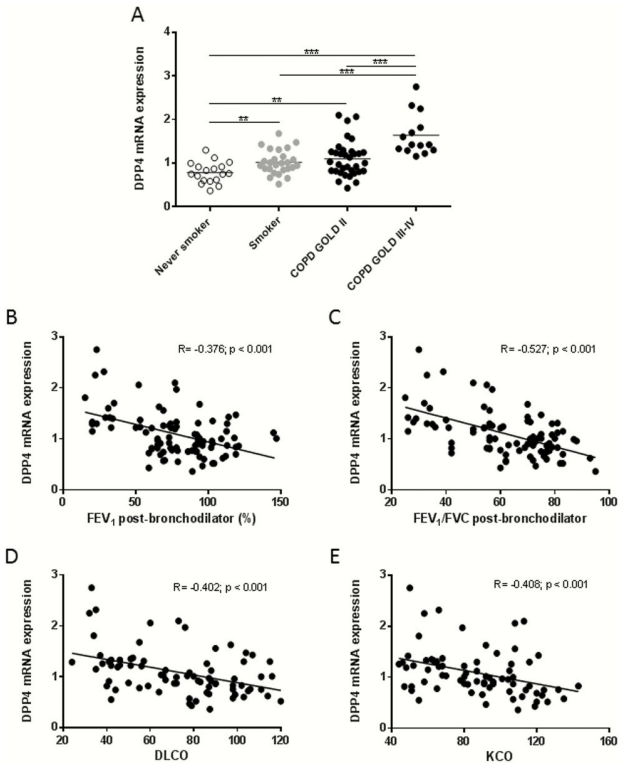


Figure 1. DPP4 mRNA expression in the lung tissues of smokers and COPD patients. A, DPP4 mRNA expression was measured by qRT-PCR and normalized to three reference genes (GAPDH, HPRT-1, SDHA). DPP4 mRNA expression in the lungs of smokers and COPD patients is significantly higher in comparison to that of never smokers. B, Correlation of DPP4 mRNA expression with post-bronchodilator FEV1 values. C, Correlation of DPP4 mRNA expression with post-bronchodilator Tiffeneau index (FEV1/FVC). D, Correlation of DPP4 mRNA expression with DLCO (diffusing capacity or transfer factor of the lung for carbon monoxide). E, Correlation of DPP4 mRNA expression with KCO (carbon monoxide transfer coefficient). **P < .01, ***P < .001. Abbreviations: COPD, chronic obstructive pulmonary disease; DLCO, diffusing capacity of the lung for carbon monoxide; FEV/FVC, forced expiratory volume in 1 second/ forced vital capacity; GOLD, global initiative for obstructive lung disease; KCO, transfer of carbon monoxide coefficient; mRNA, messenger RNA; qRT-PCR, quantitative reverse-transcription polymerase chain reaction.

Additionally, because dendritic cells (DCs) play a crucial role in antiviral immunity, we investigated whether DPP4 mRNA expression differs between DC subsets. Three DC subsets were sorted: langerin-positive DCs, DC-SIGN-positive DCs, and plasmacytoid DCs (pDCs). DPP4 mRNA was merely detected in pDCs (Supplementary Figure S2).

DPP4 Protein Expression is Upregulated in Lungs of Smokers and COPD Patients

DPP4 protein expression was studied in lung tissue of never-smokers, smokers

without airflow limitation, and COPD patients by using immunohistochemistry (IHC) staining. DPP4 was detected on the apical surface of bronchiolar epithelium and in the alveolar epithelial cells. In the alveoli, we observed that DPP4 protein was gradually increased from never-smokers to COPD GOLD stage III–IV (Figure 2). Additionally, we performed immunohistochemical staining of DPP4 with both aquaporin 5 (marker of type I alveolar epithelial cells) and pro-surfactant C (marker of type II alveolar epithelial cells), confirming that the upregulation of DPP4 protein can mainly be contributed to the alveolar epithelial cells (Figure 2I–L). In contrast, this increment was not observed in the bronchiolar epithelium (Figure 2A), as well as in the proximal bronchial epithelium (Figure 3). Furthermore, DPP4 was also detected in the endothelial cells, alveolar macrophages, immune cells in the submucosal region of airway epithelium, and lymphoid aggregates (Supplementary Figure S3). We further quantified DPP4 signals in the lung tissues of 98 subjects using the Axiovision software (Zeiss). Characteristics of these patients are presented in supplementary Table 2.

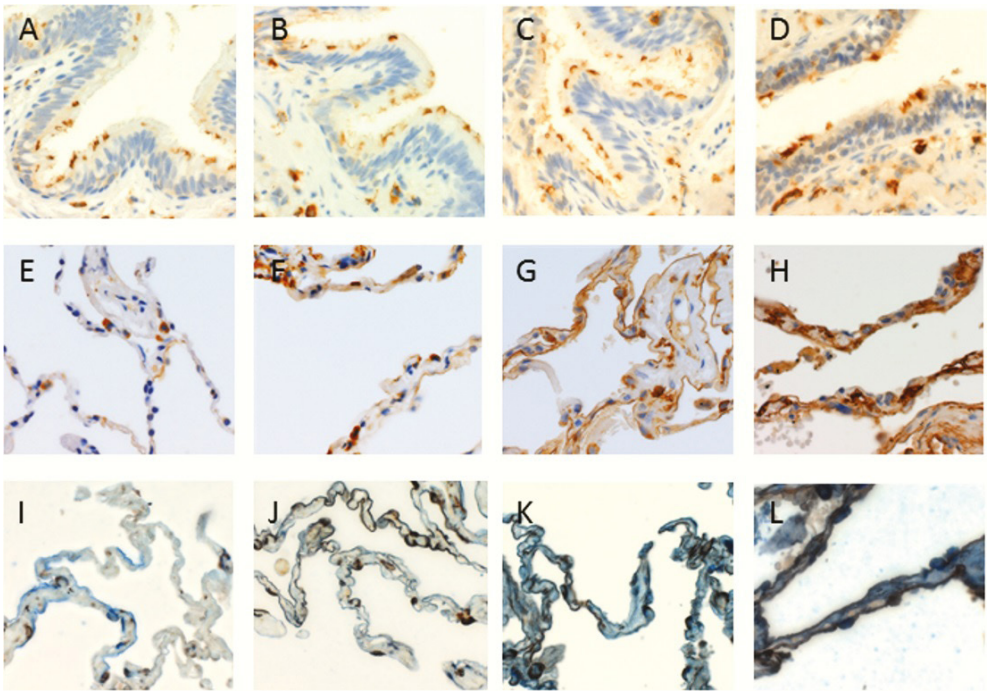


Figure 2. DPP4 protein expression in the bronchiolar epithelium and the alveolar tissues of never smoker, smoker, and COPD patients. Representative images of DPP4 staining in the bronchiolar epithelium (top row) and alveoli (middle and bottom row) of A,E,I, never-smoker, B,F,J, smoker without airflow limitation, C,G,K, subject with COPD GOLD stage II and D,H,L, subject with COPD GOLD stage III–IV. I—L, are immunohistochemical stainings of DPP4 (brown) and aquaporin 5 (marker of type I alveolar epithelial cells) and pro-surfactant C (marker of type II alveolar epithelial cells) (both in blue). Co-staining of DPP4 with either one of the alveolar epithelial cell types results in a dark brown stain. DPP4 was mainly expressed in the alveolar epithelial cells and expressed the most intense in the COPD GOLD stage III–IV group. A 400× magnification was used for all photomicrographs in this figure. Abbreviation: COPD, chronic obstructive pulmonary disease; GOLD, global initiative for chronic obstructive lung disease.

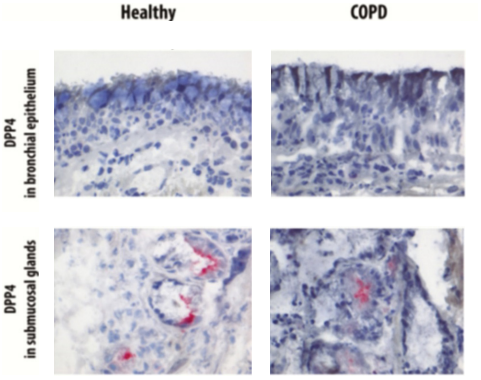


Figure 3. DPP4 staining in the proximal bronchi epithelium. Representative images of DPP4 staining in proximal bronchial epithelium and submucosal glands of the healthy control subject with COPD GOLD stage II. DPP4 was hardly detected in the apical surface of the proximal bronchi epithelium of both healthy control and COPD patients. Submucosal glands here served as positive control for DPP4 staining. Abbreviation: COPD, chronic obstructive pulmonary disease.

Compared to the never-smokers, DPP4 protein expression was significantly increased in the alveolar epithelial cells of smokers and patients with COPD. DPP4 protein expression was the highest in patients with COPD GOLD stage III–IV (Figure 4A). Quantification of DPP4 protein expression according to smoking status (ex- vs. current smoking) is shown in Supplementary Figure S4. Similar to DPP4 mRNA expression, DPP4 protein was also inversely correlated with lung function parameters FEV1 (R = −0.567, P < .001) and FEV1/FVC ratio (R = −0.689, P < .001); as well as diffusing capacity parameters DLCO (R = −0.603, P < .001) and KCO (R = −0.563, P < .001). Linear regression analysis revealed that the association of alveolar DPP4 expression with the presence of COPD was significant even when corrected for age, gender, pack-years, and use of inhaled corticosteroids (Supplementary Table S4).

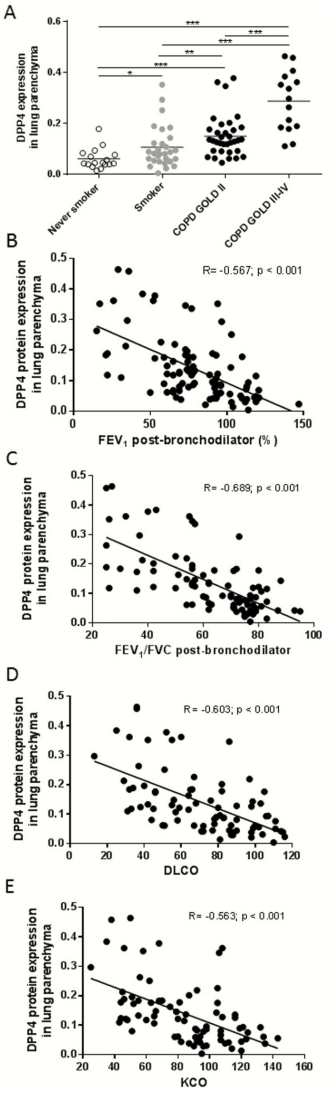


Figure 4. DPP4 protein expression in the lung tissues of smokers and COPD patients. , DPP4 protein expression was analyzed by using Axiovision software (Zeiss). The area of DPP4 positive signal was normalized to the total area of cells present in each analyzed image. DPP4 protein expression in the lungs of smokers and COPD patients is significantly higher in comparison to that of never smokers. B, Correlation of alveolar DPP4 protein expression with post-bronchodilator FEV1 values. C, Correlation of alveolar DPP4 protein expression with post-bronchodilator Tiffeneau index (FEV1/FVC). D, Correlation of alveolar DPP4 protein expression with DLCO (diffusing capacity or transfer factor of the lung for carbon monoxide). E, Correlation of alveolar DPP4 protein expression with KCO (carbon monoxide transfer coefficient). **P < .01, ***P < .001. Abbreviation: COPD, chronic obstructive pulmonary disease.

Discussion

Our study investigated the expression of the MERS-CoV receptor, DPP4, in lung tissues of smokers without airflow limitation and COPD patients in comparison to never-smokers. As previously reported, DPP4 is mainly detected in the alveolar epithelial cells of the lungs, the main target of MERS-CoV infection[13,16]. Among the dendritic cells, we found that DPP4 mRNA is mainly expressed in pDCs; confirming *in vitro* data showing that among the antigen presenting cells, pDCs produce large amounts of type I and III interferon upon contact with MERS-CoV[14]. Most importantly, we provide evidence that DPP4 is upregulated in the lungs, both at mRNA and protein level, not only in COPD patients but also in that of smokers. This indicates that these individuals may be more susceptible to MERS-CoV, supporting both smoking and COPD as risk factors for MERS-CoV infection[18]. These results are in line with a recent study describing a higher DPP4 expression in lungs of 4 COPD patients compared to 16 control subjects of different ages[13].

In this study, we did not find any evidence of DPP4 upregulation in the bronchial and bronchiolar epithelium in the lungs of smokers and COPD patients, suggesting that DPP4 upregulation in pulmonary epithelia is restricted to the alveolar epithelial cells. Previous studies have shown that DPP4 is limitedly expressed in the bronchial and bronchiolar epithelium, and even absent at the apical surface of the nasal respiratory and olfactory epithelium of humans[13,16]. Future studies are needed to assess whether DPP4 upregulation is specific for the alveolar epithelial cells or also occurs in the upper respiratory tract epithelium. Additionally, the importance of alveolar macrophages in the pathogenesis of MERS-CoV needs further research as these cells also express DPP4 and patrol the alveoli while being in close contact with the alveolar epithelial cells.

It is currently unclear how DPP4 is upregulated in the lungs of smokers and COPD patients. Several cytokines have been reported to upregulate DPP4 *in vitro*. TGF-β2, for instance, could upregulate DPP4 protein expression and enzymatic activity in primary human endothelial cells[27], whereas interleukin 13 (IL-13) has been reported to increase DPP4 mRNA expression in human primary bronchial epithelial cells[28]. On the other hand, in COPD pathogenesis, several cytokines—such as IL-6, IL-8, and the TGF-β superfamily—have been described to play important roles[29,30]. Further studies are needed to identify cytokines that could both upregulate DPP4 in the lung and influence COPD pathogenesis.

We also showed that DPP4 mRNA and protein expression were inversely correlated with lung function and diffusing capacity parameters. These data suggest a possible

role of DPP4 in COPD pathogenesis. DPP4 is an exopeptidase responsible for cleaving chemokines and this alters the biological function. Moreover, DPP4 is able to activate T cells and induce production of pro-inflammatory cytokines, which later could affect the development of COPD[19,31-33]. Furthermore, DPP4 is also capable of influencing migration of immune cells by activating or deactivating chemokines in an inflammatory or tumor environment[9,11]. Interestingly, soluble DPP4 in the serum of COPD patients has been reported to be significantly lower compared to that of non-COPD controls[34,35]. It remains possible that in COPD patients, DPP4 concentration is low in the serum and high in the lungs to facilitate migration of certain immune cells into or out of the lungs.

Our study has several strengths; first, we included a large number of patients which have been thoroughly characterized. Second, to eliminate the possible interference of the presence of malignancy in our patients, we also included lung tissue derived from explant lungs of end-stage COPD patients, devoid of malignancy. A possible limitation of our study might be the sex imbalance in the groups with, respectively, a male predominance in the COPD groups and a female predominance in the never-smokers. However, it should be noted that linear regression analyses indicated that sex does not significantly contribute to the differences in expression of membrane-bound DPP4. Our data are in line with recent analyses of soluble DPP4 in serum in patients with COPD versus non-COPD controls indicating that there is no relationship between sex and DPP4 levels[34]. It is also important to acknowledge that there are other factors related to COPD that could contribute to the increased MERS-CoV susceptibility independent of DPP4. For instance, COPD patients are mostly in advanced age and more prone to many other pulmonary infections during hospitalization[36]. Besides that, COPD is associated with systemic inflammation, which might also cause insufficient host immune response against pathogens[19,37].

In conclusion, because smoking is the most common etiology of COPD[20], our data highlight the association between chronic exposure to cigarette smoking and DPP4 upregulation in the lungs, as well as partially explain the increased susceptibility of smokers and COPD patients to MERS-CoV infection[18]. It is imperative to try replicating this observation in an animal model in order to further dissect the molecular pathway of DPP4 upregulation in the lungs.

Acknowledgements

The authors thank Greet Barbier, Indra De Borle, Katleen De Saedeleer, Anouck Goethals, Marie-Rose Mouton, and Ann Neesen, from the laboratory for Translational Research in Obstructive Pulmonary Diseases, Department of Respiratory Medicine (Ghent University Hospital, Ghent, Belgium) for their excellent technical assistance. Furthermore, we thank Dr. Geert Van Pottelberge (Maatschap Longartsen Zeeuws-Vlaanderen / Dept. of Respiratory Medicine, ZorgSaam Ziekenhuis Zeeuws-Vlaanderen, Terneuzen, The Netherlands) for his

contribution to the sorting of human DC and Prof. Bart Vanaudenaerde and Dr. Stijn Verleden (Department of Pneumology, Leuven) for providing us with the explant lungs of patients with severe chronic obstructive pulmonary disease.

This work was supported by the Concerted Research Action of the Ghent University [grant BOF/GOA 01G02714]; the Fund for Scientific Research in Flanders (FWO Vlaanderen) and the Interuniversity Attraction Poles program [grant IUAP P7/30]; and the TOP Project grant from the Netherlands Organization for Health Research and Development (ZonMW) [grantr 91213066].

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Supplementary Information

Supplementary table S1. Characteristics of study population (RT-PCR study) (n = 92)

	Never-smokers	Smokers[ç]	COPD II[%]	COPD III-IV[$]
Number	18	26	34	14
Gender ratio (M/F)	6/12 #	19/7 #	31/3 #	8/6 #
Age (years)	65 (56-70)	63 (55-70)	66 (58-69)§	56 (54-60)*§†
Current- / ex-smoker	-	16/10	22/12	0/14
Smoking history (PY)	0 (0-0)	28 (15-45)*	45 (40-60)*§	30 (25-30)*†
FEV_1 post (L)	2,7 (2,3-3,2)	2,7 (2,3-3,3)	2,0 (1,8-2,4)*§	0,7 (0,7-0,9)*§†
FEV_1 post (% predicted)	102 (92-116)	95 (93-112)	68 (61-75)*§	26 (20-32)*§†
FEV_1 / FVC post (%)	78 (75-83)	75 (71-79)*	56 (53-60)*§	32 (27-35)*§†
DLCO (% predicted)	90 (80-105)	80 (61-102)	67 (51-87)*	35 (33-41)*§†
KCO (% predicted)	103 (88-123)	91 (68-107)*	87 (62-108)*	59 (50-65)*§†
ICS (yes/no)	0/18 #	1/25 #	15/19 #	13/1 #

Footnote:
M/F (male/female); PY (pack years); FEV_1 (forced expiratory volume in 1 second); FVC (forced vital capacity); DLCO (diffusing capacity of the lung for carbon monoxide); KCO (transfer of carbon monoxide coefficient), ICS (inhaled corticosteroids), [ç]smokers without airflow limitation, [%]subjects with COPD stage II as defined by the Global initiative for Obstructive Lung Disease (GOLD), [$]subjects with COPD stage III-IV as defined by GOLD
Data are presented as median (IQR), Mann-Whitney U test: * P < 0,05 versus never smokers; § P < 0,05 versus smokers without COPD; † P < 0,05 versus COPD GOLD I-II
Fisher's exact test: # P < 0,001

Supplementary table S2. Characteristics of study population (IHC study) (n = 98)

	Never-smokers	Smokers[ç]	COPD II[%]	COPD III-IV[$]
Number	19	30	36	13
Gender ratio (male/female)	5/14 #	22/8 #	34/2 #	5/8 #
Age (years)	64 (57-69)	64 (55-70)	65 (59-69)§	59 (56-62)*§†
Current- / ex-smoker	-	18/12	23/13	0/13
Smoking history (PY)	0 (0-0)	34 (16-58)*	46 (39-60)*§	36 (30-45)*†
FEV_1 post (L)	2,6 (2,3-3,0)	3.1 (2,4-3,4)	2,0 (1,8-2,4)*§	0,6 (0,5-1)*§†
FEV_1 post (% predicted)	103 (91-115)	95 (93-114)	69 (60-75)*§	29 (21-35)*§†
FEV_1 / FVC post (%)	78 (74-83)	74 (71-78)	56 (50-58)*§	27 (25-36)*§†
D_{LCO} (% predicted)	88 (80-103)	83 (59-105)	70 (54-87)*§	33 (28-36)*§†
K_{CO} (% predicted)	95 (86-121)	90 (70-98)	89 (69-107)*	46 (25-55)*§†
ICS (yes/no)	1/18 #	2/28 #	15/21 #	13/0 #

Footnote:
M/F (male/female); PY (pack years); FEV_1 (forced expiratory volume in 1 second); FVC (forced vital capacity); DLCO (diffusing capacity of the lung for carbon monoxide); KCO (transfer of carbon monoxide coefficient), ICS (inhaled corticosteroids), [ç] smokers without airflow limitation, [%]subjects with COPD stage II as defined by the Global initiative for Obstructive Lung Disease (GOLD), [$]subjects with COPD stage III-IV as defined by GOLD
Data are presented as median (IQR), Mann-Whitney U test: * P < 0,05 versus never smokers; § P < 0,05 versus smokers without COPD; † P < 0,05 versus COPD GOLD I-II
Fisher's exact test: # P < 0,001

Supplementary table S3. Linear regression model with DPP4 mRNA expression as the dependent variable

Parameter	B	Std. Error	t	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
Intercept	-,261	,270	-,965	,337	-,798	,277
COPD GOLD III-IV	,837	,165	5,059	,000	,508	1,166

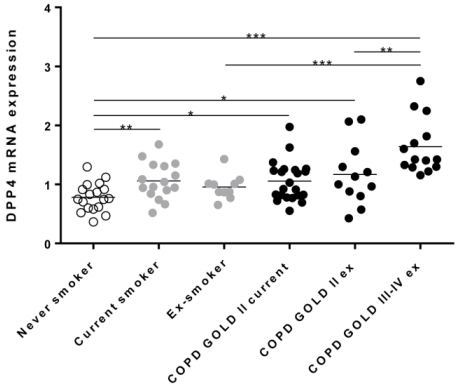
COPD GOLD II	,328	,164	1,995	,049	,001	,656
Smoker*	,252	,131	1,916	,059	-,010	,514
Never-smoker	0a	.	.	.	.	.
Male gender	,032	,085	,380	,705	-,137	,202
Female gender	0a	.	.	.	.	.
ICS yes	-,115	,110	-1,046	,299	-,334	,104
ICS no	0a	.	.	.	.	.
Pack-years	,001	,002	,271	,787	-,004	,006
Age	-,001	,004	-,195	,846	-,009	,008

Footnote:
a. This parameter is set to zero because it is redundant. COPD stage III-IV : severe and very severe COPD according to the guidelines of the Global initiative for Obstructive Lung Disease (GOLD). COPD stage II: moderate COPD according to the guidelines of GOLD. *Smoker without airflow limitation. ICS: inhaled corticosteroids.

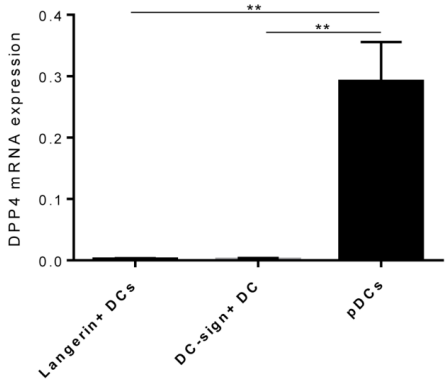
Supplementary table S4. Linear regression model with alveolar DPP4 protein expression as the dependent variable.

Parameter	B	Std. Error	t	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
Intercept	-3,203	,644	-4,977	,000	-4,487	-1,920
COPD GOLD III-IV	1,652	,392	4,213	,000	,870	2,435
COPD GOLD II	1,036	,342	3,032	,003	,354	1,717
Smoker*	,413	,293	1,410	,163	-,171	,998
Never-smoker	0[a]	.	.	.	.	.
Male gender	-,156	,209	-,744	,459	-,572	,261
Female gender	0[a]	.	.	.	.	.
ICS yes	-,153	,241	-,632	,529	-,634	,329
ICS no	0[a]	.	.	.	.	.
Pack-years	,002	,004	,409	,684	-,007	,011
Age	,004	,010	,427	,671	-,016	,024

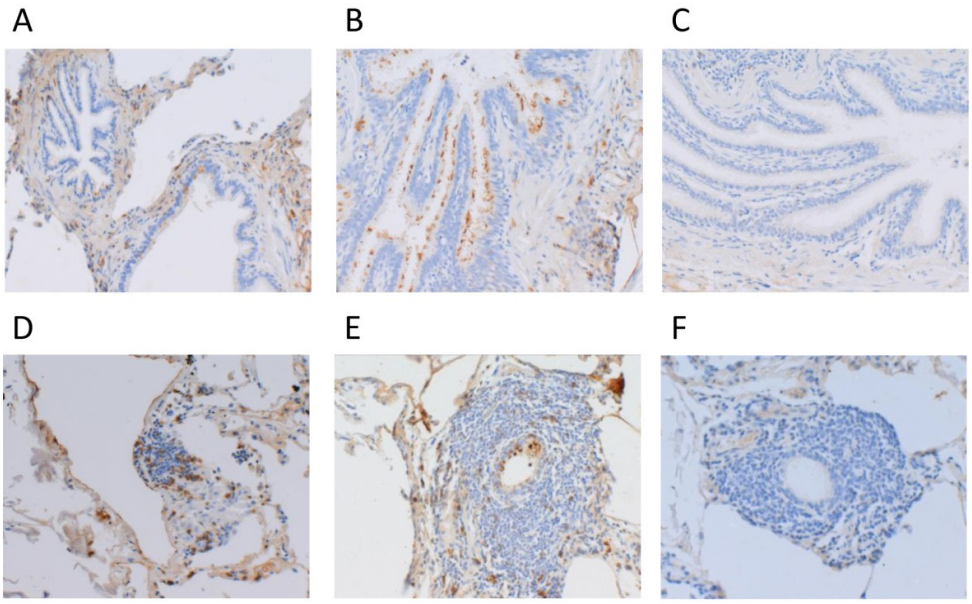
Footnote:
a. This parameter is set to zero because it is redundant. COPD stage III-IV : severe and very severe COPD according to the guidelines of the Global initiative for Obstructive Lung Disease (GOLD). COPD stage II: moderate COPD according to the guidelines of GOLD. *Smoker without airflow limitation. ICS: inhaled corticosteroids.



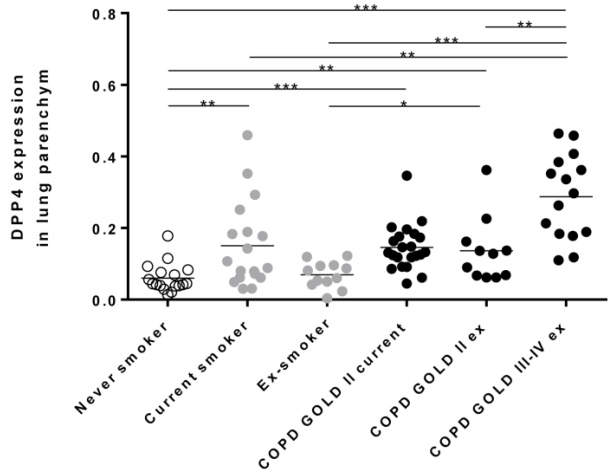
Supplementary Figure 1. mRNA expression of DPP4 in lung tissue of 92 subjects according to smoking status. DPP4 mRNA expression measured by qRT-PCR and normalized to 3 reference genes (GAPDH, HPRT-1, SDHA). *p<0.05, **p<0.01, ***p<0.001.



Supplementary figure S2. mRNA expression of DPP4 in dendritic cell subsets. DPP4 mRNA expression measured by qRT-PCR and normalized to 3 reference genes (GAPDH, HPRT-1, PPIA). **p<0.01.



Supplementary figure S3. Localisation of DPP4 expression in the human lungs. DPP4 was detected with anti-DPP4 polyclonal goat anti-human antibody and visualized in brown with diaminobenzidine. DPP4 was found in the immune cells located in the submucosal region of the airway (A), the apical surface of bronchiolar epithelium (A, B), the endothelium and the lymphoid aggregate (D), as well as the lymphoid follicle (E). Negative controls were stained with isotype antibody (goat IgG). Negative control staining on bronchiolar epithelium and lymphoid follicle were presented here as a representative (C, F).



Supplementary figure S4. DPP4 protein expression in alveolar tissue of 98 subjects according to smoking status. Analyses of DPP4 protein expression in the lung sections was performed with the Axiovision software (Zeiss). The amount of DPP4 positive cells was normalized to the total amount of cells present in the analysed image.

CHAPTER 8

DPP4-expressing Type I Pneumocytes in a Fatal Human MERS-coronavirus Case

Manuscript in preparation

Abstract

Background: MERS-coronavirus (MERS-CoV) causes lower respiratory disease in humans that may be fatal in some. The pathogenesis of MERS-CoV in humans, however, is ill-defined, partly due to the lack of autopsies performed. Here we aimed to gain insight into MERS-CoV pathogenesis using the lungs obtained from a fatal human MERS-CoV case and experiments in non-human primates. **Methods :** We analyzed histopathological lesions and the expression of MERS-CoV receptor, dipeptidyl peptidase-4 (DPP4), in the lungs of a fatal human case. We then investigated DPP4 expression in different non-human primate species infected with MERS-CoV or other viruses. **Results:** MERS-CoV was detected in the type I and II pneumocytes in the lungs of a fatal human MERS-CoV case, consistent with DPP4 expression in these cells. In contrast, in healthy human lungs, DPP4 is almost exclusively expressed in type II pneumocytes. In cynomolgus macaques, we observed a similar restricted DPP4 localization, consistent with virus replication in type II pneumocytes. Virus replication in the lungs was mainly detected at day 1 post inoculation but no upregulation of DPP4 in type I cells was observed. Instead, DPP4-expressing type I pneumocytes were found in cynomolgous macaques that developed severe pneumonia due to influenza A pH1N1, H5N1, and SARS-CoV. **Conclusions:** DPP4-expressing type I pneumocytes are present in the lungs of a fatal MERS-CoV case and in that of influenza A pH1N1, H5N1, and SARS-CoV infected macaques, but almost absent in the lungs of healthy humans and macaques. These cells may broaden the tropism of MERS-CoV in the lungs.

Introduction

Coronaviruses (CoVs) are known to cause respiratory and enteric infections in diverse animal species and humans [1-3]. Among six CoVs known to infect humans, four of them, i.e. HKU1, 229E, OC43, and NL63-CoV, mainly cause mild upper respiratory tract infection. The other two, i.e. severe acute respiratory syndrome coronavirus (SARS-CoV) and Middle East respiratory syndrome coronavirus (MERS-CoV), result in lower respiratory tract infection, ranging in clinical manifestations from asymptomatic to severe [3-5]. Severe SARS-CoV and MERS-CoV infections are often characterized by diffuse alveolar damage (DAD) lesions in the lungs, acute respiratory distress syndrome and fatal outcome [6,7].

Studies on SARS-CoV pathogenesis have revealed that DAD is a multifactorial phenomenon requiring interplay between viral and host factors. Adaptations within the S protein seen during the outbreak, have been shown to result in increased affinity of this virus to its receptor, angiotensin converting enzyme 2 (ACE2) – and may have

improved replication of this virus as well as contributed to the unusual severity of SARS [8,9]. Since ACE2 is expressed in both type I and II pneumocytes in the lung, SARS-CoV is able to replicate efficiently in both cell types [10-14]. Studies in macaques indicated that especially type I cells may be an important target during SARS, and protecting these cells from infection could reduce DAD as well as improve clinical outcome [10]. Another crucial factor in the pathogenesis of SARS-CoV-associated DAD is the delay in type I interferon (IFN) response. Inability to rapidly mount sufficient type I IFN upon SARS-CoV infection, for example due to aging, promotes the accumulation of pathogenic inflammatory monocyte-macrophages and induces DAD [13,15-17].

Unlike SARS-CoV, no major changes have been observed in the receptor binding domain of the MERS-CoV spike protein that interacts with dipeptidyl peptidase-4 (DPP4) after zoonotic transmission from dromedaries to humans [18]. In addition, different from SARS, MERS-CoV infection may cause asymptomatic to mild clinical manifestations in a substantial fraction of individuals infected [19-21]. This suggests that host factors rather than viral factors may have a determining role in the clinical outcome of MERS-CoV infection. Partly due to the limited number of autopsy reports on fatal MERS-CoV cases, there is currently a gap in our understanding of the pathogenesis of MERS-CoV in humans [22,23]. Our study aimed to gain insight in the host factors-associated with MERS-CoV pathogenesis by using post-mortem lung samples obtained from a fatal human MERS-CoV case [24], and by performing experimental MERS-CoV infection in cynomolgus macaques.

Methods

Lung tissue samples from a fatal human MERS-CoV case and healthy controls

This case was once briefly reported in PROMED mail [24]. Family consent was granted for limited postmortem tissue retrieval, consisting of a 20-cm-long midline incision in the lower chest and upper abdomen, from which tissue samples were collected from both lungs. These tissues were then fixated in 10% formalin and embedded in paraffin for further analysis (see Supplementary Methods). As healthy controls, we used lung tissue samples with minimal histopathological lesions obtained from healthy donors or patients with nonmalignant tumors, as described previously [25].

Tissues samples of different non-human primate species

Respiratory tract tissues from cynomolgus macaques, both young-adult (3-5 years old) and aged (10-19 years old), three animals each, were obtained from mock-infected animals from previous experiments [13,14]. Lung tissues of three rhesus macaques were

acquired from simian varicella virus-infected macaques that were sacrificed at day 21 post inoculation (pi). These rhesus macaques developed an asymptomatic infection and no virus detected in bronchial alveolar lavage samples. Lung tissues of two mock-infected African green monkeys were taken from past experiment [12]. Three common marmoset's lung tissues were received from Biomedical Primate Research Center in Rijswijk, the Netherlands. One was a newborn baby, another one was 2 years old female marmoset, and the last one was 4 years old male marmoset. Lung tissues from cynomolgus macaques infected with SARS-CoV, influenza A H5N1 virus, seasonal influenza A H1N1 virus, and pandemic influenza A H1N1 virus were obtained from previous studies [6,12,13,26,27]. These tissues were analyzed with immunohistochemistry and immunofluorescence-based techniques (see Supplementary Methods).

In vivo and *ex vivo* infection experiment in cynomolgus macaques

In vivo MERS-CoV infection experiments were performed under BSL 3 conditions at the Erasmus Medical Centre in Rotterdam using an animal research protocol approved by the Institutional Animal Welfare Committee (EMC2808, nr 122-12-32). Ten immunocompetent young-adult cynomolgus macaques were inoculated with MERS-CoV (EMC/2012 isolate) [7] through intranasal and intratracheal inoculation. Four animals were sacrificed at day 1 pi, four at day 4, and two at day 14. Nasal and throat swabs were collected at multiple time points. Various tissue samples were collected upon necropsy. Presence of viral RNA, virus nucleoprotein, and infectious virions were analyzed in these samples. IFN-β, IL-6, IL-8, CCL3, and CCL20 mRNA expression in the lungs were measured. As a contrast to understand MERS-CoV pathogenesis, we performed experimental NL63-CoV infection in four macaques (EMC1519, nr 122-08-17) and *ex vivo* MERS-CoV infection on nasal and lung tissues of young-adult cynomolgus macaques, as well as reanalyzed samples obtained from previous SARS-CoV (HKU-39849 strain) infection experiment in similar animal models sacrificed at day 4 pi [12]. Details are described in supplementary methods.

Results

Histopathological analysis of postmortem lung tissue samples from a fatal human MERS-CoV case

This human case was once briefly reported earlier in a PROMED mail [24]. A 56-year-old male was admitted to the hospital due to acute respiratory illness. The patient was continuously deteriorating thus required assisted ventilation. Nose and throat swabs were positive for influenza A subtype H1N1pdm09 upon admission. MERS-CoV viral RNA was detected starting at day 14 post onset. The patient developed multi-organ

failure and succumbed to a fatal outcome. Complete history of this patient is described in the supplementary information.

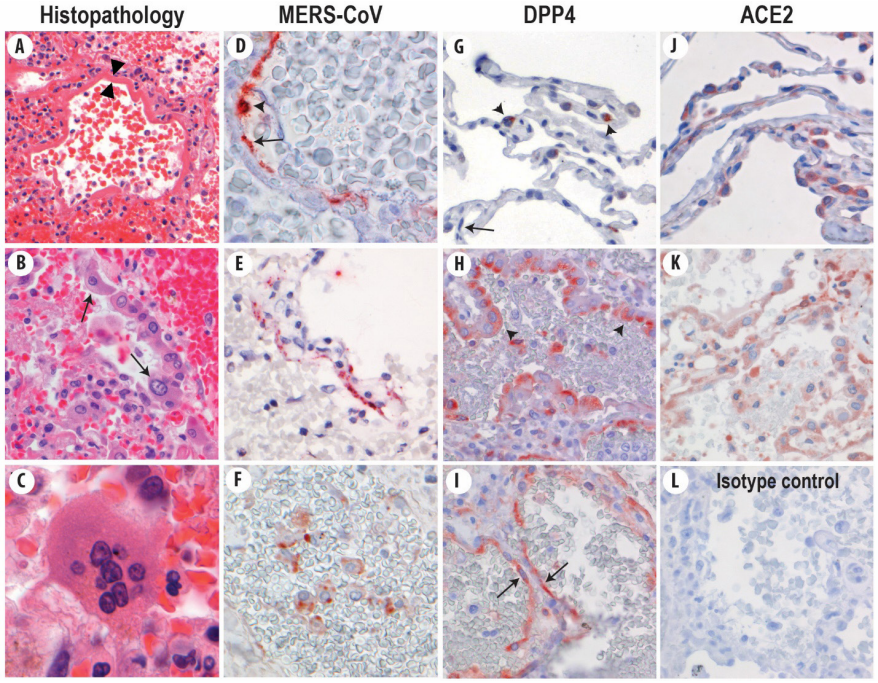


Figure 1. Histopathology and histochemistry analysis on the lungs of a fatal MERS-CoV case. Hyaline membrane (A, arrowheads), hypertrophy and hyperplasia of type II pneumocytes (B, arrows), and syncytia (C) are observed. MERS-CoV nucleocapsid protein is detected in both type I (arrow) and type II pneumocytes (arrowhead) (D). MERS-CoV RNA is detected in epithelial cells (E). MERS-CoV is also detected in alveolar macrophages (F). In healthy human lungs, DPP4 is expressed in type II pneumocytes (arrow) and hardly detected in type I pneumocytes (arrowhead) (G). While in the lungs of the fatal MERS-CoV case, DPP4 is expressed in both type II (H, arrow) and type I cells (I, arrowhead). ACE2 is expressed abundantly in the healthy lungs (J) and the lungs of the fatal MERS-CoV case (K). Isotype control for all antibodies used in this analysis were clean (represented in L). DPP4 and ACE2 are indicated in red. Figure A is taken in 100x magnification; figure B, D-L in 400x; while figure C in 1000x.

Upon histopathological analysis, we found marked alveolar haemorrhage, thickened fibrillar alveolar walls missing epithelial lining, the presence of hyaline membranes, and flooding of alveolar lumina by neutrophils, macrophages, and sloughed epithelial cells, mixed with oedema fluid and fibrin. There was hypertrophy and hyperplasia of type II pneumocytes and bronchiolar epithelial cells. Both cell types occasionally formed syncytial cells pathognomonic for virus infection (Figure 1A-C). MERS-CoV nucleocapsid protein was detected in both type I pneumocytes, the thin squamous epithelial cells, and type II pneumocytes, the larger and more cuboidal epithelial cells (Figure 1D). MERS-CoV RNA was also detected in these keratin positive pneumocytes (Figure 1E, supplementary figure S1A). Besides pneumocytes, MERS-CoV was also detected in CD68 positive alveolar macrophages (Figure 1F, supplementary figure S1B), likely due to phagocytosis of infected cellular debris based on the granular

cytoplasmic expression of virus antigen and the advanced stage of the pathological changes. These results support diffuse alveolar damage as the main histopathology finding in severe MERS-CoV infection and pneumocytes as the replication site of this virus.

We subsequently analyzed DPP4 expression in the lungs of this patient. In healthy human lungs, DPP4 is almost exclusively expressed in type II pneumocytes (Figure 1G), in line with previous reports [25,28]. In contrast, in the lungs of the fatal MERS-CoV case, we observed that DPP4 is expressed both in type I and II pneumocytes (Figure 1H,I). Unlike DPP4, ACE2 is abundantly expressed in healthy lungs (Figure 1J) as well as in the lungs of the fatal human MERS-CoV case (Figure 1K), consistent with previous studies [11,29]. The isotype controls for each of the staining showed no background signal as represented in figure 1L.

DPP4 expression in the lungs of different non-human primate species

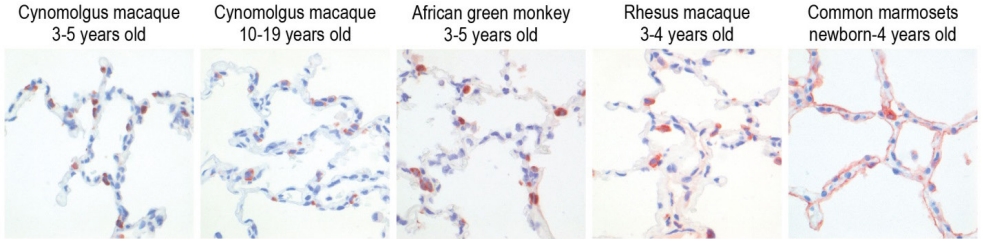


Figure 2. DPP4 expression in the lungs of different non-human primates. DPP4 is expressed in both type I and II pneumocytes in the lungs of common marmosets, while in other non-human primate species, it is almost exclusively expressed in type II pneumocytes. DPP4 is indicated in red. Pictures are made in 400x magnification..

We further studied DPP4 expression in more detail by investigating lungs of different non-human primate species. We found that DPP4 is expressed in both type I and II pneumocytes in the lungs of common marmosets, while in that of other non-human primate species, DPP4-expressing type I pneumocytes were hardly detected (Figure 2). In rhesus macaques, MERS-CoV causes mild-to-moderate pneumonia [30,31], while in common marmosets this virus may induce severe-to-fatal pneumonia [32,33]. We did not find a distinct difference in DPP4 protein expression between the lungs of young-adult and advanced-age cynomolgus macaques (Figure 2), but DPP4 mRNA level in the lungs of advanced-age cynomolgus macaques was significantly higher compared to the young-adults (Supplementary figure S2).

MERS-CoV infection hardly causes clinical manifestations in cynomolgus macaques

We then performed a MERS-CoV infection experiment in young-adult cynomolgus

macaques to better understand MERS-CoV tropism and replication in the lungs. All animals developed a mild increase in body temperature during the experiment (Supplementary Figure S3A). Upon autopsy, the macroscopic lung lesions were found to be mild, except for one macaque sacrificed at day 4 pi that developed severe suppurative bronchopneumonia due to Escherichia coli coinfection (Supplementary Figure S3B). The bronchial lymph nodes and tonsils were enlarged in all animals. Seroconversion and neutralizing antibodies were detected in the animals sacrificed at day 14 pi.

MERS-CoV does not replicate in the DPP4 negative upper respiratory tract epithelium

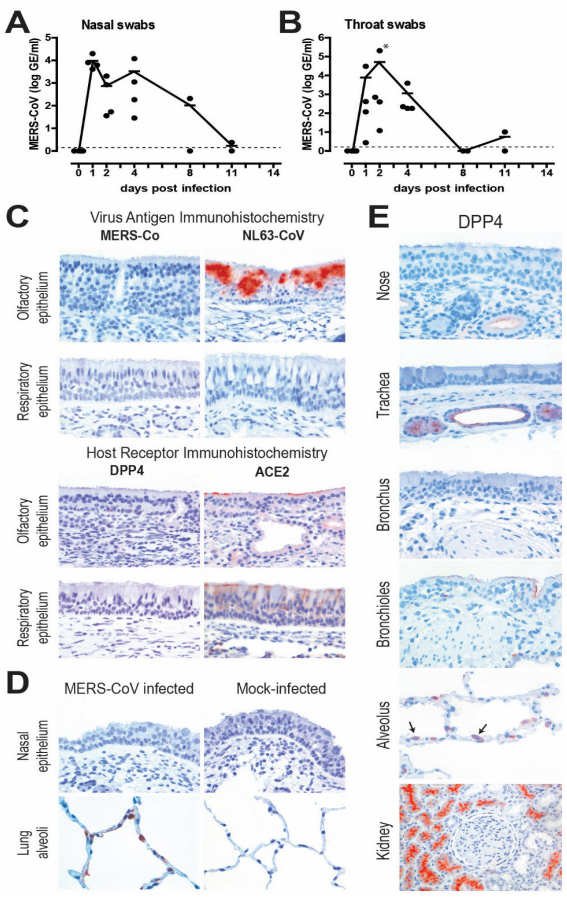


Figure 3. MERS-CoV replication and DPP4 expression in the upper respiratory tract. MERS-CoV RNA is detected in the nasal and throat swabs of all macaques up to day 11 pi (A,B). None of the samples showed detectable infectious virus titres, except for one throat swab (*) taken from the macaque that developed bacterial co-infection. Dotted lines indicated detection limit of the assays. Data from each sample is indicated by black circles and the horizontal line represents the median value. Neither MERS-CoV nucleocapsid protein nor MERS-CoV receptor, DPP4, are detected in the nasal epithelium of cynomolgus macaques (C). In contrast, NL63-CoV nucleocapsid pro-tein is detected in the nasal olfactory epithelium, while NL63-CoV receptor, ACE2, is detected in both nasal olfactory and respiratory epithelium (C). In the *ex vivo* MERS-CoV infection experiment, MERS-CoV nucleocapsid protein is detected in the pneumocytes but not in the nasal epithelial cells (D). Non-infected tissues (mock) are used as negative control. Figures C and D are taken at 400x. DPP4 is mainly detected in the type II pneumocytes (arrows) and gradually became unde-tected from lower to the upper part of the respiratory tract (E). The kidney is used as a control tissue due to its high DPP4 expression. Pictures of the terminal bronchioles and alveolus are taken at 400x magnification, while the othersat 200x.

In the nasal and throat swab samples, MERS-CoV RNA was detected from day 1 pi, but the levels were generally low (Fig 3A,B). No infectious virus was detected, except in one throat swab sample from the macaque with bacterial co-infection (10 $TCID_{50}$/ml, labelled with an asterisk in Figure 3B). We detected no MERS-CoV antigen in the upper

respiratory tract tissues even at day 1 pi, supporting the virus titration result. Unlike MERS-CoV, NL63-CoV that uses ACE2 as its receptor [34], can infect nasal epithelium. NL63-CoV-infected macaques developed mild clinical signs and did seroconvert (virus neutralization titres of 40-80), mimicking clinical manifestations in humans [35]. NL63-CoV preference to infect nasal olfactory epithelium is currently unclear. However, NL63-CoV has been described to use heparan sulfate as attachment factor; and in mice this polysaccharide is selectively expressed at the apical surface of the olfactory but not the respiratory epithelium [36,37]. In contrast to ACE2, DPP4 is not expressed in the nasal epithelium (Figure 3C). Using *ex vivo* infection of respiratory tract tissues we also observed that nasal epithelium is not susceptible to MERS-CoV infection (Figure 3D).

MERS-CoV replication is confined to type II pneumocytes that express DPP4

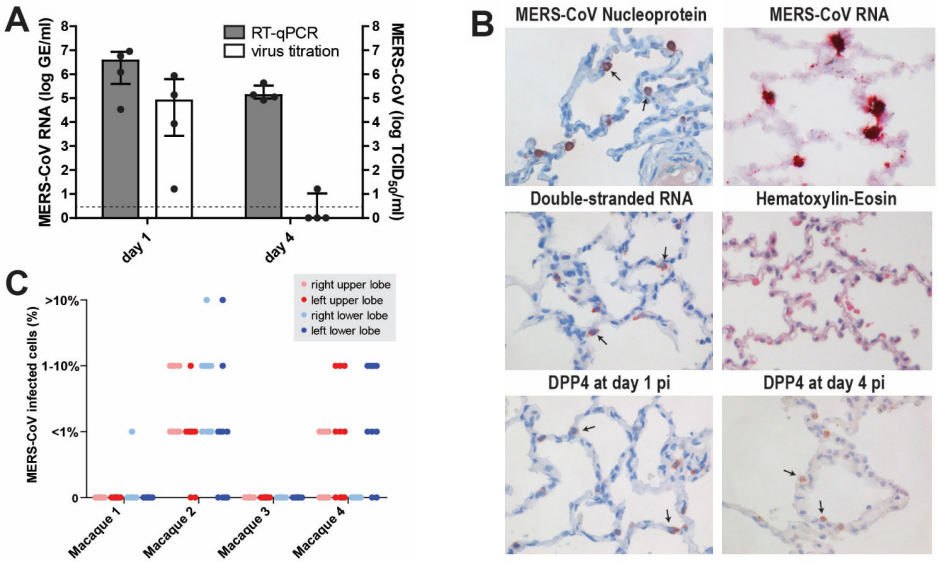


Figure 4. MERS-CoV replication and DPP4 expression in the lungs. In the lung, large amounts of MERS-CoV RNA were detected at day 1 and 4 pi, but infectious MERS-CoV is only largely detected at day 1 pi (A). Three out of four lung homogenate samples from day 4 pi are negative in virus titration. Dotted lines indicated detection limit of the assays. The data is presented in median ± interquartile range, while data from each sample is shown as black circles. MERS-CoV nucleoprotein, MERS-CoV RNA, and double-stranded RNA are detected mainly in the type II pneumocytes at day 1 pi (B). HE staining showed hardly any histopathological changes in the lungs of these macaques. DPP4 is mainly expressed in type II pneumocytes (arrows) at both time points (B). All these pictures are taken at 400x. The percentage of MERS-CoV nucleoprotein positive cells in the lungs of macaques sacrificed at day 1 pi is scored qualitatively. The percentage is generally low since most of the areas are uninfected (D).

In the respiratory tract of cynomolgus macaques, DPP4 is absent in the nasal epithelium but predominantly expressed in type II pneumocytes (Figure 3E). Its expression in the lungs is relatively low compared to that in the kidney and small intestine (Figure 3E and Supplementary Figure S4). This DPP4 distribution is comparable to humans, in which DPP4 is absent in the nasal epithelium, while its expression in the lungs, liver,

and colon are lower compared to that in kidney and small intestine [25,38].

Upon MERS-CoV inoculation, both viral RNA and infectious virus were detected in the lungs of cynomolgus macaques at day 1 pi, but infectious virus was hardly detected at day 4 pi (Figure 4A). No infectious virus was found in other respiratory and extra-respiratory tissues, while virus RNA was detected in the urine, large intestines, and kidneys of only one macaque sacrificed at day 1 pi. MERS-CoV nucleoprotein was detected in the lungs at day 4 pi in very few cells, supporting rapid clearance of the virus. At day 1 pi, MERS-CoV nucleoprotein, MERS-CoV RNA, and double-stranded RNA were mainly found in the type II pneumocytes. Histopathological lesions and immune cell infiltration were minimal in these lungs (Figure 4B). DPP4 was still mainly expressed in type II pneumocytes both at day 1 and day 4 pi (Figure 4B). Together, these data indicate that MERS-CoV replication is confined to type II pneumocytes where DPP4 is predominantly expressed and upregulation of DPP4 on type I cells was not observed during the infection.

Type I interferon is locally expressed in the lungs of MERS-CoV-infected macaques

We subsequently investigated the extent of MERS-CoV infection in the lungs of these macaques at day 1 pi. We found relatively few MERS-CoV infected cells in the lungs, with two out of four macaques, having almost no MERS-CoV-infected cells (Figure 4C). These data may indicate that MERS-CoV replication is already restricted at day 1 pi, possibly due to early innate immune response. In line with confined MERS-CoV replication, IFN-β, IL8, IL6, CCL3, and CCL20 mRNA expression was detected in several lung samples at day 1 pi (Figure 5A). We have previously reported that MERS-CoV is sensitive towards type I IFN and does not block pSTAT1 translocation, a crucial step for host cells to initiate IFN production via JAK-STAT signaling pathway [39]. In the lungs of MERS-CoV-infected macaques at day 1 pi, pSTAT1 was expressed in pneumocytes and alveolar macrophages, in areas surrounding virus-infected pneumocytes and blood vessels. Similar findings were observed in the lungs of SARS-CoV-infected macaques obtained from previous experiment [14], but not in the lungs of mock-infected macaques and MERS-CoV-infected lung explants (Figure 5B). This suggests that type I IFN is initially produced by infiltrating rather than residential immune cells. pSTAT1 colocalized with MERS-CoV nucleoprotein in several pneumocytes, but not with SARS-CoV nucleoprotein (Figure 5B), supporting that SARS-CoV is more resistant to type I IFN compared to SARS-CoV [39,40]. Upon reanalyzing past SARS-CoV infection samples [12,13], we found that at day 4 pi, viral RNA levels, IFN-β, IL-6, and CCL3expression were significantly higher in the lungs of SARS-CoV-infected macaques compared to that of MERS-CoV-infected macaques (Figure 5C, Supplementary figure S5). SARS-CoV infectious virions were also still

abundantly detected at day 4 pi [12], supporting the ongoing SARS-CoV replication in the lungs at this time point, while MERS-CoV is more rapidly contained.

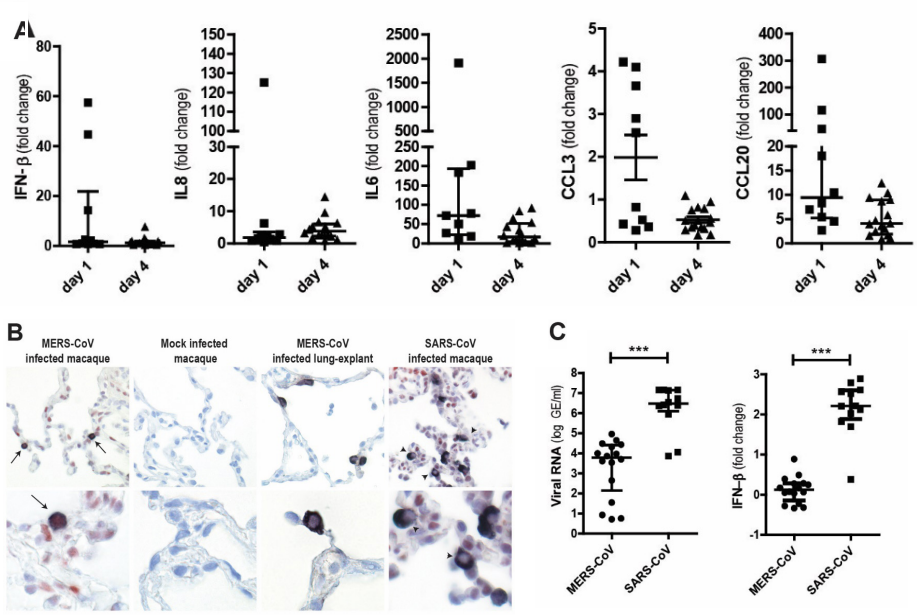


Figure 5. Cytokine expression in the lungs of MERS-CoV-infected macaques. IFN-β, IL8, IL6, CCL3, and CCL20 mRNA expression were relatively high in some of the lung tissues at day 1 pi (A). Data are presented in fold change unit as described in the supplementary methods and displayed based on their data distribution, i.e. IFN-β, IL8, IL6, and CCL20 are presented in median ± interquartile range, while CCL3 in mean ± standard error. pSTAT1 is detected in the lungs of MERS-CoV and SARS-CoV infected macaques at day 1 pi, but not in the lungs mock-infected macaques and MERS-CoV infected lung explants (B). pSTAT1 colocalizes with MERS-CoV nucleoprotein (arrows) in some of the pneumocytes, but not with SARS-CoV nucleoprotein (arrowheads) (B). Pictures in first row are taken in 400x magnification, while those in second row in 1000x magnification. Viral RNA and IFNβ expression in the lungs of SARS-CoV infected macaques are significantly higher compared to MERS-CoV at day 4 pi (C). Both are displayed in median ± interquartile range and analyzed with Mann-Whitney test. Significant difference was marked with asterisks (***p value ≤ 0,001).

Differential DPP4 expression in the lungs of cynomolgus macaques

Our data revealed that in the respiratory tract of cynomolgus macaques, DPP4 expression and MERS-CoV replication were confined mainly to type II pneumocytes, unlike the lungs of a fatal MERS-CoV case that was positive for influenza A subtype H1N1pdm09 upon admission. Next, we investigated whether other viruses that induce severe respiratory infection in the lungs upregulate DPP4 in type I pneumocytes. We found that DPP4-expressing type I pneumocytes were readily detected in several areas of the lungs of cynomolgus macaques that developed diffuse alveolar damage due to influenza A pH1N1, H5N1, and SARS-CoV (Figure 6A). These DPP4-expressing type I pneumocytes were mostly located nearby actively proliferating type II pneumocytes, as indicated by Ki67 staining (Figure 6B). Likely, these DPP4 positive type I pneumocytes recently differentiated from proliferating type II pneumocytes.

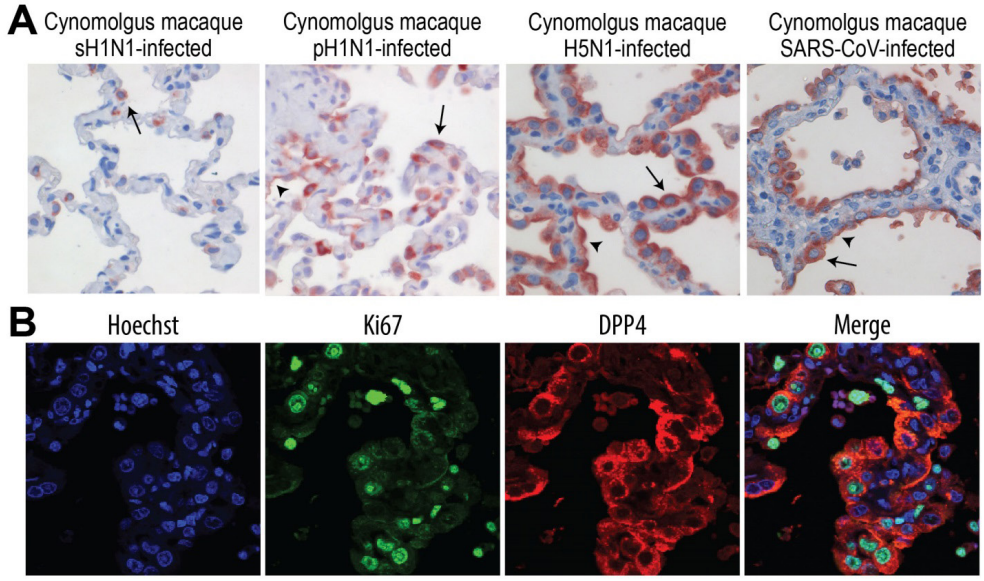


Figure 6. DPP4 expression in the lungs of cynomolgus macaques infected with different viruses. DPP4 is almost exclusively detected in type II pneumocytes in the lungs of cynomolgus macaques infected with influenza A sH1N1. However, it is prominently detected in type I and II pneumocytes in the lungs with diffuse alveolar damage, obtained from cynomolgus macaques infected with either influenza A pH1N1, influenza A H5N1 or SARS-CoV (A). Colocalization between DPP4 (red) and Ki67 (green), a cell proliferation marker, was detected in the type II pneumocytes within the lungs of H5N1-infected cynomolgus macaques (B). Nuclei were stained with Hoechst and visualized in blue. All pictures are taken at 400x magnification.

Discussion

Both MERS-CoV and SARS-CoV are known to cause severe pneumonia in humans [5]. The severe cases develop DAD-associated lesions in the lungs and have a high risk to succumb to fatal outcome [6,7,22]. For SARS-CoV, three factors have been suggested to be essential for inducing DAD and severe clinical outcome, i.e. SARS-CoV adaptation to increase its affinity to its receptor in humans, viral tropism for type I pneumocytes, and a delayed host type I interferon response [9,10,15]. Unlike SARS-CoV, MERS-CoV from dromedary camels already binds with moderate to high affinity to its receptor, DPP4, thus suggesting a difference in pathogenesis between both viruses [18].

We observed that unlike healthy lung tissues where DPP4 is almost exclusively expressed in type II pneumocytes, in the lungs of a fatal MERS-CoV case DPP4 is prominently expressed in the type I pneumocytes [25,28]. These DPP4 positive type I pneumocytes are also readily detected in the lungs of smokers and chronic lung disease patients, and in that of common marmosets [28,41]. Smoking and chronic lung diseases are known risk factors for primary and fatal MERS-CoV infection, whereas common marmosets have been reported to exhibit severe clinical manifestations upon MERS-CoV inoculation [32,33,42,43]. In genetically modified susceptible mice, MERS-CoV

induces mild infection when DPP4 is exclusively expressed in type II pneumocytes [44]. Severe clinical manifestations are found in these mice when the virus is adapted through serial passaging or DPP4 expression is upregulated throughout the airway epithelium [44,45]. These data suggest that variation in DPP4 expression may influence the severity of MERS-CoV infection.

Our experimental MERS-CoV infection in healthy young-adult cynomolgus macaques, which have a similar DPP4 distribution compared to humans [25,28], generally resulted in minimal clinical manifestations. MERS-CoV does not replicate in the DPP4 negative nasal epithelium and mainly targets type II pneumocytes where DPP4 is predominantly expressed. However, only a few cells were infected and infectious virus was rapidly eliminated within four days, similar to what has been observed in the lungs of MERS-CoV-infected rhesus macaques [30]. Limited DPP4 expression in the lungs, MERS-CoV sensitivity towards type I IFN, and MERS-CoV inability to block pSTAT1 translocation are several possible factors that limit MERS-CoV replication in these animals [38,39]. DPP4-expressing type I pneumocytes were not detected in the lungs of our macaques. In contrast, these were prominently found in the lungs of macaques that developed DAD due to other viral infections such as SARS-CoV, influenza A pH1N1 and H5N1 viruses [6,12,13,26,27]. We further showed that these DPP4-expressing type I pneumocytes are most likely those that are recently differentiated from type II cells, linking DPP4 upregulation with pneumocyte regeneration and differentiation. Interestingly, the fatal MERS-CoV case was originally admitted due to influenza A pH1N1 infection suggesting coinfection in this patient.

In general, our experimental infection study in macaques may indicate that MERS-CoV infection in healthy young individuals also results in limited replication and asymptomatic to mild clinical manifestations. In contrast, SARS-CoV-infected young-adult macaques shed infectious virus in their nasal swabs up to day 7 pi and can productively replicate in the lungs up to day 10 pi [8,12,13,46,47]. This suggests that MERS-CoV has a narrower tropism and replication window in macaques compared to SARS-CoV, hence is less pathogenic. Thus, DPP4 upregulation in type I pneumocytes either due to comorbidities [41] or coinfections might be crucial to enhance MERS-CoV replication, induce DAD lesions, and yield a fatal outcome. Further investigations to assess the importance of DPP4-expressing type I pneumocytes in MERS-CoV pathogenesis are still necessary, yet challenging to perform. These studies would for example require a reliable and physiologically representative *in vitro* model for type I pneumocytes. Recent advances in the field of lung organoids seem to offer the possibility to develop such model in the near future [48,49]. Alternatively, performing these studies *in vivo* would require the generation of a mice model that specifically expresses DPP4 in type I pneumocytes. Upregulating DPP4 expression by introducing

an underlying comorbidity or by coinfection would likely influence other factors, for example host immune response. Further studies remain necessary to understand the pathogenesis of fatal MERS-CoV infection and characterize the factors responsible for such a devastating outcome.

Notes

Acknowledgments

We are grateful for the help of F. van der Panne with microphotographs; L.M. Leijten for her advice on optimizing the immunohistochemistry techniques performed in this study; P.R. van Run, A.F Altenburg, and C.E van de Sandt for assistance in the *ex vivo* infection experiments; L.L. Provacia and C.M.E. Schapendonk for technical assistance throughout this study; Lia van den Hoek for kindly providing rabbit serum against NL63-CoV nucleocapsid protein; and G. van Amerongen and R. Dias D'Ullois for their help with the animal experiments.

Financial support

This study was funded by Nederlandse Organisatie voor Wetenschappelijk Onderzoek (grant 91213066) and Zoonotic Anticipation and Preparedness Initiative (Innovative Medicines Initiative grant 115760), with assistance and financial support from Innovative Medicines Initiative and the European Commission and contributions from European Federation of Pharmaceutical Industries and Associations partners.

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Supplementary Methods

Virus preparation

MERS-CoV (EMC/2012 isolate) [1] was passaged six times on Vero E6 cells. NL63-CoV was passaged four times on Vero E6 cells. All cell cultures were performed at biosafety level 3 facility.

In vivo infection experiment in cynomolgus macaques

Young adult cynomolgus macaques (Macaca fascicularis) seronegative for coronaviruses were purchased from commercial breeders (Harlan, Indianapolis, IN, USA), maintained in standard housing and provided with commercial food pellets and water ad libitum until the start of the experiment. Two weeks prior to inoculation with MERS-CoV, a telemetric sensor (DST micro-T ultra-small temperature logger, Star-Oddi, Reykjavik, Iceland) was placed in the peritoneal cavity of only three macaques in the MERS-CoV inoculated group to record body temperature every 15 minutes. Before the inoculation, the macaques were examined clinically and determined as healthy by a registered veterinarian and were placed in negatively pressurized glove boxes. During the experiment, animals were checked daily for clinical signs, including assessment of appearance, behavior, weight, presence of any nasal or ocular secretions, food, and water consumption.

All inoculations were performed under anesthesia ((ketamine® (Nimatek, Eurovet Animal Health BV, Bladel, the Netherlands) and domitor® (Orion Pharma, Espoo, Finland)). Ten macaques were inoculated intratracheally (4.5 ml) and intranasally (0.5 ml) with 1 x 10^6 50% Tissue Culture Infectious Dose ($TCID_{50}$) MERS-CoV. Prior to infection and at days 1, 2, 4, 8, and 11 pi, animals were anesthetized with ketamine, and oral and nasal swabs were taken and placed in 1 ml Dulbecco's modified Eagle's medium supplemented with 100 IU penicillin/ml and 100 µg streptomycin/ml (virus transport medium). The swabs were frozen at −70°C until analysis. Four macaques were euthanized at each of days 1 and 4 pi, while the last two were euthanized at day 21 pi by exsanguination under anesthesia. Autopsies were performed according to a standard protocol. One lung from each monkey was inflated with 10% neutral-buffered formalin by intrabronchial intubation and suspended in 10% neutral-buffered formalin overnight. Samples were collected in a standard manner, embedded in paraffin, cut at 3 µm, and used for immunohistochemistry, in situ hybridization, or stained with hematoxylin-eosin for examination by light microscopy. Tissue samples were also collected in virus transport medium (Hanks balanced salt solution supplemented with 10% glycerol, 200 U of penicillin per ml, 200 µg of streptomycin per ml, 100 U of polymyxin B sulphate per ml, 250 µg of gentamycin per ml, and 50 U of nystatin per ml). Tissue samples were weighed prior to homogenization in 2 ml virus transport medium, using Polytron PT2100 tissue grinders (Kinematica). After centrifugation, the homogenates were frozen at −70°C until virus titration and RT-qPCR.

NL63-CoV and SARS-CoV infections were used as a contrast to further understand the pathogenesis of MERS-CoV infection. Experimental NL63-CoV infection was performed in four macaques. They were inoculated intratracheally (2.5 ml) and intranasally (0.5 ml) with 1 x 10^5 $TCID_{50}$ of NL63-CoV. Two animals were euthanized at each of days 4 and 21 pi. All animals were euthanized by exsanguination under anesthesia. Meanwhile, the experimental SARS-CoV infection had been previously performed in our center also in healthy young adult cynomolgus macaques [2]. They were infected intratracheally with 1 × 10^6 $TCID_{50}$ of one of the late SARS-CoV strains (HKU-39849) and euthanized at day 4 pi. Leftover lung tissue samples from this experiment were analyzed together with lung tissue samples from MERS-CoV experiment.

Ex vivo infection experiment in tissues of cynomolgus macaques

Nasal tissue and one entire lung lobe were harvested from a healthy 8-year-old cynomolgus macaque and transferred immediately to ice. Lung tissue was prepared as described previously [3]. Nose and lung tissue were sliced and transferred to 24-well plates (Corning, Wiesbaden, Germany) containing culture medium and incubated overnight at 37°C in 5% (v/v) CO2. Each tissue was prepared in triplicate. Either 3 x 10^6 TCID50 MERS-CoV or culture medium (mock-infected group) was added to the wells under BSL3 conditions. After 1 hour incubation at 37°C, 500 µl fresh medium was added to each well and incubated overnight at 37°C in 5% (v/v) CO_2. All tissue pieces were then fixed in formalin and embedded in paraffin for further analysis.

Virological analysis

Viral RNA was isolated using viral RNA Mini kit (Qiagen, Hilden, Germany) and subsequently measured for a viral load using reverse transcription-quantitative polymerase chain reaction (RT-qPCR) (Roche, Mannheim, Germany). Each MERS-CoV samples was only considered positive if both E and N gene were detected [4]. Meanwhile, NL63-CoV samples were detected by RT-qPCR targeting N gene [5]. Samples were also titrated on Vero E6 cells to further confirm the results of RT-qPCR. Ten-fold serial dilutions of the homogenates were prepared in Lonza IMDM medium containing 5% fetal bovine serum (FBS) for virus titration assay on Vero E6 cells. The amount of infectious virus in lung homogenates was calculated according to the Spearman-Karber standard equation [6]. The limit of viral detection for this assay was 0.5 log10 $TCID_{50}$/ml.

Microbiology analysis

One macaque developed severe suppurative bronchopneumonia in our study. Lung tissue samples of this macaque were used

to isolate RNA and bacterial typing as described previously [7].

Immunohistochemistry and Immunofluorescence analysis

MERS-CoV antigen was detected either with rabbit-anti-SARS-CoV NSP4, which cross-reacts with MERS-CoV [8] or with mouse monoclonal antibodies against MERS-CoV nucleocapsid protein (clone 10, SinoBiological, Beijing, China). Additionally, MERS-CoV RNA in tissues was detected using in situ hybridisation designed by Advanced Cell Diagnostics (Hayward, CA, USA) with procedures suggested by the manufacturer. DPP4 was detected with polyclonal goat anti-human DPP4 (clone AF1180, R&D systems, Abingdon, UK). Angiotensin converting enzyme 2 (ACE2) with polyclonal rabbit anti-human ACE2 (clone ab15348, Abcam, Cambridge, UK). Pankeratin, an epithelial marker, with mouse monoclonal antibodies (clone AE1/AE3, Neomarkers, Fremont, CA, USA). CD68, a macrophage marker, with mouse monoclonal antibodies (clone KP-1, Dako, Glostrup, Denmark). The presence of double-stranded RNA was observed with mouse monoclonal antibody J2 (Scicons, English and Scientific Consulting, Hungary) [9]. SARS-CoV nucleocapsid protein was identified with mouse monoclonal antibody (Imgenex, Littleton, USA). NL63-CoV antigen was detected using rabbit serum against its nucleocapsid protein (a kind gift from Lia van den Hoek). CEACAM5 was checked using polyclonal rabbit IgG anti-human CEACAM5 (Abcam, Cambridge, UK). pSTAT1 protein was detected using rabbit anti-human pSTAT1 (ThermoScientific, Rockford, USA). Normal goat serum (MP Biomedicals, Bruxelles, Belgium), rabbit IgG and isotype antibodies (DAKO, Glostrup, Denmark) were used as negative controls. The dark blue signal was stained with alkaline phosphatase-labelled secondary antibody and revealed with BCIP/NBT substrate (DAKO, Glostrup, Denmark). The red signals were stained with peroxidase-labelled secondary antibody and revealed using 3-amino-9-ethylcarbazole and the 3,3'-diaminobenzidine substrate, respectively. All slides were counterstained with hematoxylin. For double-immunofluorescence staining, each slide was incubated with mouse monoclonal anti-Ki67 (DAKO, Glostrup, Denmark), and polyclonal goat IgG anti-human DPP4. Secondary staining was performed with rabbit anti-mouse IgG conjugated with Alexa Fluor 488 and rabbit-anti goat IgG conjugated with Alexa Fluor 568, while cell nuclei were detected with Hoechst (all products from Life Technologies, Rockford, USA).

In situ hybridization

In situ hybridization was performed according to the RNAScope platform suggested by the manufacturer [10]. Probes targeting the nucleocapsid gene of MERS-CoV and NL63-CoV were designed by Advanced Cell Diagnostics (Hayward, CA, USA).

Microarray processing

The original experiments and generation of microarray expression data were performed by Smits et al (PMID: 20140198; ref 9). The array data were quantile normalized and summarized using median polish (i.e., RMA, ref Irizarry et al PMID 12925520). Affymetrix rhesus macaque array annotation (available from the Affymetrix website) was used for annotating probesets with gene symbols. Statistical analysis was performed using limma [Smythe 2004 PMID: 16646809]. Log2-expressions were plotted for selected probesets.

Cytokine measurements

RT-qPCR was performed as described previously to detect cellular gene expression changes for IFN-β, IL-6, IL-8, CCL3, and CCL20 [9, 10]. Differences in gene expression are represented as the fold change in gene expression relative to a calibrator and normalized to a reference, using the $2^{-\Delta\Delta Ct}$ method [11]. GAPDH (glyceraldehyde-3-phosphate dehydrogenase) was used as a reference endogenous control. The samples from the mock-infected macaques were used for calibration. Data distribution was evaluated with D'Agustino and Pearson omnibus normality test. The data were presented either in mean ± standard error of mean or median ± interquartile range. Statistical analysis was performed with unpaired t test or Mann-Whitney test.

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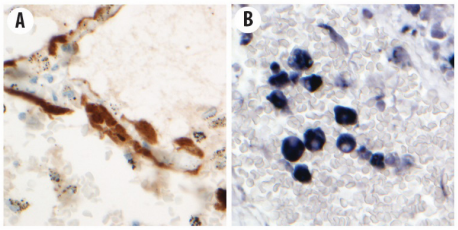
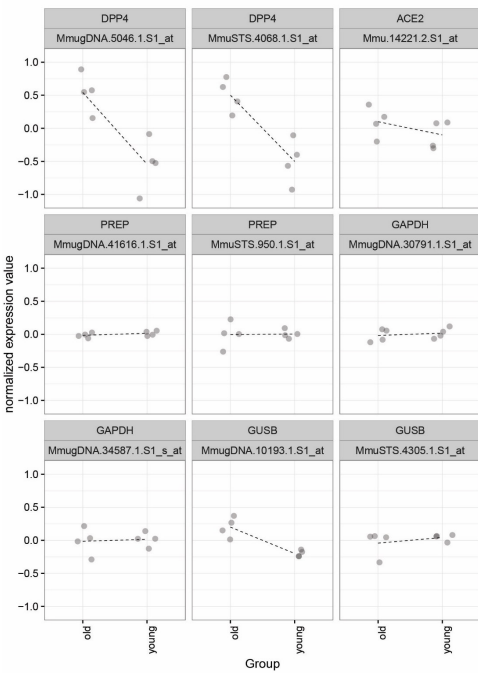
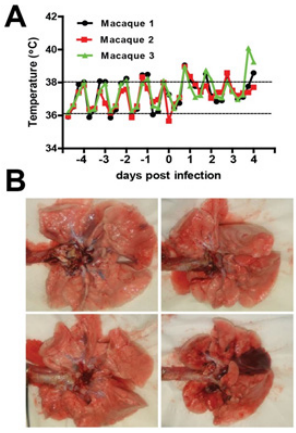
Supplementary Figures



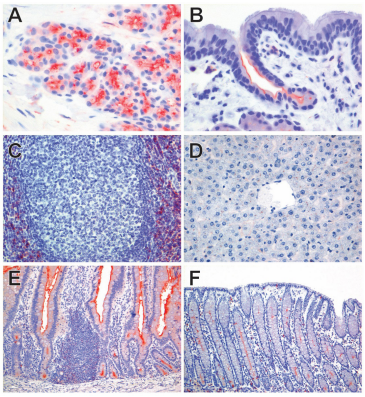
Supplementary figure S1. Pankeratin and CD68 staining on the sequential slides of the fatal MERS-CoV case lungs displayed in figure 1. Pankeratin positive epithelial cells are found in the sequential slides where MERS-CoV RNA is detected (A). CD68 alveolar macrophages are found in the sequential slides where MERS-CoV antigen is detected (B). Both figures are taken in 400x magnification.



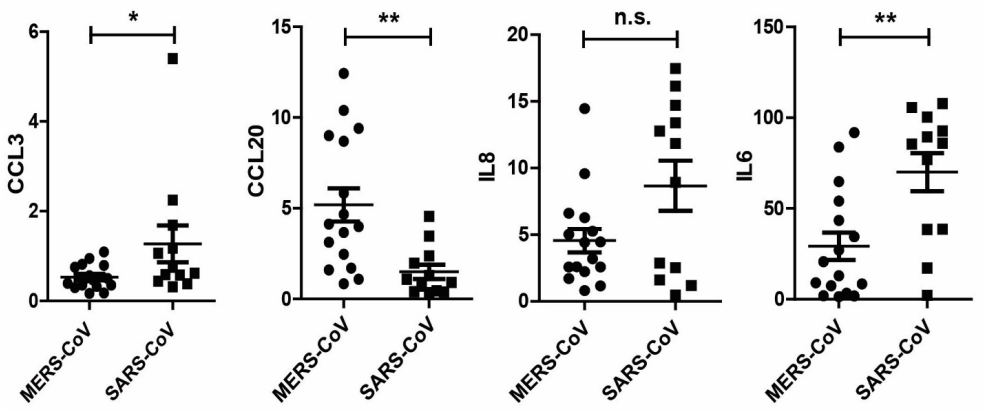
Supplementary figure S2. DPP4, ACE2, CTSC, PREP, GUSB, and GAPDH mRNA expression in the lungs of young and advance-age cynomolgus macaques. DPP4 was significantly higher in the lungs of advance-age cynomolgus macaques. ACE2, CTSC, PREP, GUSB and GAPDH were not significantly different in the lungs of young and advance-age cynomolgus macaques. Data were expressed in log2 expression values. Statistical analysis was performed using limma (FDR < 0.05). All available probeset were shown for DPP4, ACE2, PREP and GUSB; two out of six probeset for CTSC were shown (those not known had similar expressions and were not signicantly different between young and advance-age macaques).



Supplementary figure S3. Body temperature and gross pathology of the MERS-CoV infected macaques' lungs. All animals developed elevated body temperature (body temperature >38°C) post MERS-CoV inoculation. One macaque (number 3) developed bacterial co-infection during the experiment. This macaque developed elevated temperature up to 40°C around day 4 pi (A). Gross lesions were hardly found in the lungs of MERS-CoV infected macaques, except in one macaque that developed bacterial coinfection (B, bottom right). Histological examination of the gross lesions revealed myriad intralesional small rod-shaped bacteria, which later identified as Eschericia coli with bacterial typing analysis.



Supplementary figure S4. DPP4 expression in other macaque tissues. DPP4 was expressed on the apical surface of the nasal submucosal glands (A). It was also found in the tracheal submucosal glands and their secretory ducts (B). In the tonsils, it was mainly detected in the paracortex and medulla, but not inside the lymphoid follicles (C). In the liver, it was predominantly found in the endothelium of the hepatic sinusoids (D). In the intestine, it was detected on the apical surface of the small intestine (E) and colonic crypts (F). Small intestine and colon pictures were taken at 100x magnification; tonsil and liver at 200x; while the rest at 400x



Supplementary figure S5. The mRNA expression of IL8, IL6, CCL3, and CCL20 in the lungs of MERS-CoV and SARS-CoV infected macaques at day 4 pi. The fold change unit of these markers are displayed in mean ± standard error and analyzed with unpaired t-test. Significant difference was marked with asterisks (**p value ≤ 0,01; *p value ≤ 0,05; n.s. = not significant).

Supplementary Information

Case description of the fatal human MERS-CoV infection

The patient was a 56 year old male with long-standing type 2 diabetes. He travelled to the Kingdom of Saudi Arabia (KSA) for ten days and developed acute respiratory illness with malaise, rigors, sore throat, sneezing, and fever at the fifth day of his stay, on 24 January 2013. No animal contact or hospital visits were reported within ten days prior to onset of illness. He then travelled back to UK and at sixth day post onset he developed breathing difficulty and admitted to the hospital. He was referred to the intensive care unit at the same day, where he was intubated and mechanically ventilated using a low tidal volume ventilation strategy. Nose and throat swabs were taken within 24 hours from which influenza A subtype H1N1pdm09 was detected. Oseltamivir treatment was then started.

Due to persistent severe refractory hypoxia, the patient was referred to another institution for veno-venous extracorporeal membrane oxygenation (ECMO) at the twelfth day post onset. Upon arrival, bronchoschopy revealed purulent and haemorrhagic sputum with airway plugging. Chest radiograph revealed almost complete opacification of both lung fields. Upper respiratory tract sample was then tested positive for MERS-CoV at the fourteenth day post onset. MERS-CoV RNA was continuously detected in respiratory samples up to 49th day post onset. MERS-CoV reactive antibodies were detected starting from 20th day post onset.

The patient developed multi-organ failure with respiratory, cardiovascular, renal, and hepatic dysfunction at 33rd day post onset. Cytomegalovirus (CMV), Epstein-Barr virus, and herpes simplex virus type 2 were repeatedly detected by PCR in blood samples afterwards. Over the next ten days, the patient developed sepsis. Klebsiella pneumoniae was grown from respiratory tract samples including broncho-alveolar lavage (BAL), and from a central venous line tip. The patient died on 19 March 2013.

CHAPTER 9

Summarizing Discussion

on Viruses 2019, 11(3), 280

Overview

Middle East respiratory syndrome coronavirus (MERS-CoV) is a novel pathogen isolated late 2012 [1]. Since then, the virus has caused multiple outbreaks and infected more than 2000 individuals [2] which may develop a respiratory infection ranging in severity from asymptomatic to fatal [3,4]. The severe-to-fatal MERS-CoV patients have a higher chance to transmit this virus since they shed a higher amount of virus progeny in comparison to the asymptomatic-to-mild ones [5-8]. Identifying and quarantining these patients in health care facilities where outbreaks occurred, together with implementing proper infection control, has been effective in reducing transmission and containing these outbreaks [9,10]. However, new MERS-CoV cases are still being reported, especially in the Arabian Peninsula [2,11]. This is partly due to the continuous zoonotic introduction of this virus to the human population in this region by dromedaries [12]. The dromedary camel is the only animal species that has been reported to transmit this virus to humans [13-16]. MERS-CoV infection in these animals merely causes mild upper respiratory tract infection [17,18], but seroepidemiological studies showed that this virus has been circulating in dromedary camels for decades, suggesting efficient transmission of MERS-CoV in this species [19-23].

Although the clinical manifestations, as well as transmission, are remarkably different in MERS-CoV-infected humans and dromedary camels, the viruses isolated from these two species are highly similar, if not indistinguishable [12,16]. This indicates that host factors play a significant role in MERS-CoV pathogenesis and transmission. However, the identity of these host factors and how they affect the pathogenesis and transmission of MERS-CoV are generally not well understood. Dipeptidyl peptidase-4 (DPP4)–the MERS-CoV receptor, sialic acids, proteases, and interferons, are examples of potentially critical host factors that have been shown to affect MERS-CoV infection *in vitro* [24-27]. Here we highlight the role of some MERS-CoV-interacting host factors, especially DPP4, in the MERS-CoV pathogenesis and transmission.

MERS-CoV-interacting Host Factors

MERS-CoV infection of a target cell is initiated by the virus attachment to the cell surface [24,28]. MERS-CoV uses the N-terminal part of its spike, the so called S1 protein (Figure 1A), to bind to two host cell surface molecules, dipeptidyl peptidase-4 (DPP4) and α2,3-sialic acids [24,25]. DPP4 is the functional receptor of MERS-CoV; its absence renders cells resistant to this virus while its transient expression in cells permits viral replication [24]. DPP4 is a serine exopeptidase which is either expressed at the cell surface or shed in a soluble form. It has the capacity to cleave-off dipeptides out of polypeptides with either L-proline or L-alanine at the penultimate position.

Accordingly, DPP4 is capable of cutting various substrates, such as hormones, cytokines, chemokines, and neuropeptides, allowing it to be involved in multiple physiological functions as well as pathophysiological conditions [29]. This enzymatic activity is mediated by the α/β hydrolase domain of DPP4, while MERS-CoV infection is mediated by the binding of S1 protein to the β-propeller domain of this exopeptidase (Figure 1B) [29-32]. There are eleven critical residues within the β-propeller domain that directly interact with the S1 protein [30-32]. These residues are quite conserved in camelids, primates, and rabbits, species shown to be susceptible to MERS-CoV [17,32-34]. In contrast, ferrets, rats, and mice resist MERS-CoV infection due to differences in some critical DPP4 residues [32,35-37]. These data illustrate that DPP4 has the capacity to determine the host range of MERS-CoV.

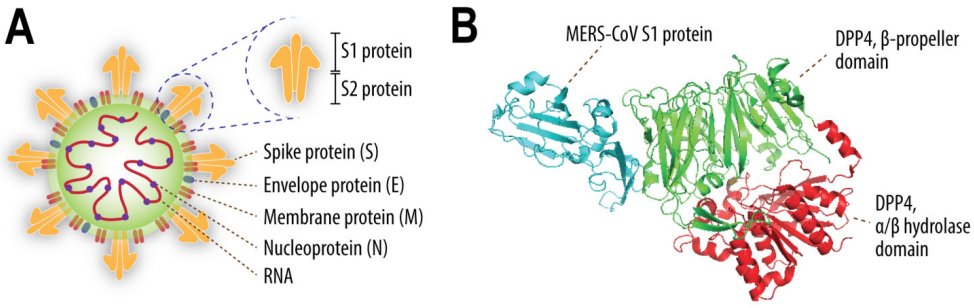


Figure 1. Schematic figure depicting four structural proteins of MERS-CoV, i.e. S, E, M, and N proteins (A); and a cartoon representation of MERS-CoV S1 protein binding to DPP4 (PDB code 4L72) (B). The S protein consists of the S1 and S2 subunit. The α/β hydrolase domain of DPP4 is indicated in red, β-propeller domain in green, while part of MERS-CoV S1 protein is shown in blue.

Other MERS-CoV-interacting host factors besides DPP4 are less extensively studied and mostly have been investigated *in vitro*. Glycotopes of α2,3-sialic acids coupled with 5-N-acetylated neuraminic acid are recognized by the S1 protein of MERS-CoV during attachment [25]. In the absence of these glycotopes, MERS-CoV entry is reduced but not abolished, indicating their function as an attachment factor rather than a receptor [25]. Besides α2,3-sialic acids, CEACAM5 and GRP78 have also been suggested to be attachment factors for MERS-CoV, but their roles *in vivo* during MERS-CoV infection are not clear at this moment [38,39]. Post attachment, MERS-CoV uses the C-terminal part of its S protein, known as S2 (Figure 1A), to interact with host proteases, such as furin, TMPRSS2, and cathepsins [40-43]. These proteases cleave the S protein and induce conformational changes allowing fusion between viral and host cellular membranes, resulting in the release of viral RNA into the cell cytoplasm [28]. TMPRSS2 and DPP4 are hold in one complex at the cell surface by a scaffolding protein, the tetraspanin CD9, leading to a rapid and efficient entry of MERS-CoV into the susceptible cells [44]. Once fusion with host cell membranes has occurred, MERS-CoV subsequently replicates its genetic material and produces viral proteins in the

cell cytoplasm to generate new virus progeny. During this stage, MERS-CoV uses its nsp3-4 polyproteins to build its replication organelles, and its accessory proteins such as the 4a and 4b proteins to inhibit host anti-viral defense mechanisms [45-55]. However, the capacity of MERS-CoV accessory proteins to impede several pathways of host immune response in the lungs may be limited. MERS-CoV inoculation of macaques and genetically modified mice generally results in limited clinical manifestations, thus adapting this virus through serial passaging or defecting the type I interferon pathway may be needed to enhance viral replication and pathogenesis in these animals [33,56-59]. These observations, together with studies showing type I interferon capacity to inhibit MERS-CoV infection *in vitro* [26,60], highlight the importance of the innate immune response, especially type I interferon, as an inhibiting factor for MERS-CoV.

Host Factors in MERS-CoV Transmission

So far MERS-CoV has been isolated from dromedary camels and humans [1,61]. Both species are not only susceptible to MERS-CoV infection, but also capable of transmitting this virus [7,12-18,23]. However, current data indicate that virus spread is more efficient in dromedary camels than in humans [5,7,20-22,62]. This difference in transmissibility could be partially due to the different tropism of MERS-CoV in these two species. In dromedaries, MERS-CoV has been shown to replicate in the nasal epithelium upon experimental *in vivo* infection [17], while in humans, MERS-CoV mainly replicates in the lower respiratory tract, particularly in the bronchiolar and alveolar epithelium [19,24,63-65]. Higher viral RNA levels in the sputum and lavage samples of MERS-CoV patients compared to nasal and throat swabs are consistent with the tropism of MERS-CoV in humans [66-68]. In chapter 2 we showed that this different MERS-CoV tropism in dromedary camels and humans is in line with the localization of DPP4 in the respiratory tract tissues of these two species. In humans, DPP4 is absent in the nasal epithelium but present in the lower respiratory tract epithelium, mainly in the type II pneumocytes [69,70]. In contrast, DPP4 is expressed in the nasal epithelium of dromedary camels [69]. This difference in DPP4 localization between humans and dromedary camels, therefore explains MERS-CoV tropism in these two species and highlights DPP4 as an essential determinant for MERS-CoV tropism.

DPP4 localization has also been investigated in many other MERS-CoV susceptible species. In chapter 3 we reported that in Gambian and Egyptian fruit bats, DPP4 is expressed in the respiratory tract and intestinal epithelium, suggesting that MERS-CoV can target both tissues [71]. In line with this finding, MERS-CoV inoculation via intranasal and intraperitoneal routes in the Jamaican fruit bats led to viral RNA shedding both in the respiratory tract and the intestinal tract [72]. In contrast to frugivorous bats, DPP4 is limitedly expressed in the respiratory tract epithelium of two insectivorous

bats, i.e. common pipistrelle and common serotine bat, but abundant in their intestinal epithelium [71]. Accordingly, sequences of MERS-like-CoVs were mainly obtained from rectal swabs and fecal samples of insectivorous bats [73-80]. These findings not only support insectivorous bats as the origin host of MERS-CoV [73-80], but also indicate the importance of intestinal tropism and fecal-oral transmission of MERS-like-CoVs in these insectivorous bats.

Besides bats, humans, and dromedary camels, other animal species have also been proposed as potential hosts of MERS-CoV. Remarkably, DPP4 of horses, llamas, alpacas, pigs, bovines, goats, sheep, and rabbits has been demonstrated to recognize the S protein of MERS-CoV [81,82]. In most of these species there is a preferential upper respiratory tract expression of DPP4 observed. Rabbits express DPP4 in the upper and lower respiratory tract epithelium, thus may allow MERS-CoV to replicate in both compartments [34,83]. We showed in chapter 4 that horses, llamas, and pigs mainly express DPP4 in the upper respiratory tract, particularly the nasal epithelium [84]. Upon intranasal MERS-CoV inoculation, llamas, alpacas, and pigs developed upper respiratory tract infection, while horses did not seroconvert and only shed infectious virus in a limited amount [84-88]. The reason why horses seem to be less permissive to MERS-CoV remains to be investigated, but a chronic co-infection in the guttural pouch, a common disease among horses, might be one of the explanations. This guttural pouch infection results in an excessive mucus production that might hinder MERS-CoV to attach and enter the nasal epithelium [84,89,90]. Sheep, on the other hand, do not seem to express significant levels of DPP4 in their respiratory tract, thus did not seroconvert nor shed infectious virus upon experimental MERS-CoV inoculation [84,88]. Comparable to sheep, goats limitedly shed infectious virus upon experimental infection and did not transmit this virus to other naïve goats upon direct contact [88]. The results of experimental MERS-CoV infection in the livestock animals are in line with data from epidemiological studies. MERS-CoV seropositive llamas and alpacas are present in the field, while horses, goats, and sheep are generally found seronegative [23,86,87,91-98].

Given the fact that experimental *in vivo* infection studies and DPP4 expression analysis in different animal species revealed that dromedary camels are not the only animals in which MERS-CoV has an upper respiratory tract tropism [17,18,83,84], it is then relevant to question whether other animals potentially can spread MERS-CoV as well. New World camelids, i.e. alpacas and llamas, are able to transmit the virus to respective naïve animals upon contact [86]. Pigs on the other hand hardly transmit the virus neither by contact nor airborne routes [99], and so does rabbits as described in chapter 5 of this thesis. Most likely this is caused by the fact that pigs and rabbits, unlike dromedary camels, shed low levels of infectious virus upon MERS-CoV inoculation

(Figure 2). This difference indicates that other host factors besides DPP4 could cause interspecies variation in MERS-CoV infection. Indeed, we reported in chapter 6 that several glycotopes of α2,3-sialic acids that function as attachment factor of MERS-CoV are present in the nasal epithelium of dromedary camels but absent in that of rabbits and pigs (Figure 3) [25,100]. The lack of these glycotopes in pigs and rabbits might limit susceptibility and transmission of MERS-CoV in these animals. Although the role of these glycotopes in MERS-CoV transmission still requires further investigations, it remains plausible that an efficient transmission of this virus might require the presence of both DPP4 and MERS-CoV-recognized glycotopes of α2,3-sialic acids (Figure 3).

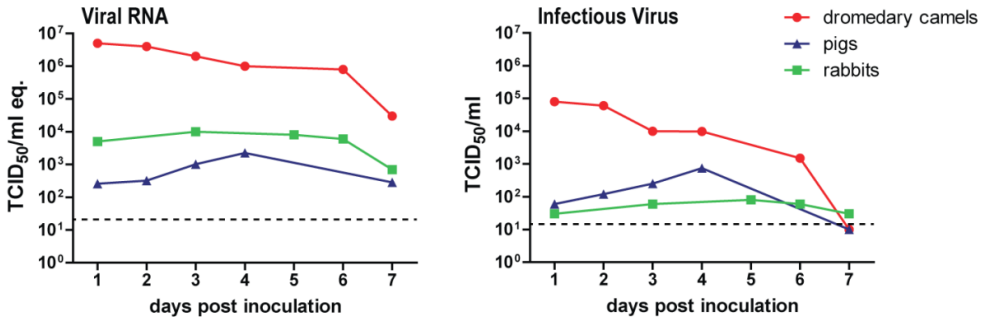


Figure 2. Schematic overview of viral RNA and infectious virus shedding of MERS-CoV-inoculated dromedary camels, pigs, and rabbits. Each data point represents the average data from previous experiments [17, 34, 84]. Viral RNA is measured in TCID50/ml genome equivalents, while infectious virus is expressed in TCID50/ml.

Besides entry and attachment receptors, MERS-CoV has been demonstrated to use both cell surface and lysosomal proteases to enter its target cells [40,41,44,101]. The preference of MERS-CoV to use certain host proteases is influenced by the type of target cells and the cleavage stage of their S protein prior to infection [41]. It has also been reported that the lysosomal proteases from bat cells support coronavirus spike-mediated virus entry more efficiently than their counterparts from human cells [40]. These observations suggest that host proteases from different host species may determine species and tissue tropism of MERS-CoV.

Because MERS-CoV has been circulating in dromedary camels for decades before emerging in the human population [19-23], it is plausible that this virus more efficiently inhibits the immune response of dromedary camels than that of other species, including pigs and rabbits. The difference in immune response among MERS-CoV susceptible species is therefore another factor that might yield interspecies variation in permissiveness to MERS-CoV. Characterizing the difference in host proteases and immune responses among MERS-CoV susceptible species, as performed for DPP4 and MERS-CoV-recognized α2,3-sialic acid glycotopes (Figure 3), has not yet been investigated. These data, however, may further explain interspecies variation in MERS-CoV infection and transmission.

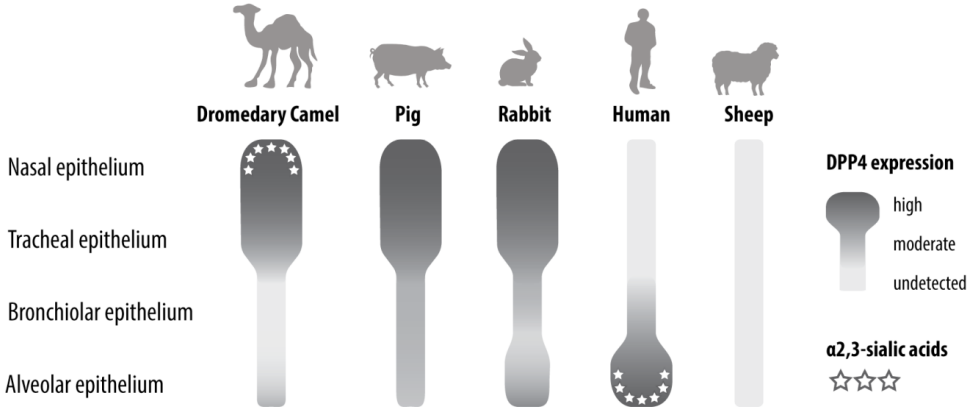


Figure 3. Schematic representation of DPP4 expression and MERS-CoV-recognized α2,3-sialic acid glycotopes in the respiratory tract of dromedary camel, pig, rabbit, human, and sheep.

Host Factors in MERS-CoV Pathogenesis

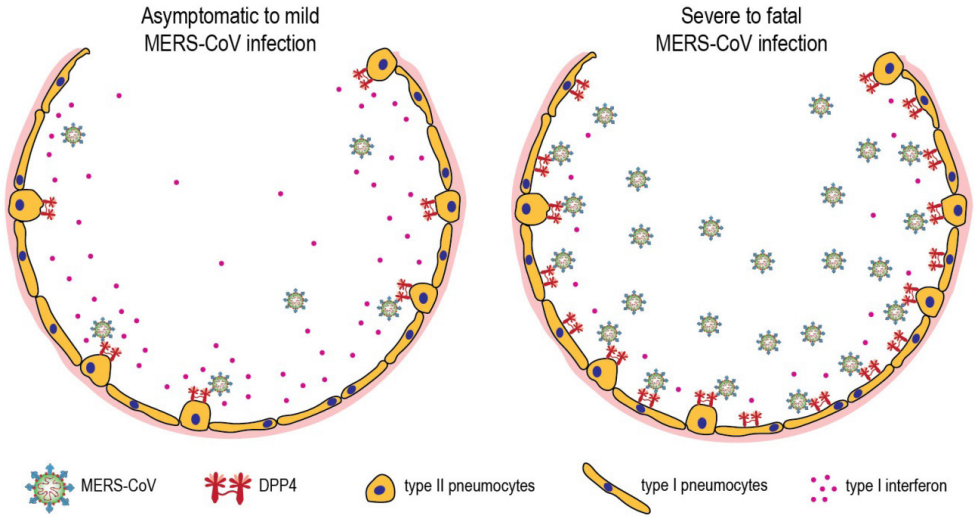


Figure 4. MERS-CoV infection in the lungs of asymptomatic-to-mild (left panel) and severe-to-fatal cases (right panel). Shown is a hypothetical model with two critical host determinants, DPP4 and interferon, differentially expressed in asymptomatic-to-mild and severe-to-fatal MERS-CoV infection.

MERS-CoV causes respiratory infection in humans ranging from asymptomatic to severe pneumonia [3,4], however, it is currently unclear what causes this intraspecies variation. Epidemiology data indicate that individuals with certain risk factors are at higher risk to develop severe MERS-CoV infection [4,102]. This implies that some host factors may dictate the outcome of MERS-CoV infection, thus rendering intraspecies variation. We reported in chapter 7 that two of the risk factors, i.e. smoking and

chronic obstructive pulmonary disease (COPD), could upregulate DPP4 expression in the lungs [70,102-104], suggesting DPP4 as a possible reason for intraspecies variation observed among MERS-CoV patients. In healthy human lungs, DPP4 is almost exclusively expressed in the type II pneumocytes [69,70]. In the lungs of smokers and COPD patients, on the other hand, DPP4 is prominently expressed in both type I and II pneumocytes, indicating upregulated expression on type I pneumocytes [104]. Autopsy reports from fatal MERS-CoV patients, including the one displayed in chapter 8 of this thesis, showed that both type I and II pneumocytes express DPP4 and became infected by MERS-CoV, proposing a role of DPP4-expressing type I pneumocytes in MERS-CoV pathogenesis [105,106]. Damage to type I cells in the lung alveoli during viral infection may lead to diffuse alveolar damage [107]. In line with observations made in human MERS cases, common marmosets, that express DPP4 in both type I and II pneumocytes, have been reported to produce more infectious virus upon experimental MERS-CoV infection compared to rhesus and cynomolgus macaques that merely express DPP4 in type II pneumocytes [59,108-111]. Accordingly, these common marmosets develop moderate to severe infection [108-110], while macaques generally develop mild transient pneumonia [33,59], as we also observed in our study described in chapter 8. Similarly, in genetically modified mice that display MERS-CoV tropism for type II pneumocytes, only mild clinical manifestations are observed upon MERS-CoV infection [57,112]. Adapting MERS-CoV through serial passaging or upregulating DPP4 expression throughout the airway epithelium in mice, however, will induce severe clinical disease [56,57]. These data altogether support the role of DPP4-expressing type I pneumocytes in the pathogenesis of severe MERS-CoV infection.

Differential expression of host factors that limit the infection should have also been taken into account. DPP4 in soluble form has been demonstrated to protect against MERS-CoV infection *in vitro* and in mice model [24,113], however, its presence in the lungs and role in MERS-CoV pathogenesis remain to be investigated. The host immune response also has the capacity to inhibit MERS-CoV infection. MERS-CoV has been shown to replicate to higher levels in immunocompromised rhesus macaques [114], consistent with the observation that immunocompromised individuals have difficulties clearing MERS-CoV upon infection [68,105,115]. The survivors of MERS-CoV infection have been shown to develop virus specific CD4+ and CD8+ T cell responses, implying the role of T cells in virus clearance [116]. However, depletion of T cells in mice can either lead to failure in MERS-CoV clearance or improvement in clinical outcome depending on the type of mouse model used [58,117]. Therefore, the role of adaptive immune response in MERS-CoV pathogenesis is currently unclear. On the other hand, one of the main components of the host innate immune response, type I interferon, inhibits MERS-CoV replication in susceptible cells, partly by inhibiting DMV formation [26,58,60,118,119]. The absence of type I interferon signaling in mice also resulted

in more severe clinical manifestations and histopathological lesions upon MERS-CoV infection [58]. Advance age, that can cause delayed type I interferon response upon viral infection, is a well-known risk factor for fatal MERS-CoV infection [4,102,120-122]. Collectively, these data highlight the role of host innate immune response as a potent inhibitor for MERS-CoV infection.

It is indubitable that severe MERS-CoV infection is not solely driven by the pathogen. Additional underlying conditions seem to be detrimental to increase MERS-CoV replication and induce severe to fatal clinical manifestations [4,11,103,123,124]. It is plausible that more than one underlying condition is needed to yield a fatal outcome [109]. DPP4 up-regulation in type I pneumocytes and insufficient type I interferon response might be crucial determinants for severe MERS-CoV infection (Figure 4). Further investigation on the host determinants of MERS-CoV pathogenesis may offer insights for developing novel therapeutic measures.

Concluding remarks and future perspectives

Although MERS-CoV has been reported to undergo some genotypic changes since it emerged in the human population [12,125-128], this did not result in distinct phenotypic changes so far [1,64,125]. Therefore host factors remain the most significant determinant in explaining inter- and intraspecies variations observed in MERS-CoV pathogenesis and transmission. DPP4 and MERS-CoV-recognized α2,3-sialic acids might partially explain these variations since their localization has been demonstrated to be variable between MERS-CoV susceptible species [69,71,84,100]. DPP4 expression in the human lungs has also been shown to vary due to certain comorbidities [70,96,104]. Nevertheless, it is undoubted that the inter- and intraspecies variation in MERS-CoV pathogenesis and transmission is a complex phenomenon influenced by more than one host factor. Current data suggest proteases and interferons as other critical host factors, but how they instigate inter- and intraspecies variations, and their role in MERS-CoV pathogenesis and transmission still remains to be further elucidated. Characterization of the host determinants of MERS-CoV pathogenesis and transmission could potentially offer insight into this virus epidemiology and guide novel therapeutic development. It may also help to identify the most vulnerable individuals to protect against MERS-CoV infection, for example by using vaccination.

Current epidemiology observations suggest that MERS-CoV has limited pathogenicity and transmissibility in individuals without risk factors [4,5,7], thus a large number of vulnerable individuals in one location that could allow this virus to efficiently spread, for example in healthcare facilities, particularly in the intensive care units, might be essential for this virus to cause an outbreak [8,62,102]. Since it is relatively more difficult

for MERS-CoV to cause outbreaks outside these facilities, one could argue that the risk of MERS-CoV causing a pandemic is considerably low. However, it is important to note that the risk is not negligible, as exemplified by a large MERS-CoV outbreak in South Korea [62]. Despite increased awareness and implementation of proper infection control in the healthcare facilities, new MERS-CoV cases are still being reported in the Arabian Peninsula [2]. MERS-CoV might also undergo adaptation to be more pathogenic and transmissible in humans, as SARS-CoV once did [129-134]. Therefore, it is still crucial to investigate whether MERS-CoV has been subjected to genotypic and phenotypic changes, by maintaining surveillance and performing *in vitro* and *in vivo* experiments comparing the earlier and the more recent virus strains. MERS-CoV transmission and pathogenesis are complex phenomena that are likely influenced by both viral and host factors.

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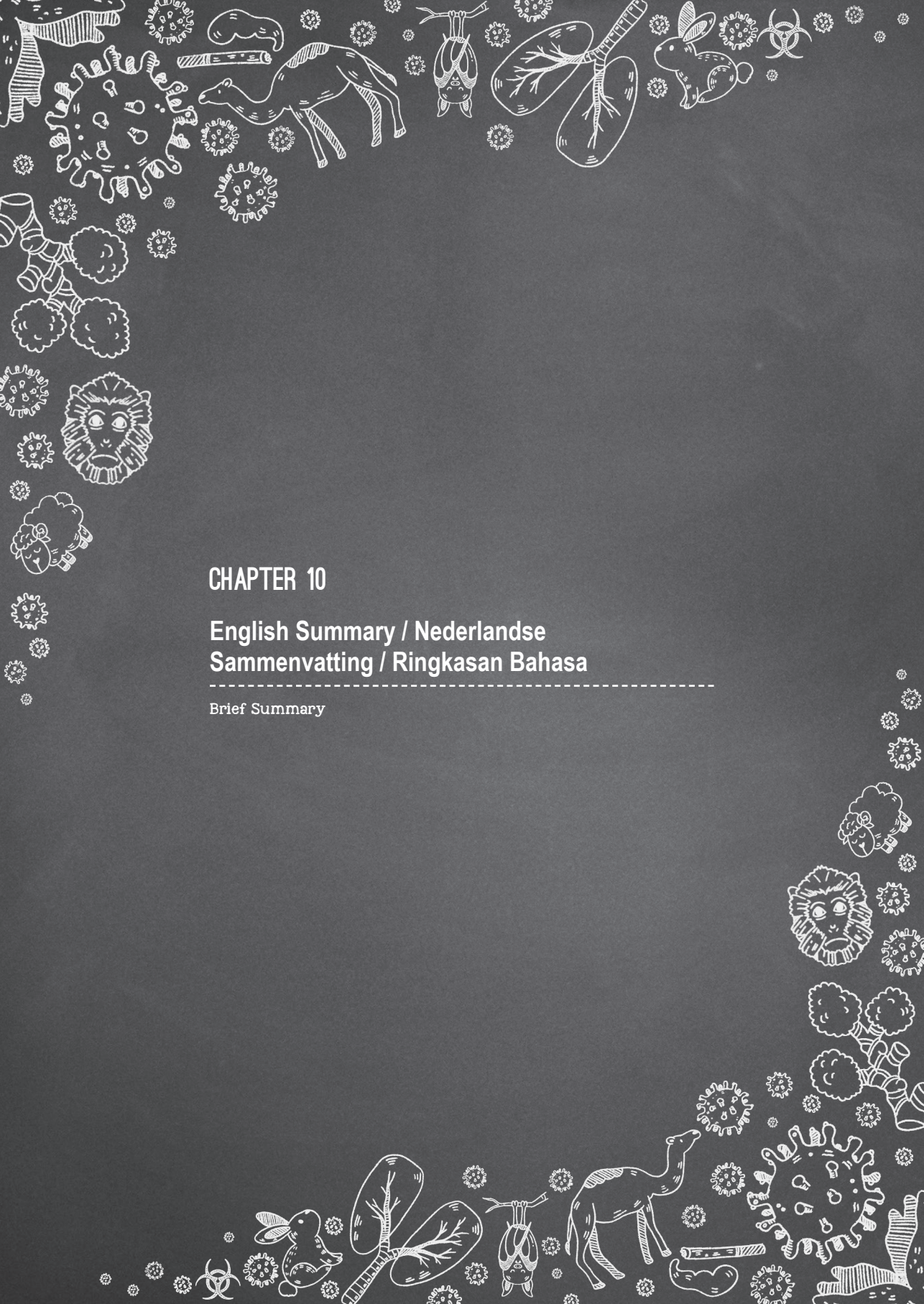


CHAPTER 10

English Summary / Nederlandse Sammenvatting / Ringkasan Bahasa

Brief Summary

ENGLISH

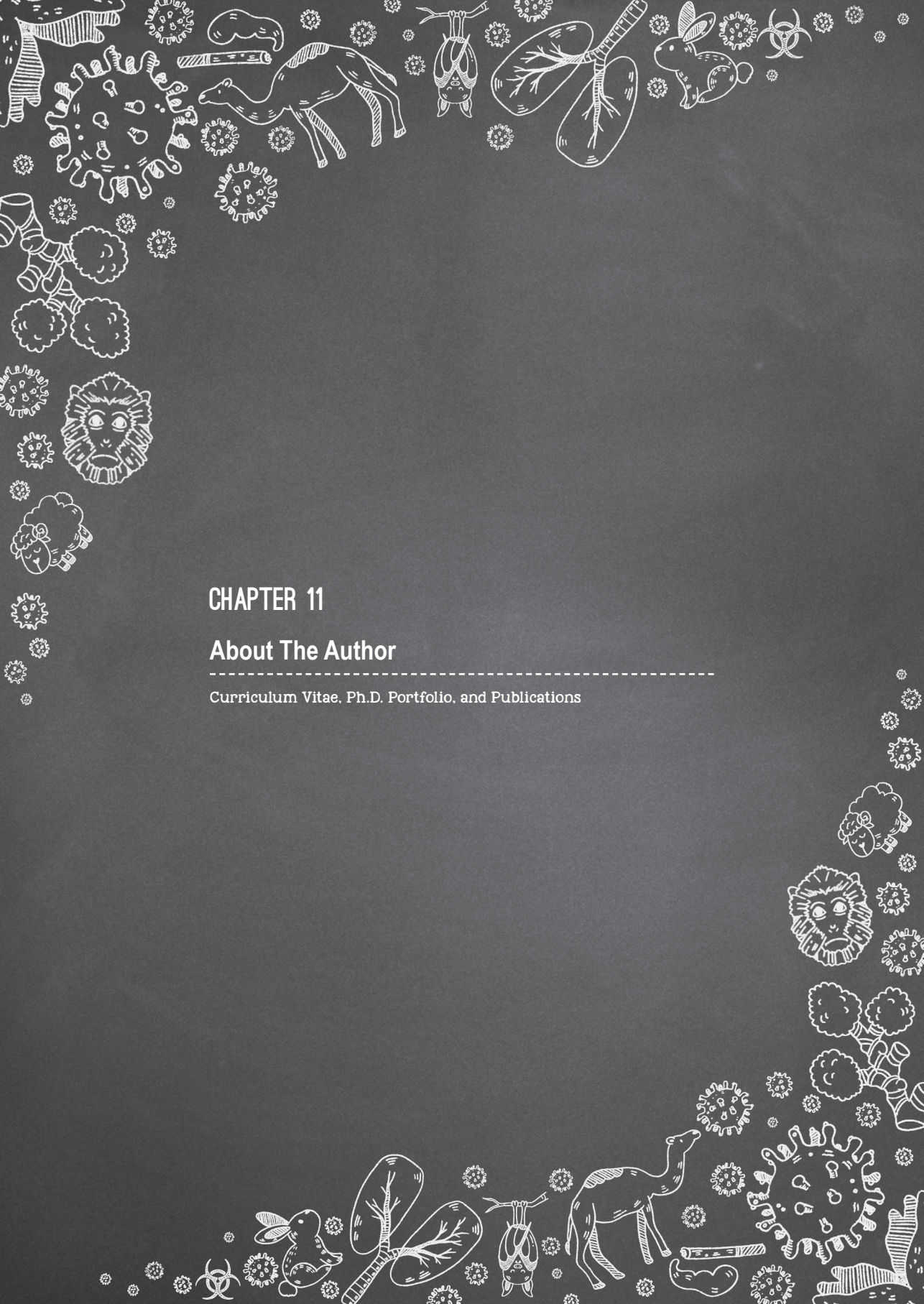
MERS-CoV is a microbe that commonly infects dromedary camels in the Arabian Peninsula and Africa. However, since the late 2012s, it is known that MERS-CoV could also cause disease in humans ranging from asymptomatic to fatal. It infects the human lungs thus render the infected individuals to develop respiratory illness known as pneumonia. During the infection, MERS-CoV enters the cells in the human lungs by first attaching to several molecules expressed at the cell surface. Two of these molecules are a protein called dipeptidyl peptidase-4 (DPP4) and a sugar structure called sialic acids. An absence of DPP4 renders the cells protected from MERS-CoV infection, while the absence of sialic acids reduces the capacity for MERS-CoV to infect the cells but does not fully protect them. Thus, DPP4 functions as the receptor for MERS-CoV, while sialic acids as an attachment factor. Our studies focused on mapping the localization of these two molecules in various tissues of animals and humans as well as get some clues on how DPP4 is regulated, in order to gain insights on how MERS-CoV is transmitted and how it causes diseases, especially in humans. We found that these two molecules are variably distributed in the tissues of different species. In human respiratory tract, DPP4 is merely expressed in the lungs, while in that of dromedary camels, it is expressed in the nasal epithelium. This means that MERS-CoV replicates in human lungs, while in camels it could replicate in the nose. Thus, it could partially explain why MERS-CoV is endemic in dromedary camels, while it is rather inefficiently transmitted between humans. We also found that DPP4 expression is relatively low in the healthy human lungs, however, chronic lung diseases and coinfections could upregulate its expression, suggesting MERS-CoV to infect a higher number of cells under these conditions. This might be one of the reasons why MERS-CoV infection causes a wide range of severity in humans, i.e. asymptomatic to fatal. Besides dromedary camels and humans, we also investigated DPP4 expression in other species as well. In insectivorous bats, that suggested to be the original host of MERS-CoV, DPP4 is hardly expressed in the respiratory tract but abundantly present in the intestine. This suggests that this virus replicates in the intestine and transmits via fecal-oral route between these bats. DPP4 is also detected in the nose of pigs and rabbits, in line with MERS-CoV replication site in these animals. However, unlike dromedary camels, these animals do not spread this virus, suggesting either the presence of inhibiting factors or the lack of supporting factors for MERS-CoV transmission in these animals. Interestingly, we also found that both pigs and rabbits are lacking an attachment factor, the sialic acids, in their nose. Collectively, the data compiled in this thesis showed that studies on MERS-CoV receptor and attachment factor could provide insights into its transmission and pathogenesis. Thus, these data are essential to better our understanding of MERS-CoV epidemiology and to inspire the development of novel interventions.

NEDERLANDS

MERS-CoV is een microbe die vaak dromedarissen infecteert in het Arabisch schiereiland en Afrika. Sinds 2012, is het ook bekend dat MERS-CoV ook bij de mens ziekte kan veroorzaken, variërend van asymptomatisch tot fataal. Het infecteert de longen, waardoor geïnfecteerde individuen longontsteking kunnen ontwikkeling. Tijdens het infectieproces bij de mens, komt MERS-CoV de cellen in de longen binnen door eerst te hechten aan verschillende moleculen die op het celoppervlak voorkomen. Twee van deze moleculen zijn de eiwitten dipeptidylpeptidase-4 (DPP-4) en een suikerstructuur genoemd siaalzuur. Een afwezigheid van DPP-4 zorgt ervoor dat de cellen beschermd zijn tegen MERS-CoV infectie, terwijl de afwezigheid van siaalzuur de infectiecapaciteit van MERS-CoV vermindert, maar de cellen niet volledig beschermd. DPP4 functioneert dus als receptor voor MERS-CoV, terwijl siaalzuur als bindingsfactor functioneert. Onze studies zijn gericht op het in kaart brengen van de lokalisatie van deze twee moleculen in verschillende weefsels van dieren en mensen. Evenals nog wat aanwijzingen over hoe DPP-4 is gereguleerd, om inzicht te krijgen over hoe MERS-CoV wordt overgedragen en hoe het ziekte veroorzaakt, voornamelijk in mensen. We vonden dat deze twee moleculen variabel worden verdeeld in de weefsels van verschillende soorten. In de menselijke luchtwegen, wordt DPP4 alleen tot expressie gebracht in de longen, terwijl bij dromedarissen wordt het uitgedrukt in het nasale epitheel. Dit betekent dat MERS-CoV zich repliceert in de menselijke longen, terwijl in kamelen repliceert in de neus. Dit verklaart gedeeltelijk waarom MERS-CoV endemisch is in dromedarissen, terwijl het nogal inefficiënt overgedragen wordt tussen de mens. We vonden ook dat DPP4 expressie relatief laag is in gezonde menselijke longen, alhoewel chronische longziekten en co-infecties zijn expressie opwaarts kunnen reguleren, wat suggereert MERS-CoV een groter aantal cellen onder deze omstandigheden te infecteerd. Dit is wellicht een van de redenen waarom MERS-CoV infectie een breed scala van de ernst bij mensen veroorzaakt, dat wil zeggen asymptomatische tot fatale. Naast dromedarissen en mensen, onderzochten we ook DPP4 expressie in andere soorten. In insectetende vleermuizen, dat de voorgesteld originele gastheren zijn van MERS-CoV, wordt DPP4 nauwelijks tot uitdrukking gebracht in de luchtwegen, maar overvloedig aanwezig is in de darm. Dit suggereert dat het virus repliceert in de ingewanden en overgebracht wordt via de fecaal-orale route tussen deze vleermuizen. DPP4 wordt ook gedetecteerd in de neus van varkens en konijnen, dat in overeenstemming komt met MERS-CoV replicatie in deze dieren. In tegenstelling tot dromedarissen, kunnen deze dieren dit virus niet verspreiden, wat suggereert naar de aanwezigheid van remmende factoren of het gebrek aan ondersteunende factoren voor MERS-CoV transmissie in deze dieren. Verder nog, vonden we ook dat zowel bij de varkens als konijnen de bindingsfactor, siaalzuur, in hun neus ontbreekt. Samengesteld, de gecompileerde data/informatie in dit proefschrift bewijzen dat onderzoek naar MERS-CoV receptoren en bindingsfactoren nieuwe inzichten kan brengen op de gebieden van transmissie en pathogenese. Deze gegevens zijn essentieel om zo ons begrip van MERS-CoV epidemiologie te verbeteren en de ontwikkeling van nieuwe interventies te inspireren.

INDONESIA

MERS-CoV adalah virus yang umumnya menginfeksi unta di jazirah Arab dan benua Afrika. Namun, di penghujung tahun 2012, virus ini diketahui dapat menginfeksi paru-paru manusia dan menyebabkan penyakit radang paru-paru ringan hingga berat, bahkan terkadang mematikan. Guna menginfeksi sel di paru-paru, virus MERS perlu terlebih dahulu menempel pada beberapa molekul di permukaan sel. Dua diantaranya adalah protein bernama dipeptidil peptidase-4 (DPP4) dan monosakarida yang disebut asam sialat. Virus MERS tidak dapat menginfeksi sel tanpa keberadaan DPP4. Tanpa keberadaan asam sialat, virus ini masih dapat menginfeksi sel, namun kemampuan infeksinya menurun. Penelitian kami bertujuan untuk mengetahui letak kedua molekul tersebut serta mempelajari regulasi produksi DPP4 di sejumlah jaringan tubuh hewan dan manusia. Data yang kami peroleh dapat memberikan petunjuk terkait penyebaran virus MERS dan bagaimana virus ini menimbulkan penyakit, khususnya pada manusia. Hasil penelitian kami menunjukkan bahwa lokasi kedua molekul ini bervariasi antar spesies. DPP4 diproduksi di paru-paru manusia, sementara di unta, DPP4 diproduksi di hidung. Hal ini menyebabkan virus MERS berkembangbiak di jaringan yang berbeda saat menginfeksi unta dan manusia. Perbedaan ini kemungkinan menyebabkan virus MERS endemik di unta, namun tidak mudah menyebar antar manusia. Kami juga menemukan bahwa DPP4 tidak banyak diproduksi di paru-paru manusia dalam kondisi sehat. Namun produksi DPP4 dapat meningkat pada penderita penyakit radang paru kronik dan radang paru akut akibat infeksi virus lain, yang diketahui beresiko menderita radang paru berat saat terinfeksi virus MERS. Hal ini dapat merupakan salah satu penyebab mengapa pasien yang terinfeksi virus MERS dapat menderita radang paru ringan hingga berat. Selain unta dan manusia, kami juga meneliti lokasi DPP4 di jaringan tubuh hewan-hewan lain. Pada kelelawar pemakan serangga, DPP4 tidak banyak diproduksi di saluran pernafasan, namun banyak ditemukan di usus. Hal ini mengindikasikan bahwa virus MERS dapat berkembang biak di usus dan menyebar lewat tinja hewan-hewan ini. DPP4 juga diproduksi di dalam hidung babi dan kelinci, sesuai dengan lokasi berkembang biak virus MERS di kedua hewan tersebut. Namun, kedua hewan tersebut tidak dapat menyebarkan virus MERS seperti layaknya unta. Kemungkinan, salah satu penyebabnya adalah tidak adanya asam sialat yang menjadi faktor pendukung infeksi virus MERS di hidung babi dan kelinci. Secara keseluruhan, data yang disajikan dalam tesis ini menunjukkan bahwa upaya mempelajari molekul-molekul yang berinteraksi dengan virus MERS dapat membantu kita mempelajari bagaimana virus MERS menyebar dan menyebabkan penyakit. Untuk itu, penelitian kami dapat pula bermanfaat untuk memahami lebih lanjut mengenai epidemiologi virus MERS, dan menginspirasi pengembangan metode intervensi terbaru.



CHAPTER 11

About The Author

Curriculum Vitae, Ph.D. Portfolio, and Publications

WIDAGDO

Research group:
Emerging Virus Group

Research department:
Viroscience, Erasmus MC

Research school:
Post-graduate school of Molecular Medicine (PGS Molmed)

Ph.D. period:
2014-2019

Promotor:
Prof. Marion PG Koopmans

Co-promotor:
Dr. Bart L Haagmans

CURRICULUM VITAE

Widagdo did his Bachelor of Medicine in the Faculty of Medicine, Diponegoro University (FMDU), Semarang, Indonesia in 2004. During the last year of his study, he started interning in the Center for Biomedical Research (CEBIOR) under the supervision of Prof. Sultana MH Faradz, as a part of a research team investigating clinical, biomolecular, and psychological aspects of multiple disorders of sexual development. For his bachelor thesis, he performed *in vivo* toxicology study of Haliclona sp and Gorgonia Isis hippuris extracts in Swiss mice, under the supervision of Dr. Neni Susilaningsih.

He then continued his clinical training to become a general practitioner in several healthcare facilities in the Central Java region in Indonesia, but mainly in the Dr. Kariadi hospital in Semarang. During this period, he was also working part-time as a data center manager at the Center for Tropical Infectious Disease (CENTRID), under the supervision of Prof. M. Hussein Gasem. He then worked as a general practitioner at a private clinic in Semarang for almost a year before receiving a scholarship called “Beasiswa Unggulan” from Indonesian Ministry of Education to enroll in a biomedical research master program majoring in genetic counseling at FMDU.

He performed a research internship at Department of Clinical Genetics, Erasmus MC, Rotterdam, the Netherlands on understanding the epigenetic silencing mechanism of fragile X syndrome under the supervision of Prof. Rob Willemsen, Dr. Celine de Esch, and Prof. Sultana MH Faradz. He then continued his education in an international research master program of Infection and Immunity at Erasmus MC with the scholarship support from the master program. His thesis focused on the development of a novel tetravalent recombinant vaccine for Dengue under the supervision of Dr. Byron EE Martina from Department of Viroscience, Erasmus MC, Rotterdam, the Netherlands.

He graduated cumlaude from his bachelor study, general practitioner training, and two master studies. He subsequently enrolled in a Ph.D. program at Department of Viroscience, Erasmus MC under the supervision of Dr. Bart L Haagmans and mentorship of Prof. Marion PG Koopmans that culminates in the writing of this book. After this Ph.D., Widagdo would like to do more translational research focusing on building bridges from bench to bedside and field.

PH.D. PORTFOLIO

AWARD

2019 | ESCMID Research Grant 2019

2018 | Travel grant from One Health Organization to follow 5th One Health congress in Saskatoon, Canada, 21st-25th June 2018

2016 | Second best poster presentation at PREDEMICS workshop, 19-22nd September 2016

Travel and accommodation grants to follow PREDEMICS FP7 Training Program, 19-22nd September 2016

ORAL PRESENTATIONS

2018 | 5th International One Health congress in Saskatoon, Canada, 21st-25th June 2018

Netherlands Centre for One Health Annual Scientific Meeting, Rotterdam, the Netherlands, 30th May 2018

Molecular Medicine Day 2018, Rotterdam, the Netherlands, 15th March 2018

2017 | Transmission of Respiratory Viruses Conference, Hong Kong, 19th-21st June 2017

XIVth International Nidovirus Symposium (Nido2017), Kansas, United States, 4-9th June 2017

2016 | PREDEMICS workshop, Paris, France, 19-22nd September 2016

POSTER PRESENTATIONS

2019 | European Congress of Virology 2019, Rotterdam, the Netherlands, 28th April-1st May 2019

2017 | Molecular Medicine Day 2017, Rotterdam, the Netherlands, March 2017

2016 | 26th European Congress of Clinical Microbiology and Infectious Diseases (ECCMID), Amsterdam, the Netherlands, 9th-12th April 2016

Molecular Medicine Day 2016, Rotterdam, the Netherlands, March 2016

COURSES

2017 | Research Integrity, provided by Erasmus MC, 2017

2016 | PREDEMICS FP7 Training Program, 19-22nd September 2016

The Course in Virology, 27th-30th June 2016

TEACHINGS

2018 | Preparation class for an Erasmus MC medical students that will perform an exchange program to several university hospitals in Indonesia, September 2018

Supervising an internship of a Bachelor student from Avans Hogeschool Breda, Breda, the Netherlands, January-August 2018

2016 | Several classes in the Master of Infection and Immunity, PGS Molmed, 2014-2016

BOOKS

2017 | Contributor of the education module and questionnaire for Viruskenner program in Indonesia (Am J Trop Med Hyg 2017;97(1):97-108. doi:10.4269/ajtmh.16-0661)

PUBLICATIONS

1. **Widagdo W**, Wiersma LCM, Smits SL, de Vries RD, Schipper D, Stalin-Raj V, van den Ham HJ, Brown R, Zambon M, Kuiken T, Osterhaus ADME, van den Brand JMA, Haagmans BL. DPP4-expressing type I pneumocytes in a fatal human MERS-coronavirus case. Manuscript in preparation.
2. **Widagdo W**, Okba NMA, Li W, de Jong A, de Swart RYL, Begeman L, van den Brand J, Bosch BJ, Haagmans BL. MERS-CoV receptor and attachment factor co-localization is species and tissue specific. Submitted.
3. **Widagdo W**, Okba NMA, Richard M, de Meulder D, Bestebroer TM, Lexmond P, Farag EABA, Al-Hajri M, Stittelaar KJ, de Waal L, van Amerongen G, van den Brand JMA, Haagmans BL, Herfst S. Middle East respiratory syndrome coronavirus transmission in rabbits. Viruses 2019, 11(4), 381.
4. Anfasa F, Goeijenbier M, **Widagdo W**, Siegers JY, Mumtaz N, van Riel D, Rockx B, Koopmans MPG, Meijers JCM, and Martina BEE. Zika virus infection on human umbilical vein endothelial cells induces activation of secondary hemostasis. Front Microbiol. doi: 10.3389/fmicb.2019.00917.
5. **Widagdo W**, Sooksawasdi-Na-Ayudhya S, Hundie GB, Haagmans BL. Host determinants of MERS-CoV transmission and pathogenesis. Viruses 2019, 11(3), 280.
6. Stalin-Raj V, Okba NMA, Gutierrez-Alvarez J, Drabek D, van Dieren B, **Widagdo W**, Lamers MM, Widjaja I, Fernandez-Delgado R, Sola I, Bensaid A, Koopmans MP, Segalés J, Osterhaus ADME, Bosch BJ, Enjuanes L, Haagmans BL. Chimeric camel/human heavy-chain antibodies protect against MERS-CoV infection. Sci Adv. 2018 Aug 8;4(8):eaas9667.
7. Haverkamp AK, Lehmbecker A, Svpitzbarth I, **Widagdo W**, Haagmans BL, Segales J, Vergara-Alert J, Bensaid A, van den Brand JMA, Osterhaus A, and Baumgartner W. Experimental infection of dromedaries with Middle East respiratory syndrome-Coronavirus is accompanied by massive ciliary loss and depletion of the cell surface receptor dipeptidyl peptidase 4. Sci Rep, 2018. 8(1): p. 9778.
8. Seys LJM, **Widagdo W**, Verhamme FM, Kleinjan A, Janssens W, Joos GF, Bracke KR, Haagmans BL, and Brusselle GG. DPP4, the Middle East Respiratory Syndrome Coronavirus Receptor, is Upregulated in Lungs of Smokers and Chronic Obstructive Pulmonary Disease Patients. Clin Infect Dis, 2018. 66(1): p. 45-53.
9. Anfasa F, Siegers JY, van der Kroeg M, Mumtaz N, Stalin-Raj V, de Vrij FMS, **Widagdo W**, Gabriel G, Salinas S, Simonin Y, Reusken C, Kushner SA, Koopmans MPG, Haagmans BL, Martina BEE, and van Riel D. Phenotypic Differences between Asian and African Lineage Zika Viruses in Human Neural Progenitor Cells. mSphere, 2017. 2(4).
10. Li W, Hulswit RJG, Widjaja I, Raj VS, McBride R, Peng W, **Widagdo W**, Tortorici MA, van Dieren B, Lang Y, van Lent JWM, Paulson JC, de Haan CAM, de Groot RJ, van Kuppeveld FJM, Haagmans BL, and Bosch BJ. Identification of sialic acid-binding function for the Middle East respiratory syndrome coronavirus spike glycoprotein. Proc Natl Acad Sci U S A, 2017. 114(40): p. E8508-E8517.
11. Vergara-Alert J*, van den Brand JMA*, **Widagdo W***, Munoz M, Raj VS, Schipper D, Solanes D, Cordon I, Bensaid A, Haagmans BL, and Segales J. Livestock Susceptibility to Infection with Middle East Respiratory Syndrome Coronavirus. Emerg Infect Dis, 2017. 23(2): p. 232-240. *equal contribution
12. **Widagdo W**, Begeman L, Schipper D, Run PRV, Cunningham AA, Kley N, Reusken CB, Haagmans BL, and van den Brand JMA, Tissue Distribution of the MERS-Coronavirus Receptor in Bats. Sci Rep, 2017. 7(1): p. 1193.
13. **Widagdo W**, Okba NMA, Stalin-Raj V, and Haagmans BL, MERS-coronavirus: From discovery to intervention. One Health, 2017. 3: p. 11-16.
14. van der Eijk AA, van Genderen PJ, Verdijk RM, Reusken CB, Mogling R, van Kampen JJ, **Widagdo W**, Aron GI, Geurts van Kessel CH, Pas SD, Raj VS, Haagmans BL, and Koopmans MP. Miscarriage Associated with Zika Virus Infection. N Engl J Med, 2016. 375(10): p. 1002-4.
15. **Widagdo W**, Raj VS, Schipper D, Kolijn K, van Leenders G, Bosch BJ, Bensaid A, Segales J, Baumgartner W, Osterhaus A, Koopmans MP, van den Brand JMA, and Haagmans BL. Differential Expression of the Middle East Respiratory Syndrome Coronavirus Receptor in the Upper Respiratory Tracts of Humans and Dromedary Camels. J Virol, 2016. 90(9): p. 4838-4842.

CHAPTER 12

Acknowledgement

Arguably, the most important part of this Thesis for the readers.

Let's be frank here. Despite the fact that the Doctoral program and the committee consider this section as trivial, this chapter is often the first one that most readers check, making it arguably the most important one. Reasons may vary, some want to see whether they are being mentioned and how they are being thanked, which are rather petty yet human nonetheless. Another reason is because this chapter is the most layman, relatable, and sometimes, the only understandable part of a dissertation. Now that we have acknowledged this sad yet truthful common fact, I can start treating this chapter with the attention it deserves. And because Dutch people are always so proud of being direct, it should not hurt if I just write candidly.

I would like to start with the most influential figure in my Ph.D., my supervisor/co-promotor, Dr. Bart L Haagmans. After a pensive contemplation, I believe that the best analogy to describe the dynamic interaction between I and Bart during the last 5 year-ish is the one between Bilbo Baggins and Gandalf (obviously the Grey, not the White) from the epic trilogy, The Hobbit. Kinda like Bilbo, I am not sure why I was chosen to venture this journey so called Ph.D. The last 5 years I often felt lost, left alone holding my pipet tightly in the corner. And kinda like Gandalf, during the desperate moments, Bart appeared before me sharing wise-sounding riddles that sometimes confusing but intentionally inspiring. On these moments, I need to bare tilting my head back almost vertically due to our height difference, or he needs to sit down to spare me from an insurance-covered-possible-neck-injury. And when all hopes seem lost, Bart cast a powerful spell or help bring an army of help in the very very very last minute. Regardless, one thing is clear, Bilbo won't succeed without the help and guidance of Gandalf, just like I won't survive this Ph.D. without Bart's support. And for that, I am deeply thankful.

To my promotor, Prof. Marion PG Koopmans – I, unfortunately, did not have as many interactions with you as I would like to. Through several brief interactions we had, you have inspired me to foresee research in various perspectives. These open-minded, outward-looking, directive yet not overbearing discussions are so refreshing as well as uplifting. I would also like to extend my gratitude to other members of my defense committee for their willingness to review my thesis and to our department secretaries that have guided me through the administrative processes at the end of this journey.

The last four years have been a very much humbling experience since I truly realized how I have been so fortunate to have learned and received so many supports from my colleagues and collaborators. Without them, I would not be where I am right now. To Prof. Guy Brusselle, Dr. Leen Seys, and Dr. Ken Bracke from Ghent, Belgium; Prof. Wolfgang Baumgartner and Dr. Annika Haverkamp from Hannover, Germany; Dr. Joaquim Segales, Dr. Julia Vergara-Alert and their research team from CReSA, Barcelona, Spain; Dr. de Waal and his team from Viroclinics Biosciences B.V., Rotterdam, the Netherlands; Dr. Annemiek van der Eijk, Dr. Jeroen van Kampen, and their research team from ViroDiagnostics of EMC; Dr. Sander Herfst, Dr. Rik de Swart and their respective research teams at Department of Viroscience, Erasmus MC, I would like to express my deepest gratitude. To our TOP project collaborators, i.e. Dr. Berend-Jan Bosch, Dr. Wentao Li, and Dr. Ruben Hulswit, thank you very much for allowing me to be a part of your fascinating research. You guys are not only brilliant but also acutely nice.

I am extremely grateful for the support of Prof. Thijs Kuiken and his research team. Thank you very much for sharing me your knowledge and allowing me to perform most of my experiments in your lab. I deeply apologize for all the messiness I cause in your lab ☺. Thijs, I never said this before, but I do adore your sense of humor, so dry and crispy like a KFC. Lonneke and Peter, thanks for sharing your wisdom with me, it does come with age and experience – total respect. Lineke, my favorite co-author/collaborator, you're a caring mother, a celebrated pathologist, and a talented scientist – the world is indeed unfair. Debby van Riel, the way you empower others around you, including your students, is nothing but inspirational. My biggest regret is that I have not got the chance to work with you during the course of my Ph.D.

To Dr. Byron Martina and the ex-members of Exotic Virus group – and to Prof. Rob Willemsen, Dr. Renate Hukema, Dr. Celine de Esch and the Fragile X syndrome group, thank you very much for prepping me for a Ph.D. journey. Rob, thanks for inspiring me to pursue science further. Byron, thanks for your tremendous support during my master. And thanks for always reminding me that science should be first and foremost, fun. You both are such a great mentor, and hopefully one day I could be one too.

To my paranymphs: Brigitta, it's almost 9 years that we know each other and you still manage to be one of the people I hate the least. I love that our friendship is built on mutual respect from the get-go, and I'd like to believe that this is why we're still friends till now. And maybe also the pitch-dark humor. Raya, beyond the facade of the elegant sunglasses, the designer coat, the tailored suit, the Tom Ford, the Gucci, and the Versace, you're actually a terribly decent human being. But in all honesty, you're like a brother I never had. You have inspired me to be a better version of myself. And I really can't thank you enough for that. And last but not least, to Adit, you're simply the life of the party. Your quotable phrases and your life-story snippets are always so funny and uplifting. It's never not fun to have you around. I am forever blessed to have your support and friendship throughout my Ph.D. To the

members of the Emerging Virus group: Nisreen, brilliance is not hard to find in our floor, but brilliance with a strong sense of humility, an absent of condescending act, and a willingness to empower others is such a rare gem – and that's what make you such a special personality to me. You have been a tremendous support during my Ph.D., and I could not express how thankful I am for everything I have learned from you, not only about science but also about faith and life. In the beginning of our Ph.D., we made a 4-years long bet and after reflecting back on this journey, I am now gladly admitting defeat. To my bro, Gadissa, I find your struggle and tenacity to do science inspiring, and I wish I could have some of your resilience moving forward in my career. To Judith, Lidewij, and Stalin thank you very much for mentoring me, sharing me your experience and expertise during my Ph.D. Debby Schipper, your name is mentioned because you demanded to be mentioned, so there I did it. But seriously, thank you for providing me with such a support during my early Ph.D. years. Mart, good luck wrapping up your Ph.D. To Tija, you should have brought food more often, thanks for those food. To Sonia, you are so much fun to work and collaborate with. Wish you luck for your future endeavor and please stop buying Givenchy if you can't afford it yet. To Kathy and Sophie, I don't really remember what you did during your internship, but I'm pretty sure you were doing great. And to so many other students that have ever been a part of our group, it's great to hear you all doing awesome now.

To all my office roommates, Gadissa, Bri, Nisreen, Laurine, Shirley, Anoushka, and Tamana, I am sorry for being a cold unapproachable jerk during the last few months. I am not saying that I am mostly Gandhi since you most likely describe me more of a neighborhood friendly Mussolini. It's the stress guys, really, well at least mostly. To the other members of Department of Viroscience: Jasmin, Nele, and Victor, every single one of you is a delightful company, and together you are a party. David Nieuwenhuisje, when you boulder, I am not sure if you are human, but you still a fun species to hang out with. Cynthia, your life journey deserves a memoir. Gijs, your humor is infectious, it's just some people are immune. Henk-Jan, you got the capacity to put a dark twist on a twisted humor, such a distorted quality that current world deserves, and for that I thank you. Rory, you are a gifted scientist, a martial-art expert, and a talented photographer – you are another living proof that life is unfair for the rest of us. Monique Spronken, you are such a walking history book of Viroscience, such an ancient and wise entity that I would never dare to cross. Jurre, you're tall. Stefan van Nieuwkoop, you're also tall, but fortunately funny. Alwin, you're diligent, smart, agile, and great with guns – you were born too little too late, you'll do much better in 1926. Oanh, the massage will cost more from June 2019, the degree makes the difference. Noreen, you have a great talent in sarcasm, please do explore it. Marjolein, you're overarching bitchiness is of platinum quality, and your humor is simply an icing on the cake, brilliant. Shanti, what can I say to a queen… of the damned – this is truly a compliment. Stephanie Popping, Laura, Wesley, Bass, Diana, and Thomas, I cannot think of anything sarcastic to say, you are all vanilla nice.

I have been blessed to be surrounded by a lot of great personality outside Ee building 17th floor Erasmus MC. These people have made my hardship through Ph.D. bearable. Syriam, you have such a delightful personality to be around with. And you introduced me to so many people with similar trait, i.e. Utt, Karn, Keng, Diu, and YC. Shatavisha, your brilliance, and sense of humor is a match made in heaven. Al and Kika – Adi and Rakhma, you are all such a graceful human beings in and out. Diah and Fuad – I personally consider you both as my idols, thanks for your support, friendship, and inspiration. To Kirsty Short, my scientific idol, thank you for everything, to me you are like an older sister I never had. And Keng Yih Chew, my brother from another mother, I really miss your cooking. Kang Opick, you have such a big heart, figuratively. Teh Dowty, thanks for being an awesome and considerate listener, and thanks for never charge me by the hours. Fasa, thanks for being a big brother to me throughout my Ph.D. Bu Yulia, your shopping skill is indeed a work of art. Mbak Rina, what more can I say, you are just such a source of inspiration. Nani, I owe you a lot and I cannot thank you enough for your support. Dai, thank you for showing me that we are indeed enough. Galuh and Fadel, you both are continuous source of fun. Mita and Nadia – thank you for crafting this book for me. And Mita, thanks for being such a loyal friend for more than a decade. To Kim Kolijn, thanks for always being there through the depressing period of my stay in Holland. To Alta, thanks for introducing me to not today Satan, not today – and for our friendship, that I am pretty sure would last a life time. To Raisa, the voice of reason and maturity for me and Alta, and a living example of getting her shit together. Keep on inspiring us and others around you, Raisa. Thanks for everything. To my recently found family in Denver, meeting you guys is one of the highlights of my Ph.D. journey. There are still so many people I have not mentioned, yet deserves my total gratitude. I am really sorry for not mentioning you all here. However, if you are one of the people that feel offended for not being mentioned here, my condolences, you should feel sorry for being super petty and start reflecting back on your life why you turned out to be this way. It may not be too late.

Last but not least, to my family – my Mom, Dad, sister, brother in law, and my niece. Thank you for all the love, support, and prayers. You have been, and always will be, my Rock.

Sincerely yours,
Widagdo