DISSERTATION

TRANSMISSION AND SHEDDING OF MIDDLE EAST RESPIRATORY SYNDROME CORONAVIRUS FROM DROMEDARY CAMELS, ALPACAS, AND DOMESTIC LIVESTOCK

Submitted by

Danielle Adney

Department of Microbiology, Immunology and Pathology

In partial fulfillment of the requirements

For the Degree of Doctor Philosophy

Colorado State University

Fort Collins, Colorado

Summer 2016

Doctoral Committee:

Advisor: Richard Bowen

Candace Mathiason
Francisco Olea-Popelka
Mark Zabel

ABSTRACT

TRANSMISSION AND SHEDDING OF MIDDLE EAST RESPIRATORY SYNDROME CORONAVIRUS FROM DROMEDARY CAMELS, ALPACAS, AND DOMESTIC LIVESTOCK

The Middle East respiratory syndrome coronavirus (MERS-CoV) is a novel betacoronavirus discovered in 2012 that causes severe respiratory disease that can result in death in infected humans. Human-to-human transmission can occur, although zoonotic transmission from dromedary camels plays an important role in transmission. A large percentage of dromedaries in the Middle East and Africa have antibodies specific for MERS-CoV, indicating widespread transmission among camels. *In vitro* studies indicate that other livestock may be susceptible to infection and transmit virus, however, field studies have not detected any seropositive goats, horses, sheep, or horses. Due to the requirement for specific high-containment facilities research on the role of dromedaries has primarily been limited to field surveys.

Here we report experimental infection of dromedary camels with a human isolate of MERS-CoV. The objectives of this study were to characterize clinical disease, shedding, and tissue burdens in infected animals. Experimental infection with the virus resulted in a mild, transient upper respiratory tract infection accompanied by shedding of large amounts of infectious virus. While infectious virus was only detected for a short time viral RNA was detected for much longer, indicating that field studies only using PCR may not be sampling animals able to transmit virus.

Due to their cost, size, and temperament, dromedaries are not conducive to high-containment studies, and we hypothesized that alpacas may be a suitable replacement for some studies. This dissertation reports shedding and transmission of MERS-CoV in experimentally infected alpacas (n = 3) or those infected by direct contact (n = 3). Infectious virus was detected in all experimentally infected animals and in 2 of 3 in-contact animals. All alpacas seroconverted and were rechallenged 70 days after the original infection in order to understand if previous infection results in protective immunity. Experimentally infected animals were protected against reinfection, and those infected by contact were partially protected. Necropsy specimens from immunologically naive animals (n = 3) obtained on day 5 postinfection showed virus in the upper respiratory tract. These data demonstrate efficient virus replication and animal-to-animal transmission and indicate that alpacas might be useful surrogates for camels in laboratory studies.

Current understanding of MERS-CoV infection suggests that camels become infected as calves or during transport to slaughterhouses, and we hypothesized that vaccinating naïve animals could decrease viral shedding upon exposure. We vaccinated three dromedary camels and two alpaca with a MERS-CoV S protein subunit vaccine. Vaccinated animals developed neutralizing antibodies to MERS CoV with titers ranging from <10 to 2560 at week 16. Vaccinated animals, as well as two control camels and two control alpaca were challenged with a human isolate of MERS-CoV and were monitored for clinical signs and viral shedding. Vaccinated animals displayed partial protection associated with low or moderate antibody titers whereas the vaccinated alpaca developed high levels of antibody and were protected against infection following viral challenge.

In vitro assays indicate that sheep, goats, and horses are susceptible to infection with MERS-CoV. However, unlike dromedaries and alpacas, infected sheep, goats, and horses all failed to shed more than trivial quantities of virus and are therefore unlikely to serve as a source of transmission to humans or other animals.

The research presented in this dissertation provides experimental evidence linking dromedaries to MERS-CoV infection. Here, we present the first experimental infection of dromedary camels with MERS-CoV. We describe clinical disease, viral shedding, organ burden, and seroconversion in these animals. It is hoped that these studies will help shape camel sampling as well as allow for a more complete understanding of current field sampling data. Because of the difficulties associated with dromedary camels, we developed a more tractable model of reservoir infection. We found that alpaca model can be a useful substitution for camels in some studies and as well as demonstrate its strengths and weaknesses in a vaccination study. Finally, the data presented here indicate that sheep, goats, and horses do not shed large amounts of infectious virus after challenge with MERS-CoV. This indicates that these animals do not play an important role in viral transmission and that surveillance and vaccine efforts should focus primarily on dromedary camels.

ACKNOWLEDGEMENTS

I would like to first acknowledge my advisor Richard Bowen for his unwavering support and encouragement throughout my program. Advisors of his caliber are rare, and I am extremely grateful for his excellent mentorship and guidance. I would also like to acknowledge my committee members, Candace Mathiason, Mark Zabel, and Francisco Olea-Popelka, with special thanks to Candace and Mark, for the extra mentorship, encouragement, and support regarding my interests in advancing women in science and science communication.

I also would like to acknowledge the members of the Bowen laboratory, whose help made my research possible. I would like to specifically thank Airn Hartwig, Angela Bosco-Lauth, Stephanie Porter, Paul Gordy, and Vienna Brown for the assistance with animal sampling. A special thanks goes to Laurie, who sacrificed more than others. I also need to recognize Rick Brandes, Amy Richardson, and Greg Harding for their assistance and patience with our dromedaries. Finally, I would like to thank Helle Bielefeldt-Ohmann for her assistance with pathology as well as her encouragement throughout my project.

All of the camel work described in this dissertation was made possible by a collaboration with Vincent Munster at Rocky Mountain Laboratory. I want to specifically thank Vincent for his mentorship both for this dissertation and for my overall career. Neeltje van Doremalen, Emmie de Wit, Trent Bushmaker, and Dana Scott were also vital to the success of their project and I am very grateful for their assistance.

The viral isolate used in these studies was provided by Bart Haagmans and Ron Fouchier, and Rocky Mountain Laboratory was kind enough to provide the rabbit

antiserum against MERS-CoV used for our immunohistochemistry. The vaccine used in chapter 4 was developed and provided by Barney Graham and Kayvon Modjarrad.

Finally, I would like to acknowledge my funding sources, which include the Infectious Diseases: Translational Research Training Program Fellowship, the Bowen laboratory, the Animal Models Core at Colorado State University, and Vincent Munster's laboratory at RML.

DEDICATION

I would like to dedicate this dissertation to my dad, Bill Adney, who made me want to be a scientist.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

CHAPTER 1: INTRODUCTION AND LITERATURE REVIEW

1.1) Discovery and Nomenclature

The Middle East respiratory syndrome coronavirus (MERS-CoV) was originally isolated by Dr. Ali Moh Zaki in June 2012 from a 60 year old patient from Saudi Arabia that died 11 days after admission (June 24, 2012) of acute respiratory disease and renal failure (1). A sputum sample tested negative for influenza, a variety of paramyxoviruses, and hantavirus by Dr. Zaki and the Erasmus Medical Center (EMC), but tested positive in a pan-coronavirus PCR. This sample was negative for SARS-CoV, indicating a novel coronavirus. Dr. Zaki reported these results on ProMED on September 15th 2012, after which he was fired and forced to flea to Cairo. After its discovery, MERS-CoV was described as novel coronavirus (NCoV), human coronavirus Erasmus Medical Center (EMC), human coronavirus England 1, human betacoronavirus 2c EMC, human betacoronavirus 2c England-Qatar, and human betacoronavirus 2C Jordan-N3 indicating the need for a consensus name. In May 2013, the Coronavirus Study Group (CSG) of the International Committee on Taxonomy of Viruses announced the consensus name Middle East respiratory syndrome coronavirus (2).

1.2) Virology

Coronaviruses (family *Coronaviridae,* subfamily *Coronavirinae*) are positive-sense, single-stranded enveloped RNA viruses. Some suggest that the diversity seen in coronaviruses are attributed to a few important factors. During replication, their RNA-dependent RNA polymerase has a mutation rate of roughly one mutation per 1000 to 10000 nucleotides, allowing for viral evolution and divergence. Among RNA viruses,

coronaviruses have the largest genomes (26.4-31.7 kb), compounding the likelihood that mutations will occur during replication. Finally, during replication random template switching known as "copy-choice mechanism" occurs allowing for homologous RNA recombination (3).

These viruses are categorized into four groups and include viruses associated with colds and pneumonia as well as livestock and diarrhea. While coronaviruses were first recognized in 1937, they were not widely studied until the discovery of the severe acute respiratory syndrome coronavirus (SARS-CoV) in 2003, after which intensive surveillance for novel viruses has occurred. Originally, only three groups were recognized: group 1, group 2, and group 3. However, these now four groups are now more commonly recognized as *Alphacoronavirus, Betacoronavirus, Gammacoronavirus,* and *Deltacoronavirus.* These viruses primarily infect mammals and birds, with alphacoronaviruses and betacoronaviruses infecting primarily mammals and gammacoronaviruses and deltacoronaviruses infecting primarily birds. Many of the novel viruses isolated from wildlife were found in both bats and birds, indicating that these animals may serve as the natural reservoir for many coronaviruses (3, 4).

Group 1 or alpahacoronaviruses are further divided into lineage 1a and lineage 1b. Group 1a clusters phylogenetically, while no such clustering is observed for group 1b; thus, group 1b is thought of as "non-group 1a" alphacoronaviruses. Perhaps the most relevant alphacoronavirus in human or veterinary medicine is porcine epidemic diarrhea virus (PEDV), which causes high morbidity and mortality rates in young piglets resulting in substantial financial losses for pig farmers (5). Group 2 or betacoronaviruses are the best understood, as it contains viruses of human importance such as MERS-CoV, and SARS-CoV

as well as mouse hepatitis virus (MHV), a commonly studied coronavirus that can cause high mortality in outbreaks of laboratory mice (6). Betacoronaviruses are further divided into four lineages: lineage A, lineage B, lineage C, and lineage D. SARS-CoV is a lineage B betacoronavirus, while MERS-CoV is a lineage C betacoronavirus. Gammacoronaviruses (group 3) are comprised of lineages A, B, and C, and are predominantly avian viruses (Figure 1.1) . Finally, deltacoronaviruses were proposed in 2012 and include seven viruses isolated from both birds and pigs (3, 4, 7). Interestingly, there is some evidence that recombination between alphacoronaviruses and deltacoronaviruses can occur in swine (8).

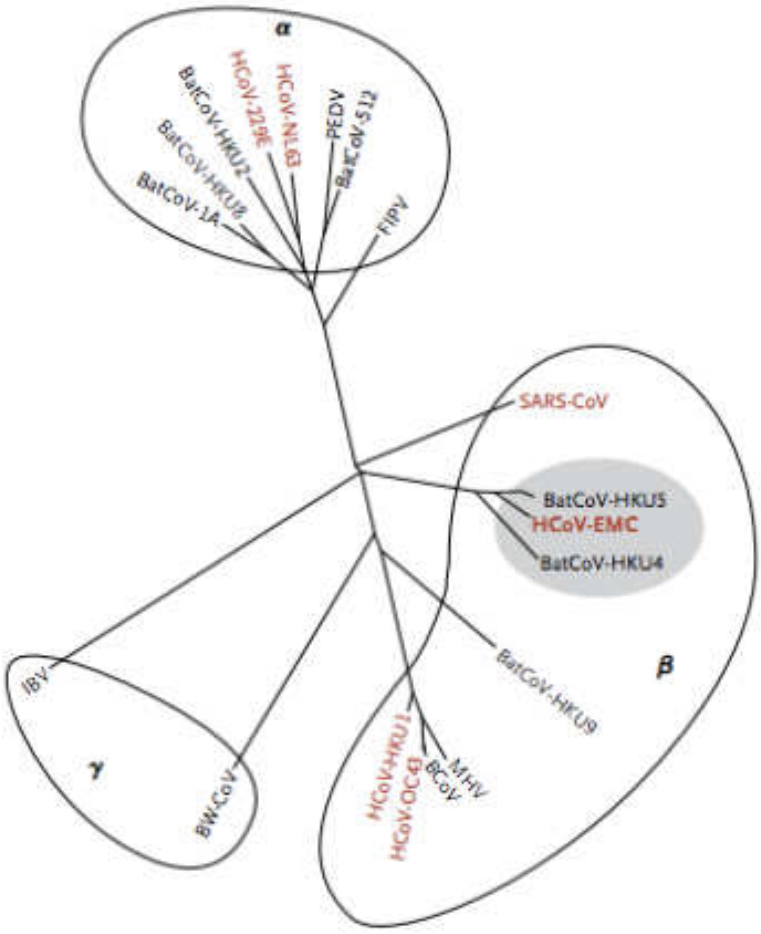

Figure 1.2. Phylogenetic tree of coronaviruses. From Zaki AM, van Boheemen S, Bestebroer TM *et al.*, 2012 (1). Red designates coronaviruses isolated from humans and grey designates Lineage C.

1.3 MERS-CoV Viral Replication

Upon infection, MERS-CoV interacts with host cell receptor dipeptidyl-peptidase 4 (DPP4) and the S protein is cleaved (likely by furin) into an N-terminal receptor binding domain (S1) and a C-terminal domain (S2). S2 then undergoes a conformational change, exposing a fusion peptide that inserts into the host membrane and mediates the fusion of the viral and cellular plasma membranes. MERS-CoV then uncoats, and the open reading frames from genomic mRNA (ORF1a and ORF1b) are immediately translated, resulting in a replicase polyprotein (RNA-dependent RNA polymerase, RdRp). ORF1a results in polyprotein 1a (pp1a) and is cleaved into 11 non-structural proteins (nsp1-11), and ORF1b is translated into polyprotein 1b (pp1b), which is cleaved into 15 nonstructural proteins (nsp1-10, 12-16). The replicase polyproteins then synthesizes an intermediate negative sense genomic RNAs, which are required for the production of positive sense RNA genomes used in progeny viruses. The virus then forms a nested set of monocistronic mRNAs that encodes the structural proteins. Structural proteins S, M, and E are transmembrane proteins, and thus are translated into the endoplasmic reticulum (ER), and move through the ER-Golgi intermediate compartment (ERGIC) where the M protein mediates virion assembly. The N protein is simultaneously translated in the cytoplasm, and attaches to the newly created full-length positive RNA that will be incorporated in progeny viruses. The newly formed viral genome and N protein complex then buds into the ERGIC and is transported through the secretory pathway in smooth-wall vesicles. Finally, these smooth-wall vesicles fuse with the plasma membrane, releasing the new viruses to infect other cells (Figure 1.2) (9-11).

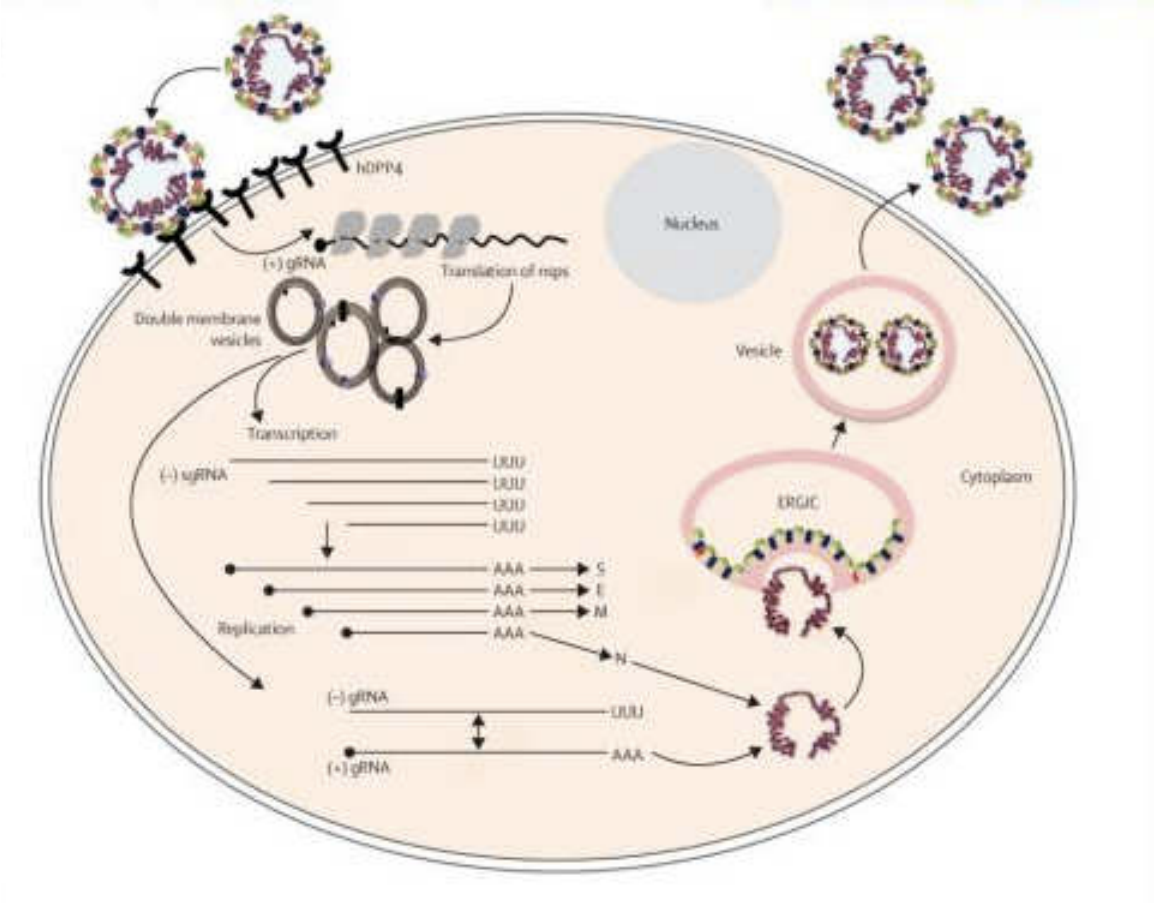

Figure 1.2. MERS CoV viral replication. From Zumla A, Hui DS, Perlman S, 2015 (12)

1.4) Human Infection and Disease

1.4a) Epidemiology of Human Infections in the Middle East

To date, every human case of MERS-CoV infection can be linked to the Middle East. Typically patients are either primary cases of infection (presumably from contact with camels or contaminated camel products) or became infected through contact with an infected patient, frequently in a hospital setting. Occasionally a patient will become infected in the Middle East and then travel to another country, where the infection is then recognized(13). To date, 26 countries have reported cases of MERS-CoV, highlighting the importance of travel-associated cases (14). The incubation period for MERS-CoV has a range of 1.9 to 14.7 days but is estimated to be 5.2 days, however, 95% of infected

individuals are symptomatic by day 12.4 (15, 16). Older men with comorbidities appear to be at a higher risk for death, but the risks reported vary with study (17-20).

1.4b) South Korean outbreak (2015)

In April 29-May 4 of 2015 a 68 year-old South Korean man traveled to Bahrain, United Arab Emirates (UAE), and Qatar but did not have any interactions with animals, patients, or hospitals (21). On May 11th he visited a local clinic with fever and chills and then was admitted to a general hospital on May 18th after visiting 4 additional hospitals with a higher fever, cough, myalgia, and dyspnea (21). This original patient infected at least 26 secondary cases is considered one of three superspreaders associated with the South Korean outbreak that involved 16 healthcare facilities and infected 185 individuals (22). This outbreak resulted in over 16,000 house quarantines, the closing of over 2700 schools and the cancellation of over 135,000 tourist visits highlighting the importance of monitoring for travel-associated cases and controlling viral transmission (22). A study evaluating environmental transmission detected viral RNA on X-ray devices, bed rails and sheets, IV fluid hangers, as well as in patient anterooms and air-ventilating equipment, highlighting the importance of controlling hospital transmissions (23). The severity of this outbreak is attributed to "doctor shopping" practices common in South Korea, family members and caretaker contact with admitted patients, lengthy close contact in multi-bed rooms and emergency rooms, poor control of healthcare associated infections, and an overall lack of awareness about the virus (24).

1.4c) Clinical Presentation of MERS in Humans

MERS-CoV infection in humans can range from an asymptomatic infection to severe respiratory disease causing death. Common clinical symptoms include fever, cough,

diarrhea, and dyspnea (25-27). Chest radiographs are typically abnormal ranging from minimal irregularities to widespread bilateral infiltrates(21, 26-28). Patients rapidly progress to hypoxemic respiratory failure and intubation can be necessary (26, 27). Neurologic disease ranging from focal motor deficits to coma been reported in very few patients, although it is documented (26). Diagnosis requires a positive RT-PCR result, typically targeting upE and ORF1a or ORF1b in respiratory tract secretions and lavage specimens, or can be diagnosed by an increase in antibody titer two weeks apart (21, 26, 27). Many patients require admission to the intensive care unit (ICU) and early supportive management is critical (26, 27). Acute kidney injury has been reported (26, 27, 29), and while there is no specific treatment for MERS-CoV infection, systemic corticosteroids are contraindicated (26, 27). Co-morbidities are associated with a negative outcome (27), and infection during pregnancy can lead to the death of both the mother and child (26, 30, 31).

1.4d) Pathology of MERS in Humans

To date there has only been one autopsy performed on a case of MERS (32). While only one patient, this autopsy is highly significant, as the pathology of animal models should faithfully recapitulate these findings. This patient was a 45 year-old Filipino man who did not smoke, was obese, and lived with 5 paramedics. He presented with fever, cough, and rhinorrhea and was diagnosed with acute bronchitis after a chest radiograph showed a left-side opacity. Four days later his chest radiograph showed additional air bronchograms and his lungs had left basal crackles and rales, at which point he was tested for MERS-CoV. He was then transferred to ICU because of respiratory distress, acute kidney injury and renal failure, and tachypnea, at which point intubation was necessary. A third chest radiograph revealed multiple patchy airspace oddities. He tested positive for MERS-

CoV by PCR the next day and died two days after his diagnosis. Over the course of his illness he was treated with prednisolone, paracetamol, levofloxacin, and received hydrocortisone (IV) and nitric oxide the day prior to his death. (32)

An autopsy was performed, which revealed pericardial effusion (150ml), pleural effusion (5ml), and abdominal effusion. His lungs were consolidated and edematous. Histology did not reveal viral inclusions and the disease was described as a "predominant(ly) pulmonary pattern." Immunohistochemistry indicated that viral antigen was primarily found in type 2 pneumocytes and epithelial syncytial cells and appeared "patchy" suggestive of immune dysfunction. There was not evidence supporting extra-pulmonary viral dissemination (32).

1.5) *In vitro* Studies on Host susceptibility

MERS-CoV S protein utilizes the host cell receptor DPP4 or CD26 for cell entry (33). *In vitro* data from cell line infections indicate that while MERS-CoV can infect a variety of cells from livestock found in the Middle East, many traditional lab animals are not susceptible to infection. Immortalized cells from a bank vole and lesser white-toothed shew express DPP4 but are refractory to infection with MERS-CoV (34). Additional *in vitro* studies with hamsters, mice (C57 mouse embryo fibroblasts and 3T3), and ferrets supported the hypothesis that the S protein requires a specific receptor binding domain (RBD) as those cells were refractory to infection despite the presence of DPP4 (35). Rat-derived DPP4 was also unable to support pseudovirus replication when expressed on hamster cells (36). Lung and tissue samples from hamsters, mice, and ferrets stained for DPP4 indicated *in vivo* expression of DPP4, suggesting that an inability to become infected stems from differences in receptor binding domains rather than receptor expression (35).

Experimental confirmation has been provided that Syrian hamsters (37), wild-type mice (38), immune-deficient mice (38), ferrets (39), and rats (40) are all unable to become infected with the MERS-CoV.

Early reports indicated that infected patients had contact with livestock prior to becoming infected; thus, cells from various livestock were evaluated for susceptibility to infection. Primary cells from horses (kidney) (41), goats (lung and kidney), alpacas (kidney), and cattle (lung and kidney) (34) all supported viral replication, as well as immortalized cells from dromedary camels, sheep, humans, common pipistrelle bat, humans, and African green monkeys (34). Replacing blades 4 and 5 of mouse DPP4 with that of human, goat, camel, horse, or bat (*Pipistrellus pipistrellus)* allowed for the successful replication of virus in mouse DBT cells (42). Additionally, expressing camel, goat, sheep, or cow DPP4 on hamster cells allows the once resistant cells to support replication of MERS-CoV (35). Overall, these *in* vitro studies suggest that camelids, sheep, goats, horses, cows could act as reservoirs for MERS-CoV.

A number of studies have attempted to determine the residues of the receptor-binding domain that control the species barrier for MERS-CoV. Of the fourteen amino acids important in binding, there are five amino acids that differ between hamster and human DPP4, and structural modeling predicts that positions 291 and 336 are the most critical for binding while positions 341 and 346 were the least critical (35). Replacing these five amino acids in hamster DPP4 with human DPP4 allows for S protein binding and cell infection (35). Hamster cells transfected with DPP4 including one human amino acid substitution were unable to replicate virus; however, if cells were transfected with human DPP4 that

included only one hamster amino acid (positions 341 or 361) these cells were susceptible (43).

1.6) Current Animal Models

1.6a) Mouse models.

Mice are not naturally susceptible to MERS-CoV infection, making small animal modeling of disease difficult. However, expression of human DPP4 in mice enables support for viral replication. Current efforts have focused on transducing mice with an adenoviral vector expressing hDPP4, transgenic mice, and knock-in mice.

One approach to making mice susceptible to MERS-CoV is to transduce them with an adenoviral vector expressing human DPP4 (Ad5-hDPP4). These mice can be infected 17-22 days after transduction depending on strain (C57BL/6 and BALB/c) (44). Young (6-12 week old) mice fail to gain weight while infected, while adult mice lose weight over the course of their infection (44). Two to three days after infection, roughly 10^7 pfu/g of MERS-CoV were detected in lungs, and the infection is cleared in 6-10 days (44). Associated pathology involved peribronchial lymphoid infiltration that associated with interstitial pneumonia (44). While these mice may not recapitulate human disease associated with MERS-CoV infection, a major advantage to this model is the ability to use different established mouse strains of transgenic mice to elucidate aspects of disease pathogenesis (44). For example, mice deficient in toll-like receptor (TLR) or type-I interferon (IFN) signaling had more severe disease than wild-type mice (44). Thus, this model may be useful for evaluating therapeutics or vaccines and has been used in a variety of such studies (44-48). However, one study reported inefficient transduction in ~40% of mice

demonstrating the need to verify the expression of human DPP4 in individual mice in challenge studies (48).

Several groups have developed conventional transgenic mice that express human DPP4. One model responded to MERS-CoV infection with multi-organ failure resulting in death after day 10 post-infection. The lungs of these mice showed mild inflammation by day 5 post-infection that progressed to severe, diffuse alveolar damage by day 9 post-infection, and was associated with activated macrophage infiltration, hemorrhage, and diffuse alveolar diffusion (49). Viral replication occurred in type I and II pnenumocytes in the lungs, and a pulmonary inflammatory response was easily detected (49). However, unlike the disease seen in humans these mice developed neurological disease by day 9 post-infection and immunohistochemistry indicated that replication occurred in neuronal cell bodies, axons, and dendrites (49). Similar models show comparable disease, and such mice can be lethally infected with as little as 10 PFU/animal (50). One transgenic model does not show neurological disease; however, high amounts of viral replication are still detected in brain tissue (51). This specific model can be used for an infection or lethal model, which may be useful for some studies (52). While these models can produce lethal disease, their major weakness is the neurological involvement, as very few human patients have had neurological disease.

One knock-in mouse model is available for use in MERS-CoV infection studies. VelociGene developed this model, in which the DPP4 gene of these mice was replaced with the human DPP4 gene, allowing expression to be under the control of mouse regulatory elements (53). Intranasal inoculation did not result in lethal disease (up to day 4 post-infection), but a viral replication occurs abundantly in the lungs (53). Histological findings

mimic radiographs taken from human patients and indicate interstitial pneumonia (53). Most importantly, viral replication in the brain is not detected and there was no brain pathology detected following infection (53).

1.6b) Non-Human primates

To date experimental infections have been preformed with rhesus macaques and the common marmoset. Rhesus macaques infected with 7 x 10^6 $TCID_{50}$ MERS=CoV via intratracheal, intranasal, oral, and ocular routes developed a transient lower respiratory tract infection with clinical disease, including rapid respiration, cough, hunched posture, elevated temperature, and reduced appetite (54, 55). Viral RNA was detected in nasal swabs and bronchoalveolar lavage samples, while urogenital swabs were almost uniformly negative (54). Pathologic lesions at day 6 post-infection were found primarily in the lung and consisted of alveolar edema, and type II pneumocyte hyperplasia. Immunohistochemisty indicated that replication primarily occurred in the pulmonary epithelium (54). Genes associated with chemotaxis, inflammation, and antiviral immunity were upregulated over the course of infection (54). In contrast, macaques infected via intertracheal inoculation of 6.5 x 10^7 $TCID_{50}$ of virus developed mild clinical disease, but viral RNA was only detected in lung samples and not from any swabs or other tissues (56).

Because infected rhesus macaques have not fully recapitulated human disease, a common marmoset model of infection was evaluated. Animo acid alignments and co-crystallography studies of both marmoset and human DPP4 indicate that marmoset DPP4 is 96.4% identical to that found in humans, and that these amino acid differences are not found in the RBD (57). Animals infected with a human isolate of virus (EMC isolate) via intranasal, oral, and ocular inoculation developed moderate to severe progressive

pneumonia that required some animals to be euthanized early (57) Viral RNA was detected in nasal swabs, throat swabs, blood, and a variety of other tissues collected at necropsy (57). Interestingly, this study indicated that in some animals, roughly 1000 times more virus was recovered from infected marmoset lungs than from infected rhesus monkeys.

Despite the similarity of disease in marmosets and humans, a few major limitations exist for a marmoset model of MERS. Because of their small size, studies are limited the data that can be collected from individual animals, as intense sampling is not recommended. Surprisingly, a similar study with animals from a different vender was unable to repeat the results from Falzarano *et al.*, highlighting the importance of standardizing animal sources (58).

1.6c) Rabbit model

Phylogenetically, rabbit DPP4 is similar to human DPP4 (39) and rabbit primary kidney cells are susceptible to infection (59) suggesting that rabbits could be a viable animal model. New Zealand White rabbits infected by intranasal and intratracheal routes with a human isolate of MERS-CoV did not develop clinical disease; however, moderate amounts of infectious virus was isolated on days 1-7 post-infection from nasal swabs (59). Viral antigen was detected by immunohistochemistry in the nasal passages and lungs; however, minimal histopathological changes were detected (59). Virus was detected in the lungs and nasal conchae at day 3 post-infection, and all 4 rabbits kept until day 21 post-infection seroconverted (59).

1.7) Therapeutic Approaches for Treatment of MERS-CoV Infection

There is no specific treatment for MERS, although several drugs may improve patient outcome. Combination therapy with ribavirin and interferon-α2b reduced clinical

disease and viral replication in experimentally infected rhesus macaques (60) and *in vitro* infections of Vero E6 cells *(61)*. Some clinical studies indicate that this drug regimen may be effective (62, 63) while others caution that slow diagnosis may negate their usefulness for MERS-CoV (64) or SARS-CoV infections (65). While not licensed for human use, other groups are investigating the possibility of small-molecule inhibitors (46, 66) and antibody therapy (45, 53, 67). Immunocompetent (BALB/c) Ad5-hDPP4 transduced mice given 200ul MERS-CoV immune camel serum (neutralizing antibody titers ranged from 1:160-1:1280) intraperitoneally 1 day prior to challenge with MERS-CoV had significantly decreased virus detection in the lungs., Ad5-hDPP4 transduced immunocompromised mice given camel sera (neutralizing antibody titer 1:1280) demonstrated accelerated virus clearance, less weight loss, and less severe histological changes as compared to untreated mice (45). A high proportion of camels sampled in the Middle East have neutralizing antibodies to MERS-CoV, and camels large size allows for a significant amount of serum to be taken at once, making it easy to obtain (45). While heterologous antibodies are associated with a low risk of a hypersensitivity reaction, recombinant camelid antibodies could potentially be developed for therapeutic or prophylactic use in human patients (45).

1.8) Vaccination Strategies for Control of MERS-CoV Infection

Vaccination strategies to control MERS-CoV infections involve two different approaches. The classical approach is development of a vaccine explicitly for humans, which can then be distributed to the population at risk. The alternative approach is vaccination of the reservoir host. Unlike several other emerging diseases (e.g. Ebola virus and Zika virus), MERS-CoV utilizes a domestic animal (dromedary camel) as a reservoir host, offering the opportunity to approach control of human infection by developing a

vaccine targeting camels. Successful control of an infectious disease of humans by controlling the infection in its zoonotic host is not unprecedented. For example, when Hendra virus outbreaks began in 1994 resulting in equine and human infection, bat depopulation was originally suggested to protect human health (68). However, because bats fill an important ecological niche, an equine vaccine was developed to prevent transmission from to humans (68). Additionally, the development of a MER CoV vaccine could be more rapidly approved for use in camels than in humans and widespread implementation could significantly decrease the incidence of human infection.

1.8a) Subunit vaccines based on the MERS-CoV receptor-binding domain

The S protein of MERS-CoV plays a critical role in viral entry into host cells, and a subunit vaccine containing the S protein may result in protective immunity. In order to determine the residues critical for viral-receptor binding several groups produced different versions of the receptor-binding domain (RBD) of the S protein and evaluated their ability to generate neutralizing antibodies. While each group divided the S protein into slightly different regions, residues 377-588 or residues 358-588 both generated the highest titer of neutralizing antibodies in mice. While other regions tested generated antibodies, the authors suggested that perhaps these regions contain non-neutralizing epitopes or epitopes that if included in candidate vaccines could dilute the neutralizing potential (69-71). Other studies confirmed the importance of this region, and indicate that residues 367-606, 377-662, and 736-761 are important in generating neutralizing antibodies (72-74)

Purified RBD used as bait to select antibodies from a nonimmune human singe-chain variable region fragment (scFv) library of *Saccharomyces cerevisiae* yielded two monoclonal antibodies capable of neutralizing MERS-CoV. Additionally, these antibodies

obstructed the RBD from successfully binding DPP4, suggesting that RBD-based subunit vaccines could potentially neutralize virus *in vivo (75).* Monoclonal antibodies purified from mice immunized with S1 also neutralized virus and specifically bound to the RBD of the S protein, preventing binding with DPP4 and host cell entry (76). Purified S nanoparticles generated by recombinant baculovirus also generated neutralizing antibodies when given intramuscularly in BALB/c mice, although antibody titers were significantly higher when given with the adjuvant alum, and higher still when given with adjuvant Matrix M1 (77).

Route of vaccine administration likely plays an important role in protection against challenge with MERS-CoV. When BALB/c mice were subcutaneously vaccinated with the RBD of S protein fused with Fc of human IgG and adjuvant Montanide ISA51 subcutaneously, neutralizing antibodies were produced. When administered intranasally with adjuvant (Poly(I:C)), a significant IgG antibody response could be detected after 5 vaccinations. Like the subcutaneous vaccination, this antibody response contained MERS-CoV S specific IgG (Th2-assocaited), IgG2a (Th1-associated) and IgG3 antibody, however it appeared that an intranasal vaccination might favor a Th1 response. Lung washings from mice vaccinated intranasally had significantly higher levels of IgA than washings from mice vaccinated subcutaneously, suggesting that intranasal vaccinations result in a stronger local mucosal response. A high frequency of IL-2 and IFN-γ-producing T cells were detected in both intranasal and subcutaneously vaccinated mice. However, intranasal vaccination resulted in a significantly higher frequency of IL-2 generating T cells and somewhat higher IFN-γ producing T cells in CD4+ and CD8+ cells, suggesting a more robust cellular immune response (70).

1.8b) Live-attenuated viral vaccines

Although MERS-CoV is a positive-sense virus with a large genome (30,119 nt), genetic manipulation can be accomplished through the assembly of contiguous cDNAs that span the entire genome, a bacterial artificial chromosome (BAC), or with a vaccinia virus vector. Contiguous cDNAs have been used to generate a variety of mutants, and while these have primarily been used to characterize the role of accessory ORFs further development could yield vaccine candidates (78). Almazán *et al.* used a BAC in order to generate a replication-competent, propagation-defective mutant lacking the envelope (E) protein (79). Because the E protein is deleted entirely, this mutant cannot revert back to a virulent strain and could be safe even for immunocompromised individuals (79). While live-attenuated virus vaccines still require a great deal of development before they can be considered candidate vaccines or tested *in vivo* in humans, they offer a promising strategy for preventing further transmission and disease.

1.8c) Viral Vectored Vaccines

Another approach toward developing a MERS-CoV vaccine has involved constructing recombinant viral vector vaccines with vaccinia virus or adenovirus vectors that express S protein. Modified vaccinia virus Ankara (MVA) is a highly attenuated vaccinia virus strain already in preclinical research and human trials for other pathogens. Thus MVA expressing MERS-CoV S protein is a logical choice as a vaccine candidate. Mice (BALB/c) vaccinated intramuscularly and boosted at 3 weeks produced substantial levels of circulating antibodies that were able to neutralized MERS-CoV *in vitro* (80). A similar modified vaccinia virus significantly lowered levels of detectable RNA in the adenovirus-transduced mouse model (47). Similarly, a camelpox-based vaccine administered

intranasally and intramuscular to dromedary camels resulted in decreased nasal shedding upon challenge, highlighting its potential utility in dromedaries (81).

Two different adenovirus vector-based vaccines expressing the S protein that were adminstered intragastrically or intramuscularly also elicited production of neutralizing antibodies. The cellular immune response was determined by counting IFN-γ-secreting T cells from the spleen of immunized mice. While intramuscular vaccination yielded a higher cellular immune response than intragastric vaccination, both candidate vaccines induced significant IFN-γ-secreting T cell responses. Finally, intramuscular vaccination with either adenoviral vaccine resulted the production of IFN-γ, IL-2, IL-10, and TNF-α, suggesting the induction of a Th1 response (82). While these vaccines successfully elicit an immune response in mice, it is currently not known if this response could protect against an actual viral challenge in either humans or dromedary camels. Pre-existing antibodies to adenovirus type 5 have no been reported *in vivo*, although camel cells can be infected by this virus, indicating they have strong potential as vaccines (83).

A measles vaccine vector has also been evaluated as a candidate vaccine. This platform has had good success in animal challenges for West Nile virus, has a good safety record, can be manufactured quickly, and has been shown to elicit humoral and cellular immune responses (48). Two candidate vaccines expressing either a full-length or truncated S protein elicited strong responses in mice and when used with the adenovirus transduced mouse model resulted in almost total reduction of viral replication (48).

1.8d) Vaccination lessons learned from SARS CoV

In 2002 the severe acute respiratory syndrome coronavirus (SARS-CoV) emerged in China and was responsible for over 8000 clinical cases of disease and nearly 800 deaths.

While related to MERS-CoV, SARS CoV utilizes a different host receptor (ACE-2), and thus antibodies against SARS-CoV are not protective against MERS-CoV. Mouse-adapted SARS-CoV variants are available, and several strains of mice are susceptible to SARS-CoV. Syrian golden and Chinese hamsters also support viral replication and develop pneumonitis accompanied by immune cell infiltration, although they do not develop severe clinical illness. Ferrets are also susceptible to infection, and although disease severity is variable, these animals could be valuable in understanding super-spreaders. Finally, a variety of non-human primate models are available, though very few vaccine candidates have been tested due to the difficulty and cost of primate experiments (84). While there is not a licensed human vaccine for SARS-CoV, vaccine candidates for SARS-CoV may offer insight for potential MERS-CoV vaccines. For example, one study suggested that in mice, vaccination enhancement of CD8+ T cells was critical to viral clearance and improved survival (85). Other studies suggested that while inactivated whole virus vaccines can induce a Th2 immune response, mice develop pulmonary immunopathology after experimental challenge (84, 86).

1.9) Bats and the Origin of MERS-CoV

Bats make up the order Chiroptera and with more than 1,300 species are incredibly diverse (87). These animals are now recognized as the reservoir host for a variety of important zoonotic pathogens including rabies virus, Nipah virus, Hendra virus, SARS-CoV, and Ebola virus (88). While their role as an excellent reservoir host is not fully understood, several features may contribute to bats' ability to become infected without clinical disease and transmit virus to other animals, including humans.

Like MERS-CoV, SARS-CoV is a betacoronavirus also causing severe respiratory disease, and evidence that SARS-CoV has originated from a bat stimulated speculation that bats might be a reservoir host for MERS-CoV and initiating a search for MERS-CoV in bats (89-91).

Fecal pellets from 62 bats in South Africa contained a novel betacoronavirus from a female *Neoromicia zuluensis* that differed from MERS-CoV by one amino acid in a 816 nucleotide fragment of the polymerase gene and was 10.9% different in the S gene (92). HKU4 and HKU5 are bat coronaviruses that were identified in 2006 in Japanese pipestrelles (*Pipistrellus abramus*) and lesser bamboo bats (*Tylonycteris pacypus*). The S protein from those viruses can bind to human DPP4 and facilitate cell entry when expressed on pseudoviruses (93). This interaction between the receptor binding domain of HKU4 and HKU5 S protein and DPP4 has a lower affinity than that of MERS-CoV, supporting the hypothesis these viruses originally circulated in bats prior to evolving into a camel virus (93). NeoCoV is another betacoronavirus identified from a bat (*Neoromicia capensis*) that is related to MERS-CoV. A full genome comparison indicated that the structural proteins E, M, and N shared the highest level of sequence identity (89-94.5%) with MERS-CoV, while the overall nucleotide identity between the MERS-CoV and NeoCoV was 85.5% (94). Bayesian phylogenies demonstrate that NeoCoV clusters with other similar CoVs isolated from a European hedgehog (95) and a *Nycteris* bat (94). While not a direct ancestor of MERS-CoV these similarities indicate the possibility of a bat origin (94).

Kidney cells from an African straw-colored fruit bat (*Eidolon helvum*), Buttikofer's epaulletted fruit bat (*Epomops buettikoferi)*, fetal kidney and lung cells from the hammer-head fruit bat (*Hypsignathus monstrosus*), and kidney and embryonic cells from the

Egyptian rousette bat (*Rousettus aegyptiacus*) all support MERS-CoV replication (96). Interestingly, both cell lines from the Egyptian rousette bat viral yield was comparable to that of Vero cells (96). Because these bats are found in Egypt, Iran, Jordan, Lebanon, Oman, Saudi Arabia, UAE, and Yemen it is possible that these animals could contribute to viral transmission either to humans or camels (96).

Field sampling from Saudi Arabia was conducted after the first human case <12 km from his home and work resulted in samples from 96 bats comprising 7 species (*Rhinopoma hardwickii, Rhinopoma microphyllum, Taphozous perforates, Pipistrellus kuhlii, Eptesicus bottae, Eidolon helvum,* and *Rossettus aegyptiacus*) (97). Extensive samples were collected from these animals; unfortunately when shipped from Saudi Arabia the shipment sat in customs at room temperature for 48 hours compromising the samples (97). Despite this challenge, a 190 nucleotide fragment with 100% identity to the isolate from the index patient was detected from *T. perfortatus*, commonly known as the Egyptian tomb bat (97); the small size of this fragment of RNA makes it difficult to judge the significance of this finding.

An experimental infection of Jamacian fruit bats (*Artibeus jamaicensis*) demonstrated *in vivo* that virus was replicated in bats and could be detected in oral and rectal swabs for up to 9 days and in tissues for up to 28 days (98). This infection was not associated with clinical disease and animals only had limited histopathology suggesting that bats could easily act as a reservoir host (98).

1.10) Camels as the Reservoir Host for MERS-CoV

1.10a) Role of Camels in the Middle East and Africa

There are an estimated 30 million of camels globally, 95% of which are dromedaries (99). Roughly 77% of these dromedaries are found in Africa (Somalia, Sudan, Kenya, Ethiopia, etc.) while 4% of these are found in the Arabian Peninsula with the highest density in UAE and Qatar (99). These animals are important sources of meat and milk, are also used for transport, trade, in races, for medicinal purposes and can also show an individual's social prestige (99). Some animals are kept on traditional camel farms, while others are housed on commercial or racing farms (99). Animals kept for leisure are frequently kept on weekend farms owned by urban dwellers (99). Understanding the cultural and economic role of these animals as well as the movement of camels within the Middle East and Africa is essential for elucidating the transmission of MERS-CoV between animals and to humans.

Camel husbandry has changed dramatically in the last fifty years, perhaps contributing to the emergence of MERS-CoV. Many communities are intricately tied to camels, highlighting the importance of approaching control of viral transmission with cultural relativism. For example, in some cultures when a male child is born, his umbilical cord is placed in a sac and tied around the neck of a female calf that he then has ownership over (100). In Somali nomads the community is split between unmarried men responsible for herding camels up to several hundred kilometers from community wells or family units that keep camels in a central location (100). These groups come together every rainy season for culturally important rituals and feasts. Raw or soured camel milk is frequently consumed, and in some cultures heat treatment of milk is not permitted (100). In Algeria

the connection with camels and camel milk is described as “water is the soul; milk is the life” (100).

However, the number of camels has more than double since 1961 and the nomadic population has shrunk to 1.5% from 10% (101). These changes have been accompanied with new hurdles for camel owners and pose distinct challenges for commercial farms. Historically camels have been superior to cattle or other ruminants in arid landscapes because of their ability to graze on a wide variety of plants, tolerate low access to water, high feeding efficiency, and high salt tolerance (102). However, as farming moves from a primarily nomadic system to a semi-intensive or intensive system the requirement for water has increased by 3.2 times and farmers have had to adjust to feeding primarily by grazing to supplying biomass (102). The increased demand for camel milk and meat has had distinct implications for camel management and food safety. Many of the markets associated with slaughterhouses are informal, based on oral agreements and are not always associated with specific camelid veterinary care (102). Thus, without better checkpoints for the movement of live camels or documentation of proper veterinary care transmissions of pathogens will remain a concern. For example, a brucellosis outbreak in Qatar was recently linked to the consumption of unpasteurized camel milk (103).

1.10b) Field surveillance

Early patient interviews indicated that contact with livestock such as goats and camels might increase risk for contracting MERS-CoV infection (104-106). Dr. Reusken is credited for the first evidence linking dromedary camels to MERS-CoV when she discovered neutralizing antibodies in 50/50 camels screened in Oman in March 2013 (107). Interestingly, herds (105 retired racing camels) sampled in the Canary Islands

yielded 15 animals (14%) seropositive for the virus (107). The Spanish camels were from a closed herd with the exception of three animals imported from Morocco over 18 years prior to the study, indicating the possibility that the virus was not currently circulating in the Canary Islands (107). A later study confirmed the presence of neutralizing antibodies, but only in older animals imported from Africa, again supporting the hypothesis that the virus is not present in the Canary Islands (108)

Field surveillance in the Middle East indicates that MERS-CoV has been circulating in dromedary herds for at least 30 years. Studies in Saudi Arabia (109-112), United Arab Emirates (UAE) (113-115), Qatar (116-118), Oman (107, 119), and Jordan (120) demonstrate the widespread presence of neutralizing antibodies and virus (RT-PCR and viral isolation) in animals. In general older animals are more likely than juveniles to be seropositive while younger animals are more likely to be actively shedding virus than adults. Investigators in UAE found virus in nasal swabs in slaughterhouses and at border sites but not with public escorts or zoos suggesting that mixing of animals is important disease transmission (113).

Attention has also been focused on camelid populations that are not circulating MERS-CoV. Feral camels (n=25) in Australia were uniformly seronegative for MERS-CoV, suggesting that these animals were negative for the virus. Over 20,000 dromedaries were imported into Australia between 1880 and 1907 and are comprise the only population of feral dromedaries in the world (121). Thus, preventing the introduction of MERS-CoV into this population is a top priority. Serologic screening in Kazakhstan of both dromedary (n= 455) and Bactrian camels (n=95) did not reveal any positive samples, suggesting that MERS-CoV is not circulating in these populations (122). Similarly, serum and nasal swabs

from Bactrian camels in the Umnougovi (n=170) and Dundgovi Province (n=30) were all negative for MERS-CoV by RT-PCR and a spike pseudoparticle neutralization test (123). Additionally, two Bactrian camels in Chile and 2 Bactrian camels in the Netherlands were also negative for MERS-CoV specific antibodies (107). One study representing 87% of the dromedaries in Japan (n=20) was unable to find any indication of MERS-CoV through serology or RT-PCR (124). Although it has not been tested *in* vivo, analysis of DPP4 indicated that while differences do occur in the receptor of dromedaries and Bactrian camels, these differences are not found in the receptor binding domain and thus these animals likely could become infected if MERS-CoV was introduced into these herds (123).

1.10c) Evidence for camel-to-human transmission

Understanding the dynamics of camel-to-human transmission is critical to prevent future primary infections. Such transmission could occur directly from handling or slaughtering infected animals or indirectly from the consumption of contaminated products such as milk or meat. Additionally, elucidating the kinetics of camel-to-camel transmission will help identify animals most likely to become infected which will likely have direct implications for those in the camel trade.

Several studies have investigated the risk of occupational exposure to dromedaries. Both camel and human isolates of MERS-CoV replicated in Vero cells and *ex* vivo bronchial and lung tissues, indicating that this transmission could be possible (125). Furthermore, MERS-CoV is stable in milk and inactivated by pasteurization, suggesting that if contaminated raw milk may be important in human transmission (126). Milk collected from five of seven camels in Qatar was positive by RT-PCR; however, these milk samples were collected traditionally and the virus could well have been the result of contamination

rather than the secretion in milk (116). Calves stimulate the let down of milk by suckling, and then milk is collected without cleaning the teats (116). As calves are frequently infected, it is likely that the viral RNA detected is the result of contamination from the calf suckling; however, this study indicates the potential of indirect transmission via unpasteurized milk. Finally, an air sample from a camel barn belonging to a Saudi patient was positive for viral RNA, indicating the potential of aerosol transmission (127).

In Saudi Arabia, serum from individuals employed at a camel abattoir, veterinary surgeons, individuals with intermittent camel contact, and herdsmen with daily contact with camels were all seronegative for MERS-CoV (128). Interestingly, dromedaries the herdsmen cared for had recently been infected with MERS-CoV indicating a low risk of infection despite daily contact and the consumption of raw milk from these animals (128). Similarly, sera from 300 animal workers (17% of these individuals had daily contact with camels) and 50 non-animal workers in Saudi Arabia were also negative for MERS-CoV specific antibodies (129). In contrast, 20 of 294 humans with dromedary contact in Qatar were seropositive while 204 individuals with no camel contact were seronegative (130). These seropositive individuals worked at slaughterhouses, animal markets, camel racing tacks, and camel farms (130). Animals at markets are frequently held for several days prior to being transferred to slaughter; thus, mixing of animals may play a significant role in transmission (118, 131). Camels from the Horn of Africa are frequently imported to camel farms in the Middle East, and in 2011 the Ministry of Environment in Qatar forbid the grazing of camels for two years prompting the movement of many camels into Saudi Arabia to feed which may have put camel farmers at risk (99).

Several studies have documented transmission of virus between camels and humans, although the direction of transmission has not been fully elucidated. In 2013, viral RNA collected from an infected Saudi individual and his camels shared a single nucleotide polymorphism and thereby support zoonotic transmission (132). Another study of the same patient also reported 100% sequencing identity between camel and patient samples. The patient was seronegative on day 1 of the study and the dromedaries all had neutralizing antibodies that transmission occurred from the camels to the patient, suggesting that the camels had become infected prior to the patient's infection (133). Another individual importing camels to UAE fro Oman and an animal worker screening dromedaries crossing the border was found to be positive for MERS-CoV by PCR at a screening checkpoint (25). Interestingly these patients were asymptomatic, and because their sera was not tested for antibodies it is possible that a previous exposure prevented clinical disease and that MERS-CoV reporting underestimates the total number of individuals infected (25).

Although there have been no human clinical cases of MERS-CoV recognized in Africa, several reports indicate that many camels are seropositive; thus, it is likely that transmission to humans has occurred (134, 135). A recent study in Kenya found that 16 of 1010 individuals tested were seropositive for MER-CoV by ELISA (136) . Of these, two had titers of 20 and 40 by plaque reduction neutralization test with a neutralization cutoff of 50%, indicating very low titers. Both of these individuals kept goats and donkeys, while one also kept sheep, suggesting that the role of other livestock reservoirs should be investigated.

1.11 Evidence for the Role of Livestock in MERS Epidemiology

Patient reports and *in vitro* assays indicate that other livestock could potentially contribute to viral transmission. In Saudi Arabia goats, sheep, cattle, and chickens have been negative for MERS-CoV by RT-PCR (nasal and rectal swabs) and/or serology (109, 112). Sera from sheep and cattle in Jordan did not contain neutralizing antibodies against MERS-CoV, although sera from 6 of 126 sheep reacted by ELISA (120). Sheep and horses sampled in UAE in 2005 also were seronegative for MERS-CoV despite a high proportion of seropositive camels in the area (41, 137). Cattle and water buffaloes at slaughterhouses as well as backyard sheep and goats in Egypt were also negative for MERS-CoV specific antibodies (138). Interestingly, 15 of 15 alpaca kept on a farm with seropositive camels had detectible neutralizing antibodies against MERS-CoV (139). Thus, despite the evidence from *in vitro* studies, it does not appear that livestock other than camelids play a major role in transmission.

CHAPTER 2: REPLICATION AND SHEDDING OF MERS-COV IN UPPER RESPIRATORY TRACT OF INOCULATED DROMEDARY CAMELS [1]

2.1) Introduction

The Middle East respiratory syndrome coronavirus (MERS-CoV) was first recognized in 2012 in a fatal human case of pneumonia in Saudi Arabia (140). Since that report, more than 800 cases of MERS have been identified, with an estimated case fatality rate of approximately 35 percent (141). Human infection is associated with a spectrum of clinical severity ranging from asymptomatic infection to severe respiratory distress and death(19, 140, 142-146). The majority of cases have been confined to the Arabian Peninsula, but a number of travel-associated cases have been reported from countries in Africa, Asia, Europe, and North America (141, 146, 147). Limited human-to-human transmission has been reported, predominantly in nosocomial settings, suggesting that the majority of human cases obtained the MERS-CoV infection directly from the reservoir source (146, 148, 149).

The close phylogenetic relationship of human MERS-CoV isolates with sequences obtained from bats initially suggested a direct link between the emergence of MERS-CoV and a putative natural reservoir, similar to severe acute respiratory syndrome coronavirus (SARS-CoV) (84, 150, 151). Anecdotal reports mentioned contact of MERS-CoV infected patients with camels and goats, suggesting that livestock might be the intermediate reservoir host for MERS-CoV (146, 152-154). Serological studies revealed widespread

[1] Chapter published as Adney DR, van Doremalen N, Brown VR, Bushmaker T, Scott D, de Wit E, Bowen RA, Munster VJ. Replication and shedding of MERS-CoV in upper respiratory tract of inoculated dromedary camels. *Emerg Infect Dis*. 2014;20:1999.2005.

prevalence of MERS-CoV specific antibodies in dromedary camels from countries with MERS-CoV cases: Saudi Arabia, Jordan, Qatar, the United Arab Emirates and Oman(109, 114, 119, 146, 155-158). MERS-CoV specific antibodies have also been detected in dromedary camels in several countries with no reported human cases of MERS-CoV in North and East Africa (138, 146, 155). Retrospective analysis of serum samples collected from dromedary camels suggest that rather than being a recent introduction from a bat reservoir, MERS-CoV has been circulating in dromedary camel populations for at least two decades(109, 114, 115). MERS-CoV specific antibodies have not yet been reported from livestock species such as sheep and goats (109, 114, 155, 157, 158). In addition to a high proportion seropositive dromedary camels, MERS-CoV RNA was detected in nasal swabs obtained from three camels on a farm linked to two human MERS-CoV cases and MERS-CoV was cultured from nasal swabs from dromedary camels in Qatar (156). MERS-CoV isolation and subsequent full genome sequencing directly linked a dromedary camel and a fatal MERS-CoV case in Saudi Arabia (132, 133). Despite all of these associations implicating dromedary camels as the prime reservoir of MERS-CoV, the role of dromedary camels as primary reservoir for MERS-CoV is still debated (159, 160). Here we report the infection of dromedary camels with a human isolate of MERS-CoV and the re-isolation of MERS-CoV up to 7 dpi from the inoculated camels, along with a description of the magnitude, sites of MERS-CoV replication, shedding, and the kinetics of the humoral response in experimentally inoculated dromedary camels.

2.2) Materials and Methods

2.2a) Ethics statement

All animal work in this study was approved by the Institutional Animal Care and Use Committee of Colorado State University and was performed in compliance with recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institute of Health with every effort to minimize animal suffering. All work with MERS-CoV was performed under BSL3 containment at Colorado State University or the Rocky Mountain Laboratories, Division of Intramural Research, National Institute of Allergy and Infectious Diseases, National Institutes of Health and was approved by those respective Institutional Biosafety Committees.

2.2b) Virus and Cells

MERS-CoV (strain HCoV-EMC/2012), provided by the Department of Viroscience, Erasmus Medical Center, Rotterdam, The Netherlands, was propagated in Vero E6 cells cultured in DMEM supplemented with 2% FBS, 2 mM L-Glutamine, 50 U/ml penicillin and 50 μg/ml of streptomycin.

2.2c) Animal study

Three male dromedary camels (*Camelus dromedarius*) seronegative for MERS-CoV and bovine coronavirus were obtained through private sale. Animals were housed in an ABSL3 facility for the duration of the experiment and fed *ad libitum*. Camels were acclimatized to the facility for two weeks prior to virus inoculation, during which they were monitored daily. Camels were inoculated with 10^7 $TCID_{50}$ of MERS-CoV (strain HCoV-EMC/2012), via the intratracheal (8 ml via transcutaneous catheter), intranasal (3.5 ml in each nostril) and conjunctival (0.2 ml in each conjunctival sac) routes while sedated with

xylazine. The animals were observed at least once daily for the duration of the experiment. Behavior, food consumption, activity level, and nasal discharge were monitored. Rectal temperature was taken daily from -2 to 7 days post inoculation (dpi), then three times a week until euthanasia. Nasal and oral swabs and feces were collected daily from day 0 to 7 dpi and then three times a week until euthanasia in virus transport medium or virus lysis buffer. Blood was collected into evacuated EDTA and serum-separating tubes daily on 0 – 7 dpi and three times weekly thereafter. On day 5, 28, and 42, animals 1, 2 and 3 respectively were sedated with intramuscular xylazine and euthanized with intravenous pentobarbital, and nasal turbinates, lungs, trachea, larynx, pharynx, liver, spleen, kidney, bladder, urine, duodenum, jejunum, colon, rectum, abomasum, reticulum, rumen, prescapular lymph node, retropharyngeal lymph node, tracheobronchial lymph node, mediastinal lymph node, mesenteric lymph node, medulla, and olfactory cortex were collected.

2.2d) RNA extraction and quantitative PCR

RNA was extracted from swabs, fecal samples and serum samples using the QiaAmp Viral RNA kit (Qiagen) according to the manufacturer's instructions. For detection of viral RNA, 5 µl of RNA was used in a one-step real-time RT-PCR upE assay(161) using the Rotor-GeneTM probe kit (Qiagen) according to manufacturer's instructions. Standard dilutions of a titered virus stock were run in parallel, to calculate $TCID_{50}$ equivalents in the samples.

2.2e) Virus titration

Swab samples in viral transport medium, whole blood and homogenized tissues (~10% w/v) were titrated for MERS-CoV virus by plaque assay. Briefly, ten-fold serial dilutions of samples were prepared in BA-1 medium containing 100 mg gentamicin, 200,000 U penicillin G, 100 mg streptomycin and 5 mg amphotericin/L, and 0.1 ml volumes

were inoculated onto confluent monolayers of VeroE6 cells grown in six-well cell culture plates in duplicate. The inoculated cells were incubated for 45 minutes at 37° C in 5% CO_2 in air and then overlaid with 2 ml/well of MEM without phenol red and containing 0.5% agarose, 2% fetal bovine serum and antibiotics as described above. Two days after the initial overlay, a second overlay was added which was identical to the first except for inclusion of neutral red (33 mg/L). Plaques were counted on days 1 and 3 after the second overlay and virus titers expressed as plaque-forming units (pfu) per ml.

2.2f) Aerosol sampling

The generation of aerosols during normal breathing was investigated by placing a funnel over the muzzle of each camel connected to a vacuum pump capturing exhaled air in tissue culture media (10 ml, DMEM + 1% FBS + 0.013% SE-15 (anti-foam) with an All Glass Impinger (Ace Glass Inc.). Aerosols were collected for ~ 2 minutes. Collected aerosols were analysed by quantitative real-time polymerase chain reaction (qRT-PCR) and virus titration.

2.2g) Histopathology and immunohistochemistry

Tissues were fixed for >7 days in 10% neutral-buffered formalin and embedded in paraffin. Tissue sections were stained with hematoxylin and eosin (H&E). To detect MERS-CoV antigen, immunohistochemistry was performed using a rabbit polyclonal antiserum against HCoV-EMC/2012 (1:1000) as a primary antibody.

2.2h) Plaque reduction neutralization assay

Camel sera were heat inactivated for 30 minutes at 56° C and two-fold dilutions beginning at 1:5 prepared in BA-1 medium. These samples were mixed with an equal volume of MERS-CoV to obtain a virus concentration of 100 pfu/0.1 ml and an initial serum

dilution of 1:10. The virus-serum mixtures were incubated at 37° C for 60 minutes, then inoculated onto VeroE6 cells as described for plaque assay. Plaque reduction neutralization assay (PRNT) titers were calculated as the reciprocal of the highest dilution that resulted in ≥90% neutralization of virus relative to no serum control samples.

2.3) Results

2.3a) Clinical signs in three dromedary camels inoculated with MERS-CoV

Three mature dromedary camels were inoculated with 10^7 $TCID_{50}$ of a human isolate of MERS-CoV (strain HCoV-EMC/2012) through a combination of intratracheal, intranasal and conjunctival inoculation. Each of the three camels exhibited only minor clinical signs of disease, which consisted of rhinorrhea (Fig 2.1A) and a mild elevation in body temperature on 2 and 5-6 dpi (Fig 2.1B); no other clinical signs were observed. All three camels developed rhinorrhea beginning on 2 (camel 1 and 3) or 5 (camel 2) dpi. The rhinorrhea persisted no longer than 2 weeks.

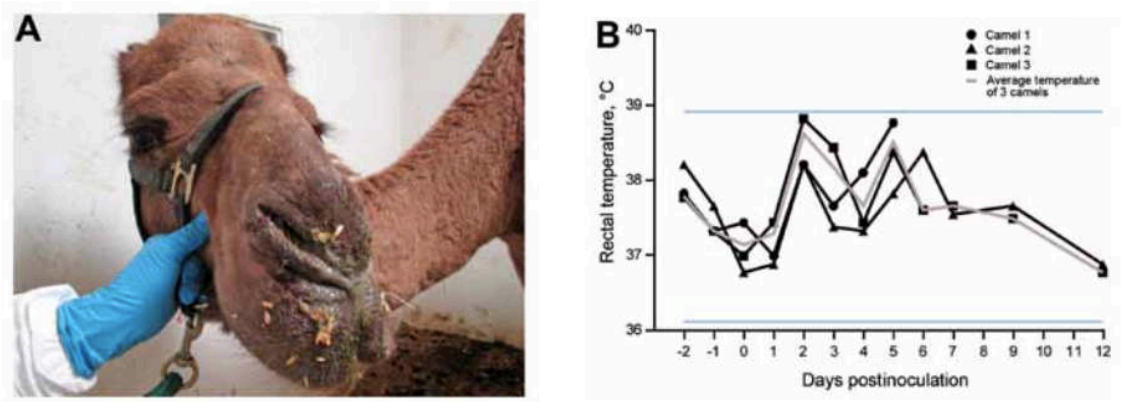

Figure 2.1. Clinical signs in dromedary camels inoculated with MERS-CoV. A) Nasal discharge observed in camel 3; all three inoculated camels displayed nasal discharge during the first 2 weeks of the experiment. B) Rectal temperatures for camel 1 (l), camel 2 (p) and camel 3 (n). The red line indicates the mean temperature of the three camels and the blue lines indicates the normal temperature range observed in these dromedary camels as calculated by mean ± 3 times the standard deviation prior to inoculation.

2.3b) MERS-CoV shedding and aerosol sampling

MERS-CoV shedding started between 1 – 2 dpi, as detected by the presence of infectious virus and viral RNA by qRT-PCR in nasal swabs. Infectious virus shedding was detected up to 7 dpi and shedding of viral RNA was detected up to 35 dpi in nasal swabs (Fig 2.2). Low concentrations of infectious virus and viral RNA were detected in oral samples, likely due to drainage from the nasal cavity (Figure 2.3). No viral RNA was detected in fecal samples or in various urine samples collected at 0, 1, 5, 14, 21, 28 and 42 dpi from the three camels. No infectious virus or viral RNA was detected in any of the serum or whole blood samples. Relatively limited amounts of MERS-CoV shedding via aerosols were detected by qRT-PCR ($10^{1.2}$ and $10^{1.4}$ $TCID_{50}$ equivalent/ml) on 3 and 5 dpi. Infectious virus was not detected in the aerosol samples during virus isolation attempts.

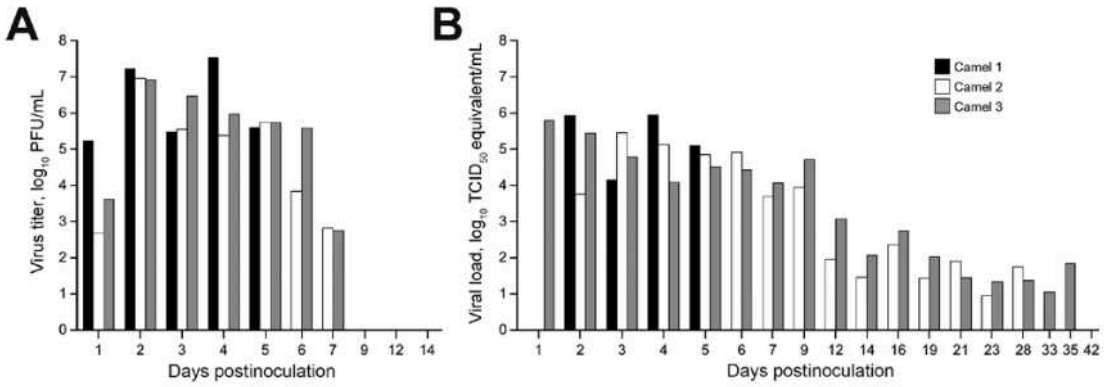

Figure 2.2. Virus shedding from the upper respiratory tract in dromedary camels inoculated with MERS-CoV. Shedding was determined by infectious titers by plaque assay (A) and viral load by qRT-PCR (B). $TCID_{50}$ equivalents were extrapolated from standard curves generated by 10-fold dilutions of a MERS-CoV stock (HCoV-EMC/2012) with known virus titer in parallel to each qRT-PCR run. Red bars indicate shedding in camel 1, blue bars indicate shedding in camel 2 and green bars indicate shedding in camel 3.

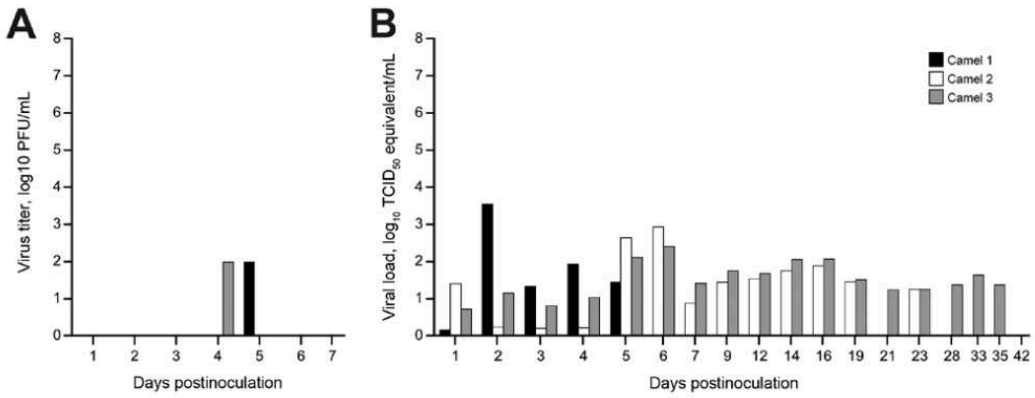

Figure 2.3. Virus shedding determined by oral swabs obtained from dromedary camels inoculated with MERS-CoV. Shedding was determined by A) determining infectious titers and B) viral RNA. Virus titers were determined by plaque assay and viral load by 2RT-PCR. $TCID_{50}$ equivalents were extrapolated from standard curves generated by 10-fold dilutions of a MERS-CoV stock (HCoV-EMC/2012) with known virus titer in parallel to each run.

2.3c) MERS-CoV infection is transient and predominantly confined to the upper respiratory tract

Tissues collected at necropsy were tested for the presence of infectious virus, and evaluated by histopathology and immunohistochemistry. Infectious virus was detected in tissues from camel 1, euthanized on 5 dpi, but not in tissues obtained from camel 2 and 3, euthanized on day 28 and 42 dpi respectively. High infectious virus titers could be detected in tissues of the upper respiratory tract (URT), including nasal turbinates, olfactory epithlium, pharynx and larynx. In the lower respiratory tract (LRT) infectious virus was detected in the trachea and right upper lung lobe. Infectious virus was also detected in the retropharyngeal, mediastinal, mesenteric and tracheobronchial lymph nodes (Fig. 2.3). The extent of MERS-CoV replication and associated lesions in dromedary camels was investigated by histopathology and immunohistochemistry. In camel 1 (necropsied 5 dpi), histological lesions were present in the pseudostratified epithelial cells in URT (nasal

turbinates and larynx) and the LRT (trachea, bronchi and bronchioles). No lesions were observed in the alveoli (Fig. 2.4). The observed lesions were characterized as mild to moderate acute intraepithelial and submucosal inflammation with multifocal necrosis and loss of pseudostratified epithelial cells, comparable to common cold in humans. Multifocal loss of epithelial polarity and cilia with squamous metaplasia were observed in these lesions. The epithelium was infiltrated by small to moderate numbers of neutrophils with fewer macrophages; similar inflammatory cells also permeated the submucosa. The submucosal glands of the trachea were multifocally necrotic and infiltrated by small numbers of neutrophils. Viral antigen was detected within the epithelial cells of the nasal turbinates, larynx, trachea, bronchi and bronchioles, but not the alveoli. In addition, the tonsils, mediastinal and retropharyncheal lymph nodes also demonstrated viral antigen at the follicular mantle zone (Fig. 2.4). The nasal turbinates, larynx and trachea of camel 2 (necropsied 28 dpi) had similar but milder lesions when compared to camel 1 (data not shown). The nasal turbinate, larynx, and bronchus showed small numbers of infiltrating neutrophils, however, in contrast with camel 1, the cilia and goblet cells were intact. The remainder of the respiratory tract of camel 2 was unaffected (data not shown)

. Immunohistochemistry revealed the presence of limited viral antigen in the nasal turbinate but not in any of the other tissues at this timepoint. No lesions or viral antigen were detected in camel 3 on 42 dpi (data not shown).

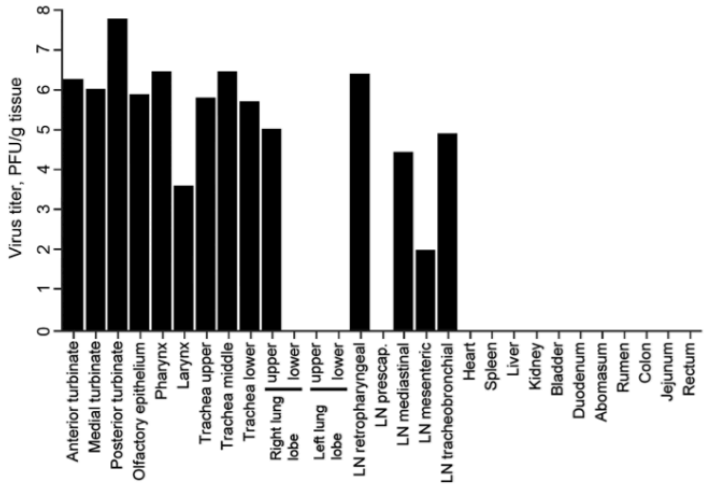

Figure 2.4). Virus titers in tissues collected from dromedary camels inoculated with MERS-CoV. Tissues were collected at 5 dpi. Nasal turbinates were sampled in three different sections: anterior, medial and posterior. LN is lymph node.

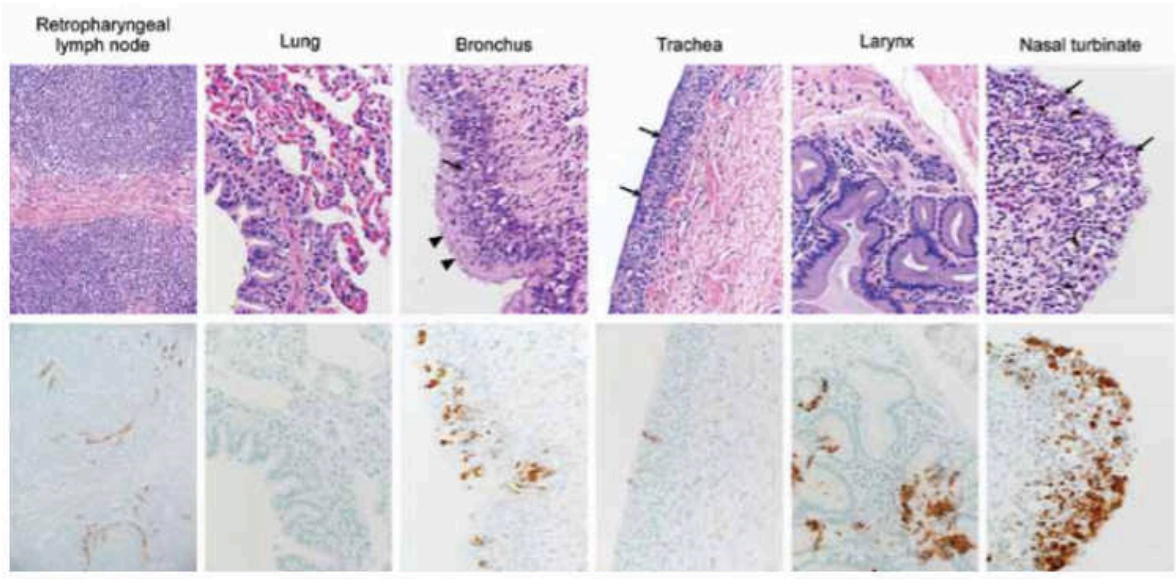

Figure 2.5). Histopathological changes at 5 dpi in camel 1 inoculated with MERS-CoV. Tissues were collected and stained with hematoxylin and eosin (H&E) and anti-MERS-CoV immunohistochemistry (IHC). MERS-CoV antigen is visible as a red brown staining in the immunohistochemistry panel. Degeneration of the pseudostratified epithelium lining the nasal turbinate, trachea and bronchus is indicated by the absence of goblet cells, cilia and nuclear regimentation with infiltration of neutrophils (arrows). The arrowheads indicate areas where the cilia remained intact. Magnification: 400x.

2.3d) Humoral response to MERS-CoV

Sera were collected weekly from the camels to monitor the generation of neutralizing antibodies specific to MERS-CoV. Each of the three camels was seronegative prior to inoculation. Camels 2 and 3 (euthanized on 28 and 42 dpi respectively) developed robust MERS-CoV specific antibody responses, detected first on 14 dpi with a PRNT titer between 20 and 40, up to a PRNT titer of 640 at 35 dpi (Table 2.1)

Table 2.1. Neutralizing antibody titers in dromedary camels inoculated with MERS-CoV as determined by 90% plaque reduction assay.

Day	Camel 1	Camel 2	Camel 3
0	<10	<10	<10
7	-	<10	<10
14	-	40	20
21	-	80	20
28	-	40	160
35	-	-	640
42	-	-	320

- Indicates that no sera were available.

2.4) Discussion

Epidemiological and surveillance data on the emergence of MERS-CoV strongly point towards a role for dromedary camels as the prime reservoir for zoonotic transmission of MERS-CoV (109, 114, 115, 119, 132, 133, 155-158). To understand the ecology of MERS-CoV in the context of the most likely reservoir host we performed an infection experiment with MERS-CoV in three dromedary camels. The disease observed in these animals was clinically benign, in agreement with the absence of morbidity reported from field surveillance studies performed in the Middle East (119, 133, 156, 158). Upon experimental inoculation, MERS-CoV was predominantly shed from the nasal turbinates of dromedary camels, again in agreement with data on naturally infected dromedary camels in the Middle East, which appear to shed MERS-CoV predominantly from the URT as

well(109-111, 133, 156). Although many coronaviruses circulating in livestock infect the intestinal tract, leading to shedding in feces, MERS-CoV was not detected in either urine or feces of dromedary camels in this study; this is in line with the rarity of detection of MERS-CoV in fecal samples obtained from animals sampled in the field (109, 110). Shedding of MERS-CoV RNA from the experimentally infected dromedary camels could consistently be detected in nasal secretions for several weeks after inoculation, although the detection of infectious virus lasted only for 7 days. Therefor, although MERS-CoV RNA can be detected up to 35 dpi, the actual infectious period of dromedary camels infected with MERS-CoV is relatively short, compared to the detection of viral RNA. Currently, no information is available on the duration of MERS-CoV shedding of dromedary camels from field studies. The relatively short duration of shedding of infectious virus will likely shape the transmission and epidemiological kinetics of MERS-CoV and should be taken into account in the design of intervention strategies. The large quantities of infectious MERS-CoV shed in the nasal secretions by each of the three camels suggest that camel-to-camel transmission can occur readily (Fig. 2.6).

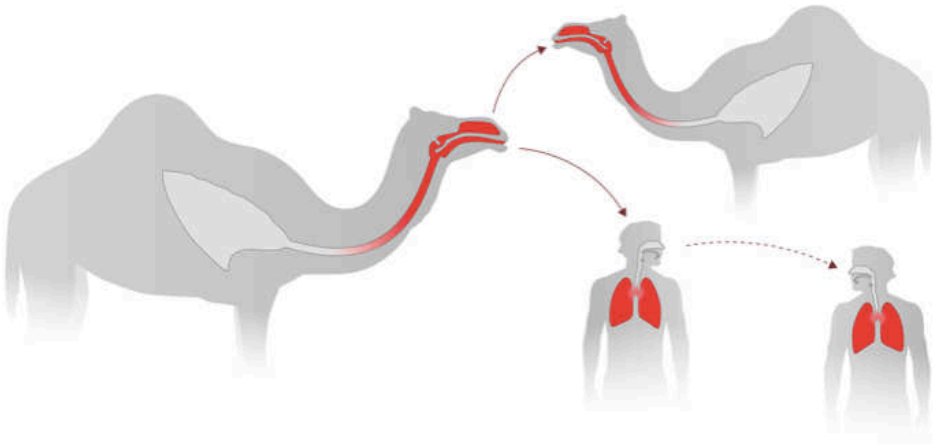

Figure 2.6. Schematic representation of the MERS-CoV tissue tropism in humans and dromedary camels and the potential relationship with transmission and severity of disease. In dromedary camels, MERS-CoV primarily targets the upper respiratory tract, which may explain the relatively benign nature of the infection and efficient camel-to-camel transmission and camel-to-human transmission (solid arrows). In humans, MERS-CoV appears to be confined to the lower respiratory tract, which might explain both the severity and the inefficient human-to-human transmission (dotted arrow). The sites of MERS-CoV replication are indicated in red.

The most likely routes of camel-to-camel transmission for MERS-CoV include direct contact and potentially fomite transmission. In additions, the presence of viral RNA in aerosols generated by the camels also suggests a potential for aerosol transmission. This is in agreement with the recent detection of MERS-CoV viral RNA in air sampled in a camel barn in Saudi Arabia (127). However, in our study (direct sampling of exhaled breathing air) and in the study in Saudi Arabia (sampling of barn air), viable MERS-CoV could not be recovered from the MERS-CoV RNA positive samples. In addition, the hot and dry conditions in the Middle East might not favor efficient aerosol and fomite transmission between camels (162). Additional studies are needed to define the exact route of camel-to-camel transmission. In addition to camel-to-camel transmission, the large quantities of MERS-CoV shed via the URT also makes it highly likely that humans handling infected dromedary camels would readily be exposed to virus (Fig. 2.5) (132, 133).

Histopathological examination revealed that the URT, specifically the respiratory epithelium in the nasal turbinates, is the predominant site of MERS-CoV replication in dromedary camels. The tropism of MERS-CoV for the URT in dromedary camels is in stark contrast to that reported in humans (Fig. 2.6). Although limited post-mortem data from human MERS-CoV cases are currently available, clinical data suggest that virus infection in humans is mostly limited to the LRT (140, 142, 148, 152, 154, 163). The predominant absence of detection of MERS-CoV in human nasal swabs compared to LRT samples such as bronchoalveolar lavages, supports the notion that MERS-CoV in humans is largely a lower respiratory tract infection(154, 164). Experimental MERS-CoV studies utilizing non-human primates as a human proxy identified type I and II pneumocytes, deep in the lower respiratory tract, as the target cells for MERS-CoV replication (165). In addition, efficient replication of MERS-CoV has been demonstrated in alveolar epithelial cells in human *ex vivo* lung tissue (166). The tropism for the lower respiratory tract in humans explains the potential for MERS-CoV to cause severe respiratory disease, although the exact mechanism through which MERS-CoV causes severe respiratory disease is still unknown. The tropism of MERS-CoV for the URT in dromedary camels explains the benign nature of the infection in these animals, similar to the common cold coronaviruses in humans, where replication in the upper respiratory tract causes symptoms such as congestion, nasal discharge and sore throat. In addition, the URT tropism in combination with the high viral load shed provides a possible explanation for the relative efficiency of the zoonotic transmission from camel-to-human, as compared to human-to-human transmission (Fig. 2.5). At 42 dpi neither infectious MERS-CoV nor viral RNA was detected in the respective tissues. This is in agreement with the histopathological and immunohistochemical data, which revealed

lesions and abundant MERS-CoV antigen on 5 dpi, less severe lesions and very limited MERS-CoV antigen on 28 dpi and no lesions and no MERS-CoV antigen on 42 dpi. Since clinical signs and virus shedding were comparable in all three animals, this suggests that MERS-CoV infection was cleared by 42 dpi and that the observed histopathological lesions on 5 and 28 dpi were resolved by then.

Neutralizing antibodies were detected from 14 dpi onwards, reaching a maximum neutralizing titer of 640 after 35 days. Serological studies in dromedary camels reported MERS-CoV neutralizing titers as high as 5120 (114, 156). These high serum titers might indicate that the strong humoral response observed in field studies would take longer than the duration of our experimental infection. An alternative explanation could be that the MERS-CoV specific humoral response in dromedary camels is boosted repeatedly, either by reinfection or continuous exposure. In addition, this would suggest that the humoral immune response to MERS-CoV observed in dromedary camels might not provide complete protection from reinfection of the upper respiratory tract.

Although considerable efforts are currently underway to develop prophylactic and therapeutic countermeasures against MERS-CoV in humans, a potentially promising way to minimize the public health impact of MERS-CoV is to prevent transmission from camel to human. Similarly to Hendra virus in Australia, where horse vaccination is implemented to stop the transmission chain of Hendra virus from fruit bat to horse to human(68), a similar strategy might be achievable for MERS-CoV by vaccinating dromedary camels against MERS-CoV. Vaccination of dromedary camels against MERS-CoV might help to reduce virus shedding and subsequently prevent zoonotic transmission and human infection. However, it will be important to determine whether the humoral and cellular responses elicited by

MERS-CoV vaccines will provide protective immunity against re-infection or, at the very least block or limit shedding of virus to the extent that zoonotic transmission is unlikely.

The data presented here indicate that dromedary camels infected with MERS-CoV develop mild disease associated with nasal shedding of high quantities of virus, and support the available epidemiological data indicating that camels are likely the most important reservoir host for MERS-CoV and can readily transmit the virus to humans.

CHATPTER 3 – INFECTION, REPLICATION, AND TRANSMISSION OF MIDDLE EAST RESPIRATORY SYNDROME CORONAVIRUS IN ALPACAS[2]

3.1) Introduction

The Middle East respiratory syndrome coronavirus (MERS-CoV) was first detected in samples from a Saudi Arabian man who presented with severe respiratory disease in 2012 (1). Since its identification, over 1600 cases have been documented with a case fatality rate of ~36% (14). While efficient human-to-human transmission has been documented, zoonotic spillover likely plays an important role in human infection (25, 130, 132, 133, 167). Dromedary camels were identified early after recognition of the virus as a possible reservoir host for the disease, although not all patients report contact with camels. Numerous investigators have reported the presence of MERS-CoV RNA and/or infectious virus from the nasal swabs of dromedary camels in Saudi Arabia (109-111, 132, 133), Qatar (116, 117, 131, 167), Oman (119), UAE (113), Nigeria (168), Egypt (169). In some areas of the Middle East and Africa nearly 100% of tested animals were serologically positive to MERS-CoV, suggesting widespread circulation among camel populations (109, 114, 170). Interestingly, historical samples demonstrated the presence of MERS-CoV specific antibodies as long ago as 1992, indicating that MERS-CoV has been circulating much longer than originally thought (134, 170). Young animals appear to be at a greater risk for productive infection, and handling practices such as weaning or shipping animals may play an important role in animal-to-animal transmission. Many of the dromedary camels tested

[2] Chapter published as: Adney DR, Bielefeldt-Ohmann H, Hartwig AE, Bowen RA. Infection, Replication, and Transmission of Middle East Respiratory Syndrome Coronavirus in Alpacas. *Emerg Infect Dis.* 2016;6:1031-7.

had very high antibody titers, suggesting that they may have been repeatedly exposed and boosted (114). However, it is currently unknown if these repeated exposures result in productive infection or if antibodies generated from a previous infection are protective.

We have previously demonstrated that dromedary camels can be experimentally infected with MERS-CoV and found that they develop mild upper respiratory tract disease associated with shedding copious amounts of virus via nasal secretions during the first week following inoculation (171). However, due to the cost of dromedaries, their size, and the requirement for specialized facilities to conduct such studies, it would be useful to identify alternative animal models that respond similarly to infection with MERS-CoV. Here we describe characterization of an alpaca model of MERS-CoV infection in which we evaluated virus shedding and pathology, transmission by contact, and protective immunity 10 weeks after initial infection. Infectious virus was detected from nasal swabs of alpacas directly inoculated with virus, although these animals did not display clinical signs of infection. Alpacas co-housed with experimentally inoculated animals seroconverted and two of three animals shed infectious virus in nasal secretions. Finally, animals were allowed to clear the virus and were re-challenged in order to test the hypothesis that a previous infection is protective. The alpacas previously inoculated with MERS-CoV did not shed virus on re-challenge and animals that had previously been infected by co-housing were partially protected. Together, these data indicate that alpacas may be a useful substitute for dromedary camels in certain types of MERS-CoV experiments.

3.2) Materials and Methods

3.2a) Ethics Statement

All animal work was conducted was approved by the Animal Care and Use Committee of Colorado State University, and every effort was made to minimize stress and suffering.

3.2b) Virus and Cells

Animals were infected with a low passage human isolate of MERS-CoV (strain HCoV-EMC/2012) propagated in Vero E6 cells cultured in Dulbecco modified Eagle medium as described previously (171).

3.2c) Animal study

Nine locally bred alpacas were purchased by private sale for use in this study. Animals were allowed to acclimate to the facility for one week prior to infection and were fed hay ad libitum. One day prior to inoculation, animals were subcutaneously injected with an identification and temperature-sensing transponder (Lifechip, Destron Fearing, Dallas-Fortworth Airport, Texas, USA) and their body temperature was monitored throughout the study. Alpacas A1-A3 were housed in together and experimentally inoculated by intranasal instillation of a total of 10^7 plaque forming units (pfu) of MERS-CoV diluted in sterile PBS (3ml per nare). Two days later, alpacas A4-A6 were introduced into the same room as alpacas A1-A3 and housed together for the duration of the study. Nasal swabs were collected by inserting and rotating sterile swabs into both nares, immediately placed in virus transport medium, and frozen until assay. Blood was collected weekly into serum-separating tubes for detection of neutralizing antibodies. Animals A1-A6 were held in the facility for seventy days post-original inoculation and then all six animals were re-

inoculated intranasally as before with 10^7 pfu of MERS-CoV. Three additional alpacas (A7-A9) were also infected to serve as infection controls and evaluate tissue distribution of virus replication. Nasal swabs were collected daily from all animals for five days, at which point animals A7-A9 were humanely euthanized. Tissues collected at necropsy for detection of infectious virus from those three animals included nasal turbinates, trachea, larynx, and all four lung lobes. These samples plus additional samples including brain, kidney, liver, skeletal muscle, heart, spleen, bladder, mesenteric lymph node, submandibular lymph node, and mediastinal lymph node were fixed in formalin for histopathological and IHC evaluation. Nasal swabs and serum were collected from A1-A6 were sampled for two weeks after the second inoculation and then those animals were humanely euthanized.

3.2d) Histopathology and immunohistochemistry

Tissues were fixed in 10% neutral-buffered formalin for >7 days and routine embedded in paraffin. Tissue sections were stained with hematoxylin and eosin and evaluated by a veterinary pathologist (HBO). Immunohistochemistry was preformed to detect MERS-CoV antigen using a rabbit polyclonal antiserum against HCoV-EMC/2012 antigen (1:1000) as a primary antibody as previously described (57).

3.2e) Viral isolation and PRNTs

MERS-CoV was titrated from nasal swabs in virus transport medium and homogenized tissue by plaque assay as described as described previously for camels (171). A 1 ml volume of viral transport medium was considered a 10^{-1}dilution, and 10-fold serial dilutions were prepared in BA1 medium. Neutralizing antibodies were detected by plaque

reduction neutralization test (PRNT) as described previously and seropositive animals were identified using a 90% neutralization cutoff (172).

3.3) Results

3.3a) Clinical signs of MERS-CoV in infected alpacas

Field studies and experimental inoculations suggest that infected camels develop a mild respiratory disease associated with nasal discharge(81, 118, 171). Like dromedaries, none of the alpacas had any appreciable rise in body temperature during challenge or rechallenge (Fig. 3.1A-B). Interestingly, unlike dromedary camels, none of the alpaca had any observable nasal discharge over the course of infection. The alpacas all maintained consistent activity level, temperament, and food intake throughout the study.

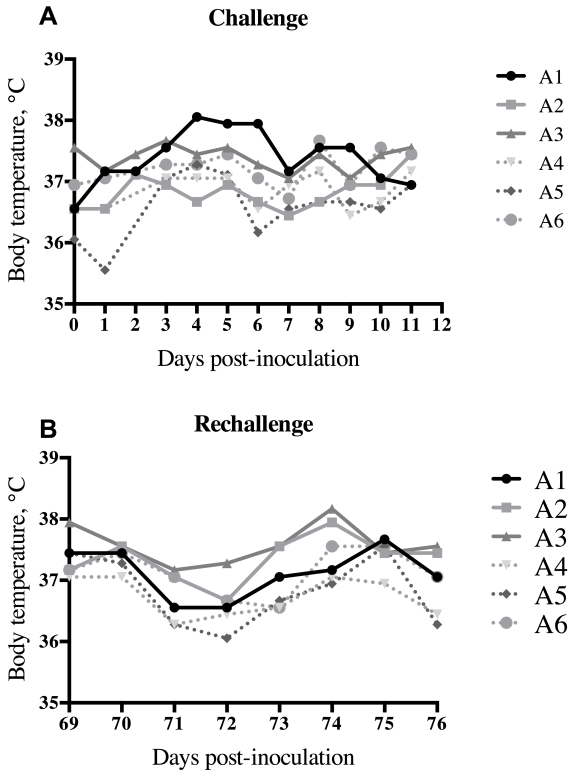

Figure 3.1. Body temperatures following initial challenge (A) and after re-challenge on day 7 (B).

3.3b) Viral shedding

Nasal swabs were collected from inoculated animals immediately prior to challenge, on days one through five post-inoculation, and then again on day ten post-inoculation. All three experimentally-inoculated animals (A1-A3) had detectable infectious virus on days one through five, but had stopped shedding by day ten (Fig. 3.2A-B). The three co-housed animals (A4-A6) were introduced into the room with the inoculated animals two days after the initial virus inoculation. Nasal swabs were collected from the co-housed animals on days three through ten after the inoculation of animals A1-3, and then three times a week through day nineteen. Infectious virus was detected from animal A6 beginning on day seven through day fourteen and from animal A4 on day fourteen only. We did not isolate infectious virus from animal A5 (Fig. 3.2A). Interestingly, while infectious virus was detected in A6 on D7, infectious virus was not detected from A4 until day 14 (Fig. 3.2A). We speculate that A4 became infected via contact with A6 after A1-A3 had cleared infection, suggesting that transmission is linked to intimate animal contact rather than aerosol transmission.

In order to test the hypothesis that previous infection was protective against subsequent viral challenge, all six original study animals (A1-A6) were allowed to clear the infection, then challenged by intranasal inoculation on day 70 post-infection, along with three naïve alpacas (A7-A9) that served as infection controls. All three naïve animals became infected and shed virus from day one to day five post-inoculation at which point they were euthanized. The three animals that became infected through contact (A4-A6) shed minimal virus between days 1-2 post re-infection, but not on days 3-5. In contrast, the

animals that had been experimentally inoculated were completely protected against re-challenge and failed to shed detectable quantities of virus (Fig. 3.2C-D).

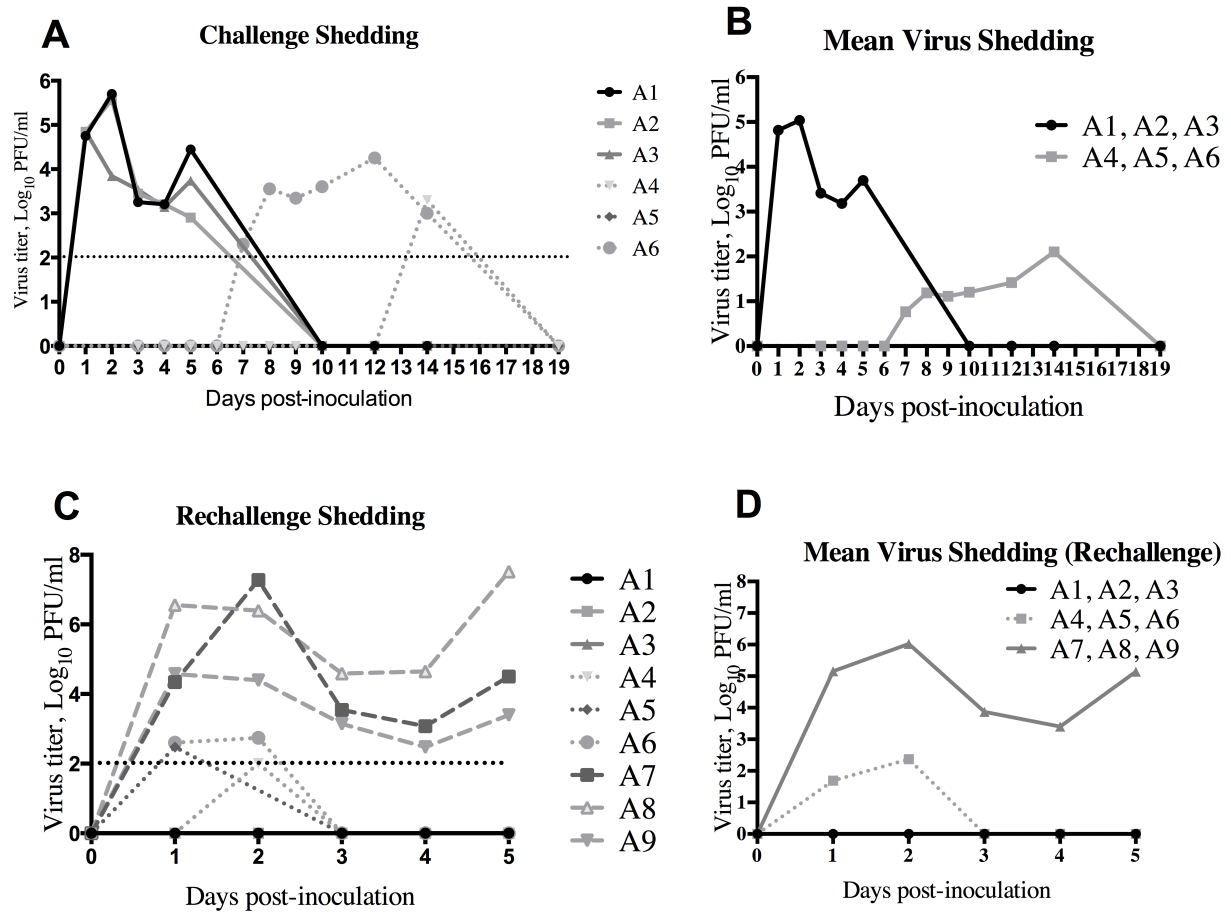

Figure 3.2. Virus shedding (nasal swabs) in alpacas directly inoculated and co-housed with inoculated animals (A, B) and following re-challenge (C,D).Individual animal (A, C) and group means (B, D) are presented. A dashed line indicates the detection limit of the assay.

3.3c) Humoral Response in alpacas infected with MERS-CoV

Serum was collected weekly and tested for the presence of neutralizing antibodies against MERS-CoV. As expected, all three experimentally infected animals (A1-A3) had detectable antibodies beginning on day fourteen (Table 3.1). Notably, although infectious virus was only isolated from two of the three co-housed animals, all three of those animals

had neutralizing antibodies detected first on day twenty one (A5 and A6) or day twenty eight (A4) (Table 3.1).

Table 3.1. Neutralizing antibody titers in alpacas in response to direct inoculation with MERS-CoV (A1-A3) or co-housing with inoculated alpacas (A4-A6), and following re-challenge on day 70. Titers were determined using a 90% cutoff. ‘

	Alpaca					
Day	**A1**	**A2**	**A3**	**A4**	**A5**	**A6**
0	<10	<10	<10	<10	<10	<10
14	40	40	40	<10	<10	<10
21	40	40	40	<10	10	20
28	40	80	80	10	160	20
35	80	160	160	20	80	40
42	160	320	160	20	40	20
49	80	320	80	20	80	80
56	80	640	160	20	80	80
63	80	640	160	40	80	80
70	160	640	80	20	40	80
77	320	640	80	160	320	80
84	320	640	160	320	320	80

3.3d) Organ burden, pathology, and immunohistochemistry

The nasal turbinate, upper trachea, lower trachea, larynx, and all four lung lobes were sampled at necropsy from alpacas A7, A8, and A9 and tested for infectious virus by

plaque assay. Virus was detected in the nasal turbinates, larynx, and trachea of all three alpaca but not in any of the lung lobes tested (Fig. 3.3).

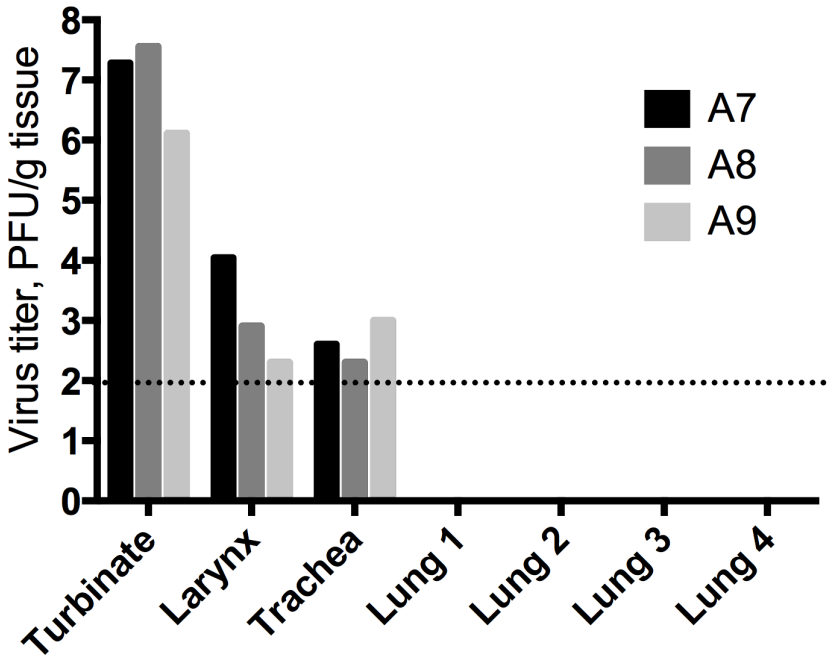

Figure 3.3. Virus titers from tissues collected from naïve animals challenged with MERS-CoV and necropsied on day 5 post-inoculation.

Gross lesions were not observed at necropsy in any of the alpacas, however, microscopy of formaldehyde-fixed tissue sections from animals A7-A9 revealed mild squamous metaplasia of the epithelium of the turbinates in A8 (Fig. 3.4A), as well as rare foci of mucosal erosion accompanied by minimal to mild subepithelial infiltration of neutrophils, macrophages and fewer lymphocytes (Fig. 3.4C). All three animals also had follicular hypertrophy and hyperplasia of the draining lymph nodes, suggestive of immune activation (not shown). By immunohistochemistry, rare, scattered virus-antigen positive cells were detected in the respiratory epithelium of the turbinates (Figure 3.4B) and in rare cells interpreted to be intraepithelial leukocytes. Viral antigen was not detected in any of

the other tissues examined. Interestingly, A7 and A9 had histopathological evidence of mild encephalitis with perivascular infiltrates of lymphocytes and monocytes and mild gliosis (Fig. 3.4D). We did not assay brain for virus, either by isolation or PCR due to the high potential of contamination from the nasal cavity during extraction. Brain tissue was negative by immunohistochemistry, but the etiology of the encephalitis observed remains unknown and may have been unrelated to MERS-CoV infection.

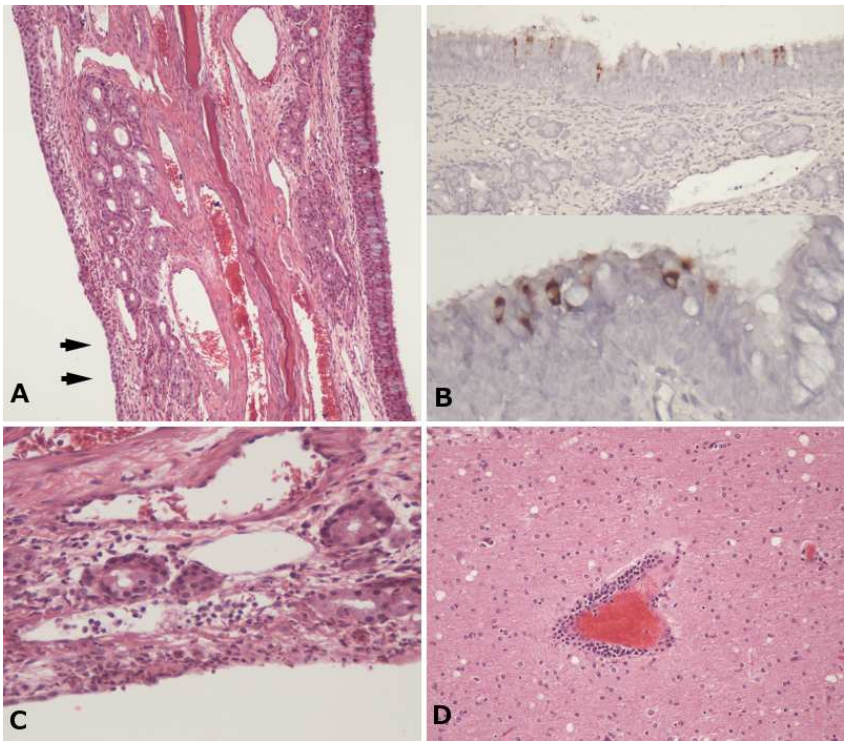

Figure 3.4. Photomicrographs of mild upper respiratory inflammation, encephalitis and virus-antigen detection in respiratory epithelium. (A) Turbinate from alpaca A8 showing normal respiratory epithelium on the right side with goblet cells evident (blue cells), while the epithelium on the left side has undergone squamous metaplasia (arrows) and is focally eroded with mild subepithelial inflammation. (B) MERS-CoV antigen detected in apparently intact respiratory epithelium of alpaca A8 by immunohistochemistry and lack of subepithelial inflammation. (C) An erosion in turbinate epithelium from alpaca A8 revealing leukocytosis in underlying blood vessels. (D) Perivascular infiltration of lymphocytes and monocytes in the brain of alpaca A9.

3.4) Discussion

A variety of difficulties are associated with high containment experiments involving dromedary camels; thus, additional animal models are necessary for MERS-CoV research. Due to their greater availability in the U.S. and smaller size, we decided to pursue an alpaca model. Here, we describe an alpaca model of MERS-CoV infection in camelids, including demonstration of animal-to-animal transmission and reinfection dynamics. Infected alpacas shed considerable quantities of infectious virus nasally, although at lower concentration than has been reported for dromedary camels (81, 171). Additionally, none of the infected alpaca displayed noticeable nasal discharge, which is distinctly different from what has been observed in camels and may explain the relatively low efficiency of contact transmission we observed with alpacas. Infectious virus was detected in nasal swabs from two out of three alpacas co-housed with experimentally infected animals and each of the three co-housed animals developed neutralizing antibodies against MERS-CoV, indicating transmission. The antibody titers observed approximate those seen from inoculated dromedaries with the exception of A4, whose antibodies titers remained low until after rechallenge (171). Finally, experimentally inoculated alpacas were completely protected against subsequent viral challenge, while contact-infected alpaca were only partially protected.

These results suggest that infection can easily spread among closely grouped camelids infected with MERS-CoV. Camels are frequently moved within the Middle East for grazing, as well as for camel shows and races. Such movement allows for the mixing and close mingling of animals, and could play an important role in MERS-CoV transmission among animals and to handlers. In a report by Khalafalla *et al.*, animals bound for slaughter

were held in a livestock market several days, transferred to an abattoir and kept for up to 24 hours before slaughter (118) . Our data suggest that not only could these handling practices promote animal-to-animal transmission, but also that at the time of slaughter, and that virus could potentially be transmitted to slaughterhouse workers.

An important question related to the pathogenesis of MERS-CoV infection in camels, and of great relevance to vaccination strategies, is whether animals that have been previously infected are resistant to reinfection and virus shedding and, if so, for how long. Our experimentally infected animals were completely protected against challenge 70 days later, suggesting that sterilizing immunity can be achieved. However, the infected animals that were infected through contact (A4-A6) shed infectious virus upon reinfection, albeit at much lower levels than the infection control animals (A7-A9). While not tested in the present study, it might be surmised that the three in-contact animals would have acquired sterilizing immunity from the second (booster) infection. These results support field data suggesting that young animals become infected and are likely repeatedly boosted; a majority of older animals have acquired immunity and are not susceptible to infection and virus shedding (173). This also highlights the possibility that widespread vaccination of dromedary camels could significantly decrease viral transmission to humans.

To date neutralizing antibodies against MERS-CoV have not been detected in camelids outside Africa or the Middle East. However, if virus were to be introduced into naïve camelid populations, it likely would be readily transmitted among animals. Many New World camelids are prized for their fiber, and such transmission has the possibility of devastating such industries (174). Thus, as travel-associated cases of MERS-CoV continue

to be documented it is important to monitor for both human-to-human transmission but also possible human-to-animal transmission.

This study had several limitations. Each of the three experimental groups had only three animals, limiting our ability to perform statistical analysis. Additionally, we evaluated protective immunity 10 weeks after the original infection, which is a relatively short time period and does not fully recapitulate "seasonal" exposures; clearly, further studies are necessary to better understand duration of immunity in both camels and alpacas.

CHAPTER 4: EFFICACY OF A MIDDLE EAST RESPIRATORY SYNDROME CORONAVIRUS SPIKE PROTEIN VACCINE IN DROMEDARY CAMELS AND ALPACAS

4.1 Introduction

Since the recognition of the Middle East respiratory syndrome coronavirus (MERS-CoV) in 2012 there have been over 1,500 confirmed human cases, with a case fatality rate of roughly 36% (14). The majority of cases have originated in the Middle East, although some travel associated cases have been documented. Recently, a travel-associated case in South Korea led to a hospital-associated outbreak with 186 confirmed cases, 36 deaths (22). This event clearly indicated the potential for efficient nosocomial human-to-human transmission of MERS-CoV. However, no continuous circulation of MERS-CoV in the human population has been observed and multiple zoonotic spillover spillover events from dromedary camels play an important role in the ongoing outbreak.

Many field studies indicate that an exceptionally large fraction of dromedary camels in the Middle East have antibodies specific to MERS-CoV and that seropositive animals in North and East Africa are common (109, 110, 112, 135, 158, 170, 175, 176). Seropositivity risks of 60-100% suggest extensive circulation of MERS-CoV in the dromedary camel population in the Middle East and Africa (109, 112). Interestingly, historical samples suggest MERS-CoV circulation for greater several decades, suggesting that the emergence of MERS-CoV in dromedary camels was not a recent event (134). Rather than a single introduction into the human population, MERS-CoV has been introduced on multiple occasions into humans (25, 127, 130, 132, 133). Experimentally inoculated camels shed infectious virus in nasal discharge for up to 7 days, associated with mild upper respiratory

tract disease and rapid seroconversion (171). Several studies from endemic regions have indicated that a large fraction of camels become infected as calves and that infection is likely associated with weaning calves and the mixing of animals in an abattoir prior to slaughter (118, 173). Additionally, people that report a high level of contact with camels (e.g. animal handlers and slaughterhouse workers) are more likely to be seropositive than individuals without contact with camels (130, 177).

Two vaccination strategies for dromedary camels have been evaluated. A synthetic DNA vaccine with the MERS-CoV S protein generated from sequences from clade A and B demonstrated strong cellular and antigen-specific neutralizing immunity in C56/BL6 mice and rhesus macaques (178). This vaccine requires electroporation and when administered to dromedary camels neutralizing antibodies were detected in two of three animals tested using a 50% TCID neutralizing cutoff (178). A modified orthopoxvirus-based vaccine expressing the MERS-CoV S protein delivered with a mucosal atomization devise and intramuscularly elicited neutralizing antibodies in four of four vaccinated dromedaries (81). These animals vaccinated, received a boost immunization four weeks after the initial vaccination and challenged after an additional three weeks (81). Upon challenge, vaccinated animals did not display nasal discharge and had significantly lower amounts of detectable viral RNA in nasal swabs (81). Interestingly, one animal had no detectable antibodies in a nasal swab and shed small amounts of infectious virus on day 6 post-infection (81). This study utilized a protein subunit S1-protein vaccine previously demonstrated to elicit neutralizing antibodies in both BALB/CJ mice and Indian rhesus macaques (179).

The high case fatality rate, continuous zoonotic introductions, and large-scale hospital outbreaks underscore the need of intervention strategies focused on preventing zoonotic transmission. A recent report suggested that an orthopoxvirus-based MERS-CoV significantly reduced viral shedding after inoculation in dromedary camels (81). Here we report on the feasibility of an intramuscular, subunit S1-protein vaccine to prevent infection or reduce shedding in dromedary camels (*Camelus dromedarius)* and alpaca (*Vicugna pacs*).

4.2) Materials and Methods

4.2a) Study design

All experiments were approved by the Colorado State University Institutional Animal Care and Use Committee. Five dromedary camels and four alpaca seronegative for MERS-CoV were purchased by private sale for use in this study. All animals were born in the United States and were a variety of ages and sexes. Animals were fed ad libitum, housed in outdoor pens during immunization and moved into an Animal Biosafety Level 3 facility one week prior to challenge to allow for acclimation to the facility. Blood was collected into serum-separating tubes weekly during immunization. Animals were infected in three groups due to facility limitations. Group 1 consisted of vaccinated camels 1-3 (CA1-CA3), group 2 consisted of unvaccinated camels 4 and 5 (CA4 and CA5) and vaccinated alpaca 1 and 2 (A1 and A2), and group 3 consisted of vaccinated camels 1 and 2 (CA1 and CA2) and unvaccinated alpaca 3 and 4 (A3 and A4). Historical samples from an previously infected camel euthanized on day 5 post inoculation (171) were included as an additional control camel (CA6). Animals were sedated with xylazine and then inoculated with a total dose of

10^7 50% tissue culture infective dose (TCID50) of a human isolate of MERS-CoV (strain HCoV-EMC/2012) as described previously. Camels were inoculated with 5ml per nare, and alpaca were inoculated with 3 ml per nare. All animals were evaluated at least once daily for temperature, nasal discharge, activity level, and food consumption. Nasal swabs were collected daily and placed immediately into viral transport medium or virus lysis buffer and then frozen until processing. Animals were sedated on day 5 post-inoculation and humanely euthanized with intravenous pentobarbital. The following tissues were collected for viral isolation and formalin-fixed for immunohistochemistry and histopathology: Lung (crainial lobe), larynx, trachea (upper, middle, lower), heart, liver, spleen, kidney, bladder, mediastinal lymph node, mesenteric lymph node, prescapular lymph node, brain (medulla and olfactory cortex) nasal turbinates, and muscle (shoulder) were collected for viral isolation and qRT-PCR.

4.2b) Vaccine development

DNA expression vectors were produced encoding the S1 portion of the Spike glycoprotein of MERS-CoV England1 strain (strain England1, GenBank ID: AFY13307). Sequences were reverse-translated and codon-optimized for human cell expression as described(179). Proteins were produced by transfecting Expi293 cells with the mammalian expression vector VRC8400 expressing the codon-optimized S1 gene. Protein was purified from transfected cell culture supernatants with HisTrap HP Hiload 16/60 Superdex columns (GE Healthcare, Piscataway, NJ, USA), and stored at -80^{0}C in PBS until use.

4.2c) Immunization and Serology

Vaccinated animals were given 400μg S1 protein combined with 40 mg Advax HCXL adjuvant in two 1 ml intramuscular injections given on each shoulder at weeks 0 and 4 and then given 400μg S1 protein in RIBI adjuvant on week 15. Serum was analyzed for neutralization activity by plaque reduction neutralization test (PRNT) as described previously (171), and by pseudotyped lentivirus reporter assay quantified as the reciprocal serum dilution resulting in 50% reduction of infected cells (IC_{50}).

4.2d) Virus titration

Nasal swabs and tissue samples were titrated by plaque assay (PA) as described previously (171).

4.2e) RNA extraction and qPCR

RNA was extracted from swab samples using the QiaAmp Viral RNA kit (Qiagen). RNA was eluted in 60 μl. Tissues (30 mg) were homogenized in RLT buffer and RNA was extracted using the RNeasy kit (Qiagen). RNA was eluted in 50 μl. For detection of viral RNA in samples, 5 μl RNA was used in a one-step real-time RT-PCR upE assay (ref) using the Rotor-Gene™ probe kit (Qiagen) according to the manufacturer instructions. In each run, standard dilutions of a titered MERS-CoV stock were run in parallel, to calculate $TCID_{50}$ equivalents in the samples.

4.2f) MERS-CoV Spike glycoprotein sequencing.

Total RNA from nasal turbinate tissue samples from dromedary camels and alpaca were extracted using the RNeasy Mini Kit (Qiagen) and cDNAs were synthesized using random hexamers and the High Capacity RNA to cDNA Kit (Thermofisher). cDNA was

subsequently used to PCR-amplify the MERS-CoV S using iProof High-Fidelity DNA Polymerase (Biorad) according to the manufacture's protocol, primer sequences are available upon request. Sequences were assembled on SeqMan Pro (DNASTAR) and analyzed on MegAlign (DNASTAR) by comparison to the MERS-CoV (strain HCoV-EMC/2012) input sequence.

4.2g) Histopathology and immunohistochemistry

Tissues were fixed for >7 days in 10% neutral-buffered formalin and embedded in paraffin. Tissue sections were stained with hematoxylin and eosin (H&E). To detect MERS-CoV antigen, immunohistochemistry was performed using a rabbit polyclonal antiserum against HCoV-EMC/2012 (1:1000) as a primary antibody as described previously (171).

4.3) Results

4.3a) Experimental design

Three dromedary camels (CA1, CA2, CA3) and two alpacas (A1, A2) were vaccinated with an adjuvated S1-protein subunit vaccine, while two additional camels (CA4, CA5) and two alpacas (A3, A4) served as unvaccinated controls. Animals were vaccinated on days 0 and 28 with 400 mg of S1 protein mixed with 40 mg Advax™ HCXL adjuvant (Vaxine Pty Ltd., South Australia, Australia) delivered as two 1 ml intramuscular injections in the shoulders. Because not all animals developed a robust antibody response, all were vaccinated a third time on day 105 with the same quantity of S protein emulsified in Sigma Adjuvant System (S6322, Sigma-Aldrich, St. Louis, MO, USA). Animals were then moved into ABSL3 containment, intranasally challenged on day 130, 131, or 132 with 10^7 $TCID_{50}$ of

MERS-CoV (strain HCoV-EMC/2012). All animals were euthanized and necropsied 5 days post-challenge.

4.3b) MERS-CoV neutralizing titers

Serum from vaccinated animals was collected and tested by plaque reduction neutralization test (PRNT) using homologous virus, as previously described (171) . Dromedary camels 1 and 2 had low titers (40 and 10) on D28, when they were revaccinated with Advax™ HCXL adjuvant. Neutralizing antibodies were not detected from these animals on day 71 or 98, but these animals had titers of 160 and 80 on day 112 after revaccination with S1 protein and Sigma Adjuvant System on day 112. These titers increased to 1280 and 320 by the day of challenge. Neutralizing antibodies were not detected in dromedary camel 3 at any point during the vaccination protocol. In contrast, alpacas 1 and 2 had titers of 640 and 40 n day 28. Alpaca 1's titer decreased to 80 on day 98 but increased to 5210 on day 112 and was 2560 on the day of challenge. Alpaca 2 did not have detectible neutralizing antibodies on days 71 or 98 and increased to 640 on day 112 (Table 4.1).

4.3c) The effect of vaccination on clinical disease upon inoculation of camelids with MERS-CoV

Animals were evaluated daily for clinical signs associated with infection such as nasal discharge. While very minor temperature fluctuations were detected in several of the camels, there was no appreciable fever associated with infection (data not shown). Temperature fluctuation was not associated vaccination status. Minor nasal discharge was detected in both control camels (CA4 and CA5) on day 2, although this resolved within a

few days. Observable nasal discharge was not detected in the vaccinated camels or in any of the alpaca.

Table 4.1. Neutralizing antibody titers in dromedary camels vaccinated against MERS-CoV as determined by 90% plaque reduction assay.

	CA1	CA2	CA3	A1	A2
D0	<10	<10	<10	<10	<10
D14	<10	ND	<10	ND	ND
D21	<10	<10	<10	<10	<10
D28	40	10	<10	640	40
D35	40	10	<10	640	20
D71	<10	<10	<10	320	<10
D98	<10	<10	<10	80	<10
D112	160	80	<10	5210	640
D130-133	1280	320	<10	2560	1280

4.3d) Protective efficacy of MERS-CoV S protein vaccination

In order to specifically test the hypothesis that an adjuvanted S1 protein subunit vaccine could prevent viral shedding we monitored both the vaccinated and unvaccinated animal groups for viral shedding from days D0-D5 post-inoculation (dpi). Infectious virus was detected from both nasal swabs collected from unvaccinated control camels (CA4-6) (Fig. 4.1 A-B). Shedding was first detected in the unvaccinated control camels on day 1

post-inoculation and was detected every day until their euthanasia at 5 dpi (Fig. 4.1 A-B). Despite the presence of neutralizing antibodies in two of the three vaccinated camels, infectious virus was detected in both nasal swabs all vaccinated camels (Fig. 4.1 A-B). On day 1 post-inoculation shedding was only detected in CA1 although infectious virus was isolated from all vaccinated camels on day 2 (Fig. 4.1 A-B). Virus was not detected in the nasal swabs of CA1 on day 3 or 4 but was detected again on day 5 and on days 2-5 from camels CA2 and CA3 (Fig. 4.1 A-B). To exclude the possibility of viral escape isolates from nasal swabs were sequenced on day 1 and day 5. In contrast to the camels, vaccination of alpaca resulted in complete protection against viral challenge. Infectious virus was not detected in any nasal swabs from vaccinated alpaca (A1, A2) or in any of the assayed tissue collected at necropsy (Fig. 4.1a, C-D).

Infectious virus was detected in the nasal turbinates, trachea, and larynx in both unvaccinated control camels, indicating a productive infection. Additionally, small amounts of virus were detected 1 of 4 lung lobes tested in CA5 and the tracheobronchial lymph node and spleen of CA4. Historical samples from a previous infection (CA6) had detectable virus in the nasal turbinate, trachea, larynx, tracheobronchial lymph node, and in 1 of 4 lung lobes tested. No MERS-CoV replication was detected by qPCR in any of the other tested tissues samples (Fig. 4.2b).

All three vaccinated camels had large amounts of virus detected in the nasal turbinates, however less virus was detected in the trachea of CA3 and not in the trachea of CA1. Trachea was not collected from CA2. Virus was also detected in the larynx of CA3, but not in the larynx of CA1 or CA2. Infectious virus was not isolated from the lung, spleen, mediastinal lymph node, (not collected from CA2), or the tracheobronchial lymph node of

CA3 (not collected in CA1 or CA2) from vaccinated camels. Both unvaccinated alpaca had infectious virus present in their nasal turbinates, trachea, and larynx but not in any other tissue tested for virus (Fig. 4.2).

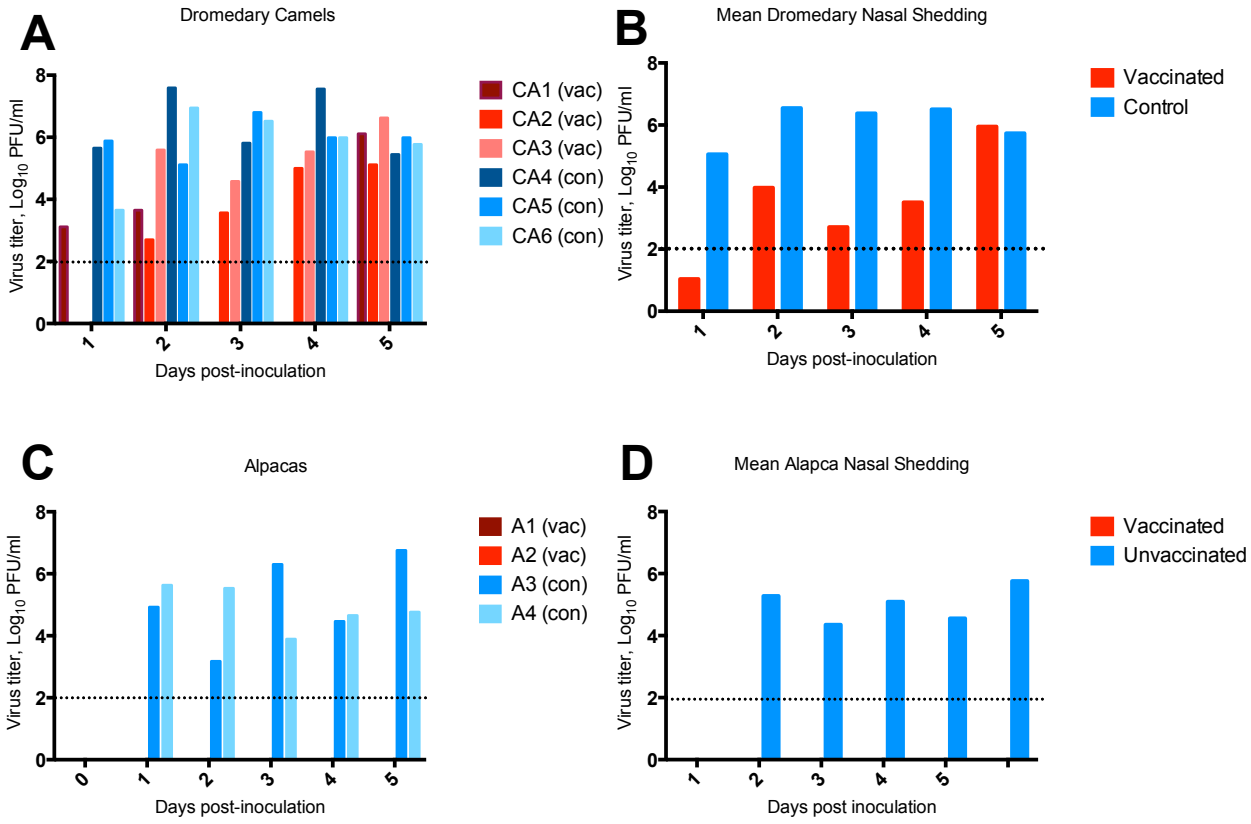

Fig. 4.1. Virus shedding (nasal swabs) in camels (A, B) and alpacas (C, D) vaccinated against MERS-CoV.Individual animal (A, C) and group means (B, D) are presented. A dashed line indicates the detection limit of the assay. Red bars denote vaccinated animals and blue bars indicate control (unvaccinated) animals.

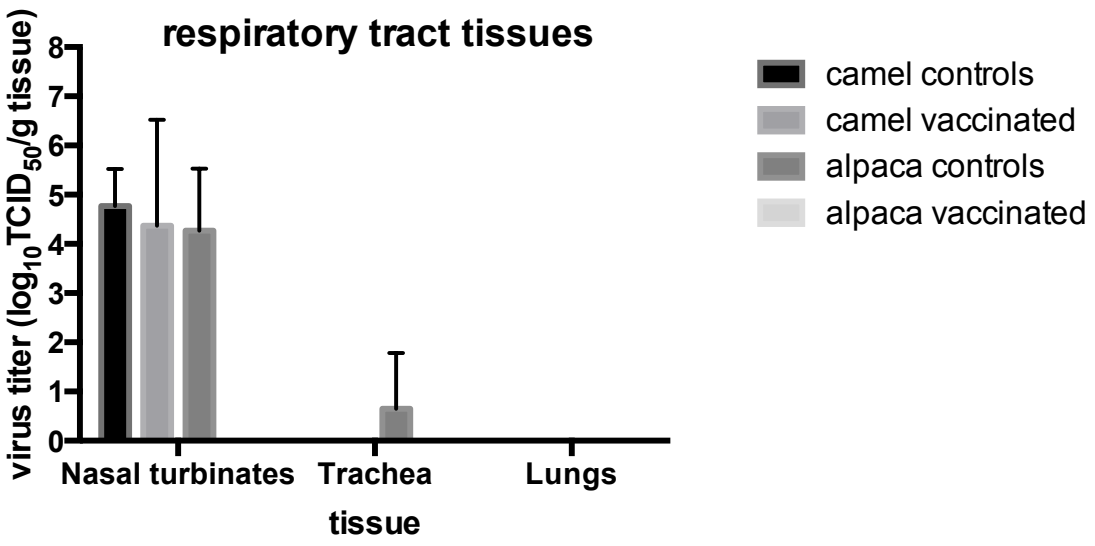

Figure 4.2. Mean viral organ burden of camels and alpacas vaccinated against MERS-CoV on day 5 post-infection.

4.4) Discussion

Although considerable efforts are currently underway to develop prophylactic and therapeutic countermeasures against MERS-CoV in humans, a potential way to minimize the public health impact of MERS-CoV is to prevent transmission from camel to human. Here we describe a proof of principle study that describes the vaccination of camelids to prevent viral shedding upon challenge. Similarly to Hendra virus in Australia, where horse vaccination is implemented to stop the transmission chain of Hendra virus from fruit bat to horse to human (68), such a strategy might be achievable for MERS-CoV by vaccinating dromedary camels against MERS-CoV. Vaccination of dromedary camels against MERS-CoV might help to reduce virus shedding and subsequently prevent zoonotic transmission and human infection.

Some seropositive animals sampled in the field have had detectable RNA in nasal swabs, indicating the possibility of reinfections (110). Nasal swabs from experimentally inoculated camels were positive for RNA for 35 days post-inoculation; thus, the animals sampled in the field could have been recently infected (110, 171). Therefore, degree to which immunity following an initial infection affords protection against reinfection and shedding is currently not known. It is likely that the force of infection is very high among camels in endemic regions and that they receive frequent natural boosters.

For this study we vaccinated three dromedary camels and two alpaca and monitored these animals for viral shedding after an experimental inoculation. Our experimental animals received an initial vaccination and two subsequent boosters and developed a range of antibody titers from <10 to 2560, highlighting the variability observed in non-inbred animals. Immunized alpaca rapidly developed high titers, peaking at 5210 on day 105 before decreasing to 2560 and 640 at the time of challenge. Interestingly, the dromedary camels did not uniformly react to the vaccine. One of our vaccinated animals (CA3) failed to develop any detectable neutralizing antibody after one boost, while all other animals had a detectable humoral response. Because of the difficulties associated with obtaining dromedaries in the United States we chose to change adjuvants for the second boost to maximize the possibility of a achieving a humoral response in CA3. Despite the change in adjuvant CA3 failed to mount a humoral response to the vaccine suggesting that the lack of response was not due to the adjuvant. CA3 was intact ten year-old male housed in a pen adjacent to a mature female, and the bulk of the vaccination schedule occurred while the male was in rut. While it is impossible to know if this affected his response to vaccination, we wonder if lack of antibody response might be correlated

with his reproductive status. The vaccinated camels and alpaca received the same amount of antigen, and so an alternative hypothesis is that dromedary camels require more antigen to mount a response than alpaca.

Upon viral challenge both immunized alpaca were completely protected from viral infection. In contrast, infectious virus was detected in nasal swabs and tissues from all three vaccinated dromedaries. Interestingly, each of the dromedary camels had slightly delayed shedding as compared to the control camels. This response does not appear to be related to neutralizing titer, as the two immunized alpaca developed neutralizing titers of 2560 and 640, while the dromedary camels developed titers of <10, 320 and 1280. One explanation for this difference is that camels and alpaca have different immunologic responses to MERS-CoV vaccination. An alternative explanation is that camels may need a much higher humoral response than alpacas to afford protection. This indicates that further refinement is required, but that a vaccination on camelids could be instrumental in preventing zoonotic viral transmission.

As with many high-containment large animal experiments, our study design had a number of limitations. It was not possible to obtain five camels of a similar age from the same source; thus, our study animals had a variety of ages, sexes, and backgrounds making comparisons between and within groups difficult. We think that some of these differences explain the range of neutralizing titers against the vaccine in dromedary camels. Unlike many traditional laboratory infections, these differences and small sample size make group comparisons inappropriate, and thus we reported descriptive results on an animal level rather than making group comparisons. However, this could allow for a better understanding of how individual animals in the field may react to vaccination. Finally, this

study only explored the humoral response to vaccination and further studies will be required to understand the role of a cell-mediated immune response to MERS-CoV infection.

In summary, we demonstrate that sterilizing immunity can be achieved against MERS-CoV in camelids after vaccination given that a robust immune response is generated. Ideally, a camel vaccine would require a small number of boosts to achieve high neutralizing titers, be easily administered in the field, and prevent or significantly decrease viral shedding. While this specific platform does not meet the requirements of a successful vaccine, we think that this study highlights the potential of a successful camel vaccine.

CHAPTER 5: INOCULATION OF GOATS, SHEEP, AND HORSES WITH MERS-COV DOES NOT RESULT IN PRODUCTIVE VIRAL SHEDDING

5.1) Introduction

The Middle East respiratory syndrome coronavirus (MERS-CoV) is an emerging pathogen first described from Saudi Arabia in 2012 (1) that can cause severe respiratory disease and death in roughly 36% of infected humans (14). There is considerable field and experimental evidence that Dromedary camels serve as an important reservoir host involved in transmission to humans (81, 109, 110, 118, 133, 171), but whether other livestock such as goats, sheep, and horses play a role in transmission has been evaluated only indirectly. Serologic testing of sheep, goats and cattle from Jordan (120) and Saudi Arabia (112) failed to identify animals with neutralizing antibodies to MERS-CoV. Similarly, horses tested in the United Arab Emirates lacked antibodies to MERS-CoV (41). In vitro assays in which replication of MERS-CoV in cultured cells was evaluated have yielded mixed results with respect to species susceptibility. Cells from goats but not sheep or cattle supported replication of MERS-CoV (34) and primary equine kidney cells supported virus replication, albeit at lower levels that with Vero cells (41). Transfection of the DPP4 receptor from goats, sheep and horses into non-permissive mouse or hamster cells allowed replication of MERS-CoV (35, 42). Collectively, these in vitro assays suggest the possibility that some livestock are susceptible to infection, but demonstration of infection in live animals is required to better assess their potential as reservoir hosts. The objective of this study was to determine if goats, sheep, and horses can be infected with MERS-CoV and shed quantities of infectious virus that might be conducive to human infection. Here we report

virologic and serologic results following experimental inoculation of goats, sheep and horses.

5.2. The study

Two goats, three sheep, and four horses were purchased locally. Both of the goats were bred on site and gave birth to either two (Doe A) or three kids (Doe B). All animals were housed in an animal biosafety level-3 facility for the duration of the experiment, were fed a complete pelleted feed supplemented with hay, and were observed at least once daily for nasal discharge, activity level, food consumption, and clinical illness. Sheep, goat kids and horses were each inoculated intranasally with 1.4 to 1.9 x 10^6 pfu of a low passage human isolate of MERS-CoV (strain HCoV-EMC/2012) propagated in Vero E6 cells as described previously(171). The goat kids were maintained at all times in a room with their mothers, who served as an in-contact controls to test for virus transmission. Rectal temperature and nasal swabs were taken daily for seven days. Samples of nasal secretions were collected by inserting and rotating a swab into each nare and were immediately placed in viral transport medium and frozen until plaque assay was performed. Serum was collected immediately prior to inoculation and weekly thereafter until necropsy. Neutralizing antibodies in sera were assayed using a plaque reduction neutralization test (PRNT) with a 90% neutralization cutoff as described previously (171).

One goat kid from each doe was euthanized 5 days post-inoculation (DPI) and the remaining kids and mother goats were euthanized on day 28 post-inoculation. The horses and sheep were monitored for viral shedding and seroconversion, and were euthanized on day 28 post-inoculation, with the exception horse H4, who was euthanized on day 17 due to an injury.

Fevers were not detected in any of the animals (data not shown) and nasal discharge was not observed in the goats or sheep. Horses 1 and 3 showed mild intermittent nasal discharge prior to inoculation and through the experiment, which seemed unrelated to the time of inoculation and was likely not the result of infection. Low levels (1.5-2.5 $\log_{10}$ PFU/ml) of infectious virus were detected in two of the inoculated goat kids (Fig. 1A), but not from either of the adult goats that had intimate contact (Fig. 1). Low levels of virus were detected in nasal swab samples from three of the four inoculated horses 1.5-3 $\log_{10}$ PFU/ml, which could well represent input virus (Fig. 1B). Similarly, a small quantity (<2.5 $\log_{10}$ PFU/ml) of virus was detected in nasal swabs from one of the three sheep (sheep 1) on days 1, 2, 3, and 6 (Fig. 1C). Plaques originating from all of these animals having low titers of virus were confirmed to be MERS-CoV by immunofluorescence.

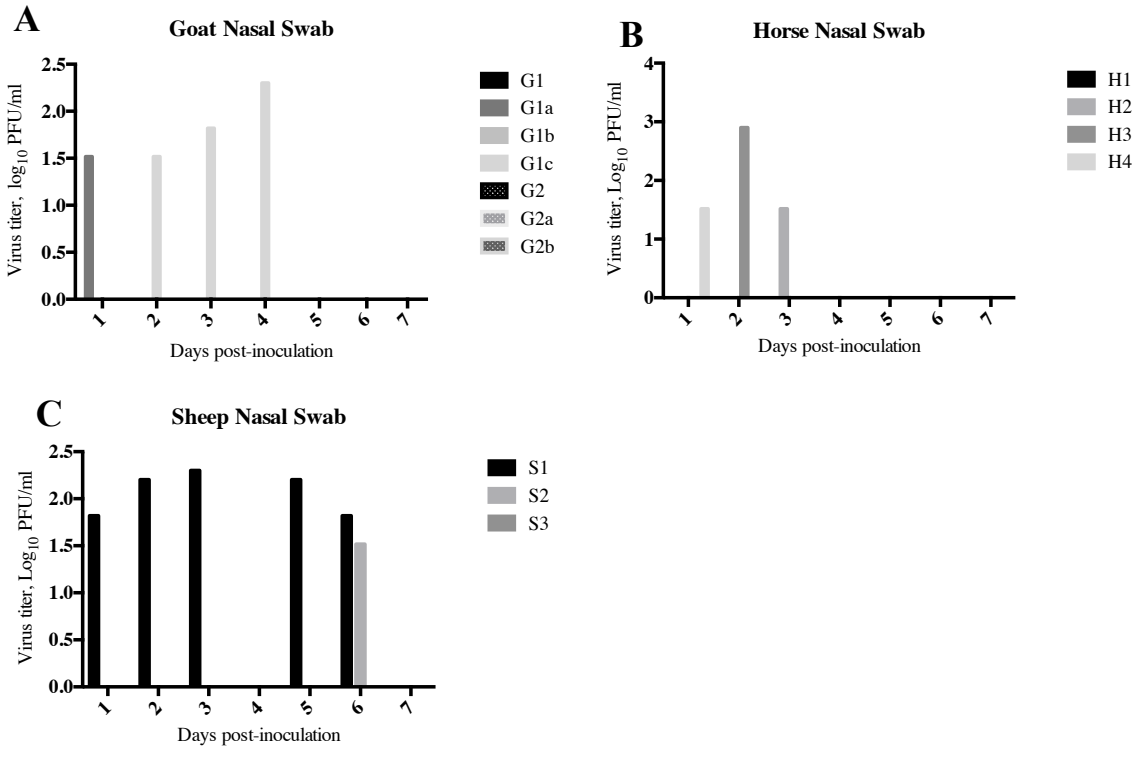

Figure 5.1. Infectious virus (nasal swabs) from goats (A), sheep (B), and horses (C).

Each of three kid goats held past day 5 seroconverted, however, neutralizing antibodies were not detected in either of their mothers (Table 5.1). Sheep 2 developed low titer of neutralizing antibody on day 14 (1:10), but neutralizing antibodies were not detected in either of the other two sheep or any of the four (data not shown).

Table 5.1. Neutralizing antibody titers in goats experimentally infected or exposed by contact to MERS-CoV. Mother goats are indicated as A or B, and their corresponded kids are indicated as Aa, Ab, Ac (Doe A), Ba, or Bb (Doe B). Titers were determined using a 90% cutoff

	A	**Aa**	**Ab**	**Ac**	**B**	**Ba**	**Bb**
D0	<10	<10	<10	<10	<10	<10	<10
D7	<10	40	80	ND	<10	ND	20
D14	<10	20	40	ND	<10	ND	40
D21	<10	20	20	ND	<10	ND	10
D28	<10	20	20	ND	<10	ND	<10

Nasal turbinates and trachea from the two goats euthanized day 5-post inoculation were collected and samples frozen for virus titration or fixed in 10% neutral-buffered formalin for greater than 7 days prior to being embedded in paraffin. Very small but confirmed quantities of virus were isolated from the turbinates of both goats (~100 pfu/gram), which may reflect input virus or very low level virus replication. Tissue sections (hematoxylin/eosin and immunohistochemistry) were prepared and evaluated by a veterinary pathologist (HBO) as previously described (180). Goat kid 1c was histologically unremarkable, however, the turbinates of goat kid 2a had multifocal areas of loss of goblet cells, epithelial necrosis or squamous metaplasia and attenuation and/or erosion of the epithelium, accompanied by mild to moderate neutrophil and monocyte/macrophage infiltration and occasional minimal hemorrhage. Small amounts of cellular debris, leukocytes and mucus (exudate) were present in the nasal cavity, mainly associated with

the aforementioned affected areas. These tissues were negative for viral antigen by IHC and the histopathologic lesions were very likely the result of trauma from daily swabbing rather than due to virus replication.

5.3 Conclusions

The purpose of this study was to determine if sheep, horses, and goats could be important in the transmission of MERS-CoV. These data suggest that goats, sheep, and horses shed very low or no MERS-CoV following experimental inoculation and are not likely to be important in the transmission cycle of MERS-CoV, nor will they be useful animal models for veterinary vaccines targeted to camelids.

CHAPTER 6: CONCLUDING REMARKS

Since its discovery, attempts to control or prevent the transmission of MERS-CoV have presented several unprecedented challenges. Due to cultural beliefs, only one autopsy has been performed. As any animal model for human disease must faithfully recapitulate human disease, associated human pathology must be well understood. This knowledge gap severely limits researches to standardize models and perform critical research. Thus, developing animal models for the reservoir host and working with the actual reservoir host are important.

However, the reservoir host for MERS-CoV presents additional hurdles. Dromedary camels are an integral part of many cultures and economies and so a balance of protecting at-risk handlers and respecting local customs must be maintained. The size, temperament, and cost of dromedary camels make laboratory studies difficult and require specialized large-animal high containment facilities. The overarching goals of this dissertation were to better understand the pathogenesis and shedding of MERS-CoV from dromedary camels, assess the potential of other camelids as a replacement for dromedaries for laboratory infections, demonstrate the application of dromedary and alpaca models for vaccine trials, and determine if other livestock could act as reservoir hosts.

The findings of these studies indicate that when infected dromedary camels develop a mild upper-respiratory tract infection that is associated with the shedding of large quantities of infectious virus via nasal secretions. Infectious virus is readily detected for roughly one week, while viral RNA can be detected for up to 35 days. Thus, special attention should be given to field studies that only evaluate the presence of viral RNA, as

these camels may not be transmitting infectious virus. Additionally, the large amount of infectious virus detected in nasal swabs from infected animals indicates that individuals handling camels are likely to be exposed and at risk for infection.

The studies in this dissertation also suggest that alpacas may be a suitable replacement for dromedary camels in some studies. Like dromedary camels, experimentally infected alpacas shed large amounts of infectious virus; however, these animals do not have any observable nasal discharge. Transmission between alpacas is inefficient, and this may be related to a lack of nasal discharge. Previously infected alpacas are refractory to further infection, supporting the hypothesis that a widespread vaccination program in dromedaries could limit viral transmission.

This dissertation also describes the application of both a camel and alpaca model in a vaccination study. While alpacas developed strong humoral responses that protected animals against challenge, the response detected in dromedaries was varied and not protective against viral challenge. Further studies should further explore the difference between alpacas and camels infected with MERS-CoV, as alpacas may not be good models for testing different some hypotheses.

Finally, despite overwhelming *in vitro* evidence that goats, horses, and sheep are susceptible to infection we were not able to detect meaningful viral replication or transmission of infected animals. These findings support field data reporting no seropositive animals in regions with infected dromedaries. As sheep, goat, and horse DPP4 all bind to the S protein, additional studies investigating host factors will be important to fully elucidate factors contributing to a productive infection.

REFERENCES

1. Zaki A, van Boheemen S, Bestebroer T, Osterhaus A, Fouchier R. Isolation of a novel coronavirus from a man with pneumonia in Saudi Arabia. The New England journal of medicine. 2012;367(19):1814-20.
2. de Groot RJ, Baker SC, Baric RS, Brown CS, Drosten C, Enjuanes L, et al. Middle East respiratory syndrome coronavirus (MERS-CoV): announcement of the Coronavirus Study Group. Journal of virology. 2013 Jul;87(14):7790-2.
3. Woo PC, Lau SK, Huang Y, Yuen KY. Coronavirus diversity, phylogeny and interspecies jumping. Experimental biology and medicine (Maywood, NJ). 2009 Oct;234(10):1117-27.
4. Wertheim JO, Chu DK, Peiris JS, Kosakovsky Pond SL, Poon LL. A case for the ancient origin of coronaviruses. Journal of virology. 2013 Jun;87(12):7039-45.
5. Wang L, Hayes J, Byrum B, Zhang Y. US variant porcine epidemic diarrhea virus: histological lesions and genetic characterization. Virus genes. 2016 Apr 8.
6. Percy DH BS. Pathology of Laboratory Rodents and Rabbits: Third Edition 2012.
7. Woo PC, Lau SK, Lam CS, Lau CC, Tsang AK, Lau JH, et al. Discovery of seven novel Mammalian and avian coronaviruses in the genus deltacoronavirus supports bat coronaviruses as the gene source of alphacoronavirus and betacoronavirus and avian coronaviruses as the gene source of gammacoronavirus and deltacoronavirus. Journal of virology. 2012 Apr;86(7):3995-4008.
8. Akimkin V, Beer M, Blome S, Hanke D, Hoper D, Jenckel M, et al. New Chimeric Porcine Coronavirus in Swine Feces, Germany, 2012. Emerging infectious diseases. 2016 Jul 15;22(7).
9. Fung TS, Liu DX. Coronavirus infection, ER stress, apoptosis and innate immunity. Frontiers in microbiology. 2014;5:296.
10. Perlman S, Netland J. Coronaviruses post-SARS: update on replication and pathogenesis. Nature reviews Microbiology. 2009 Jun;7(6):439-50.
11. Denison MR, Graham RL, Donaldson EF, Eckerle LD, Baric RS. Coronaviruses: an RNA proofreading machine regulates replication fidelity and diversity. RNA biology. 2011 Mar-Apr;8(2):270-9.
12. Zumla A, Hui DS, Perlman S. Middle East respiratory syndrome. Lancet (London, England). 2015 Sep 5;386(9997):995-1007.
13. Chan JF, Lau SK, To KK, Cheng VC, Woo PC, Yuen KY. Middle East respiratory syndrome coronavirus: another zoonotic betacoronavirus causing SARS-like disease. Clinical microbiology reviews. 2015 Apr;28(2):465-522.
14. WHO. Middle East respiratory syndrome coronavirus (MERS-CoV). 2015 [cited; Available from: http://www.who.int/emergencies/mers-cov/en/
15. Assiri A, McGeer A, Perl T, Price C, Al Rabeeah A, Cummings D, et al. Hospital outbreak of Middle East respiratory syndrome coronavirus. The New England journal of medicine. 2013;369(5):407-16.
16. Assiri A, Al-Tawfiq JA, Al-Rabeeah AA, Al-Rabiah FA, Al-Hajjar S, Al-Barrak A, et al. Epidemiological, demographic, and clinical characteristics of 47 cases of Middle East

respiratory syndrome coronavirus disease from Saudi Arabia: a descriptive study. The Lancet Infectious diseases. 2013 Sep;13(9):752-61.
17. Assiri A, Al-Tawfiq J, Al-Rabeeah A, Al-Rabiah F, Al-Hajjar S, Al-Barrak A, et al. Epidemiological, demographic, and clinical characteristics of 47 cases of Middle East respiratory syndrome coronavirus disease from Saudi Arabia: a descriptive study. The Lancet infectious diseases. 2013;13(9):752-61.
18. Al-Abdallat MM, Payne DC, Alqasrawi S, Rha B, Tohme RA, Abedi GR, et al. Hospital-associated outbreak of Middle East respiratory syndrome coronavirus: a serologic, epidemiologic, and clinical description. Clinical infectious diseases : an official publication of the Infectious Diseases Society of America. 2014 Nov;59(9):1225-33.
19. Al-Tawfiq JA, Hinedi K, Ghandour J, Khairalla H, Musleh S, Ujayli A, et al. Middle East respiratory syndrome coronavirus: a case-control study of hospitalized patients. Clinical infectious diseases : an official publication of the Infectious Diseases Society of America. 2014 Jul 15;59(2):160-5.
20. Arabi YM, Arifi AA, Balkhy HH, Najm H, Aldawood AS, Ghabashi A, et al. Clinical course and outcomes of critically ill patients with Middle East respiratory syndrome coronavirus infection. Ann Intern Med. 2014 Mar 18;160(6):389-97.
21. Yang JS, Park S, Kim YJ, Kang HJ, Kim H, Han YW, et al. Middle East Respiratory Syndrome in 3 Persons, South Korea, 2015. Emerging infectious diseases. 2015 Nov;21(11):2084-7.
22. Middle East Respiratory Syndrome Coronavirus Outbreak in the Republic of Korea, 2015. Osong public health and research perspectives. 2015 Aug;6(4):269-78.
23. Bin SY, Heo JY, Song MS, Lee J, Kim EH, Park SJ, et al. Environmental Contamination and Viral Shedding in MERS Patients During MERS-CoV Outbreak in South Korea. Clinical infectious diseases : an official publication of the Infectious Diseases Society of America. 2016 Mar 15;62(6):755-60.
24. Lee SI. Costly Lessons From the 2015 Middle East Respiratory Syndrome Coronavirus Outbreak in Korea. Journal of preventive medicine and public health = Yebang Uihakhoe chi. 2015 Nov;48(6):274-6.
25. Al Hammadi ZM, Chu DK, Eltahir YM, Al Hosani F, Al Mulla M, Tarnini W, et al. Asymptomatic MERS-CoV Infection in Humans Possibly Linked to Infected Dromedaries Imported from Oman to United Arab Emirates, May 2015. Emerging infectious diseases. 2015 Dec;21(12):2197-200.
26. Al-Dorzi HM, Alsolamy S, Arabi YM. Critically ill patients with Middle East respiratory syndrome coronavirus infection. Critical care (London, England). 2016;20(1):65.
27. Sampathkumar P. Middle East respiratory syndrome: what clinicians need to know. Mayo Clinic proceedings. 2014 Aug;89(8):1153-8.
28. Rhee JY, Hong G, Ryu KM. Clinical implications of five cases of Middle East respiratory syndrome coronavirus infection in South Korea Outbreak. Japanese journal of infectious diseases. 2016 Jan 8.
29. Cha RH, Yang SH, Moon KC, Joh JS, Lee JY, Shin HS, et al. A Case Report of a Middle East Respiratory Syndrome Survivor with Kidney Biopsy Results. Journal of Korean medical science. 2016 Apr;31(4):635-40.

30. Malik A, El Masry KM, Ravi M, Sayed F. Middle East Respiratory Syndrome Coronavirus during Pregnancy, Abu Dhabi, United Arab Emirates, 2013. Emerging infectious diseases. 2016 Mar;22(3).
31. Payne DC, Iblan I, Alqasrawi S, Al Nsour M, Rha B, Tohme RA, et al. Stillbirth during infection with Middle East respiratory syndrome coronavirus. The Journal of infectious diseases. 2014 Jun 15;209(12):1870-2.
32. Ng DL, Al Hosani F, Keating MK, Gerber SI, Jones TL, Metcalfe MG, et al. Clinicopathologic, Immunohistochemical, and Ultrastructural Findings of a Fatal Case of Middle East Respiratory Syndrome Coronavirus Infection in the United Arab Emirates, April 2014. The American journal of pathology. 2016 Mar;186(3):652-8.
33. Raj VS, Mou HH, Smits SL, Dekkers DHW, Muller MA, Dijkman R, et al. Dipeptidyl peptidase 4 is a functional receptor for the emerging human coronavirus-EMC. Nature. 2013 Mar 14;495(7440):251-4.
34. Eckerle I, Corman VM, Muller MA, Lenk M, Ulrich RG, Drosten C. Replicative Capacity of MERS Coronavirus in Livestock Cell Lines. Emerging infectious diseases. 2014 Feb;20(2):276-9.
35. van Doremalen N, Miazgowicz KL, Milne-Price S, Bushmaker T, Robertson S, Scott D, et al. Host species restriction of Middle East respiratory syndrome coronavirus through its receptor, dipeptidyl peptidase 4. Journal of virology. 2014 Aug;88(16):9220-32.
36. Fukuma A, Tani H, Taniguchi S, Shimojima M, Saijo M, Fukushi S. Inability of rat DPP4 to allow MERS-CoV infection revealed by using a VSV pseudotype bearing truncated MERS-CoV spike protein. Archives of virology. 2015 Sep;160(9):2293-300.
37. de Wit E, Prescott J, Baseler L, Bushmaker T, Thomas T, Lackemeyer MG, et al. The Middle East respiratory syndrome coronavirus (MERS-CoV) does not replicate in Syrian hamsters. PloS one. 2013;8(7):e69127.
38. Coleman C, Matthews K, Goicochea L, Frieman M. Wild type and innate immune deficient mice are not susceptible to the Middle East Respiratory Syndrome Coronavirus. The Journal of general virology. 2013.
39. Raj VS, Smits SL, Provacia LB, van den Brand JM, Wiersma L, Ouwendijk WJ, et al. Adenosine deaminase acts as a natural antagonist for dipeptidyl peptidase 4-mediated entry of the Middle East respiratory syndrome coronavirus. Journal of virology. 2014 Feb;88(3):1834-8.
40. Iwata-Yoshikawa N, Fukushi S, Fukuma A, Suzuki T, Takeda M, Tashiro M, et al. No susceptibility of neonatal and adult rats against the Middle East respiratory syndrome coronavirus. Japanese journal of infectious diseases. 2016 Mar 18.
41. Meyer B, Garcia-Bocanegra I, Wernery U, Wernery R, Sieberg A, Muller MA, et al. Serologic assessment of possibility for MERS-CoV infection in equids. Emerging infectious diseases. 2015 Jan;21(1):181-2.
42. Barlan A, Zhao J, Sarkar MK, Li K, McCray PB, Jr., Perlman S, et al. Receptor variation and susceptibility to Middle East respiratory syndrome coronavirus infection. Journal of virology. 2014 May;88(9):4953-61.
43. van Doremalen N, Miazgowicz KL, Munster VJ. Mapping the specific amino acid residues to confer hamster DPP4 into a functional receptor for Middle East respiratory syndrome coronavirus. Journal of virology. 2016 Mar 30.

44. Zhao J, Li K, Wohlford-Lenane C, Agnihothram SS, Fett C, Zhao J, et al. Rapid generation of a mouse model for Middle East respiratory syndrome. Proceedings of the National Academy of Sciences of the United States of America. 2014 Apr 1;111(13):4970-5.
45. Zhao J, Perera RA, Kayali G, Meyerholz D, Perlman S, Peiris M. Passive immunotherapy with dromedary immune serum in an experimental animal model for Middle East respiratory syndrome coronavirus infection. Journal of virology. 2015 Jun;89(11):6117-20.
46. Channappanavar R, Lu L, Xia S, Du L, Meyerholz DK, Perlman S, et al. Protective Effect of Intranasal Regimens Containing Peptidic Middle East Respiratory Syndrome Coronavirus Fusion Inhibitor Against MERS-CoV Infection. The Journal of infectious diseases. 2015 Dec 15;212(12):1894-903.
47. Volz A, Kupke A, Song F, Jany S, Fux R, Shams-Eldin H, et al. Protective Efficacy of Recombinant Modified Vaccinia Virus Ankara Delivering Middle East Respiratory Syndrome Coronavirus Spike Glycoprotein. Journal of virology. 2015 Aug;89(16):8651-6.
48. Malczyk AH, Kupke A, Prufer S, Scheuplein VA, Hutzler S, Kreuz D, et al. A Highly Immunogenic and Protective Middle East Respiratory Syndrome Coronavirus Vaccine Based on a Recombinant Measles Virus Vaccine Platform. Journal of virology. 2015 Nov;89(22):11654-67.
49. Zhao G, Jiang Y, Qiu H, Gao T, Zeng Y, Guo Y, et al. Multi-Organ Damage in Human Dipeptidyl Peptidase 4 Transgenic Mice Infected with Middle East Respiratory Syndrome-Coronavirus. PloS one. 2015;10(12):e0145561.
50. Li K, Wohlford-Lenane C, Perlman S, Zhao J, Jewell AK, Reznikov LR, et al. Middle East Respiratory Syndrome Coronavirus Causes Multiple Organ Damage and Lethal Disease in Mice Transgenic for Human Dipeptidyl Peptidase 4. The Journal of infectious diseases. 2016 Mar 1;213(5):712-22.
51. Agrawal AS, Garron T, Tao X, Peng BH, Wakamiya M, Chan TS, et al. Generation of a transgenic mouse model of middle East respiratory syndrome coronavirus infection and disease. Journal of virology. 2015 Apr 1;89(7):3659-70.
52. Tao X, Garron T, Agrawal AS, Algaissi A, Peng BH, Wakamiya M, et al. Characterization and Demonstration of the Value of a Lethal Mouse Model of Middle East Respiratory Syndrome Coronavirus Infection and Disease. Journal of virology. 2015;90(1):57-67.
53. Pascal KE, Coleman CM, Mujica AO, Kamat V, Badithe A, Fairhurst J, et al. Pre- and postexposure efficacy of fully human antibodies against Spike protein in a novel humanized mouse model of MERS-CoV infection. Proceedings of the National Academy of Sciences of the United States of America. 2015 Jul 14;112(28):8738-43.
54. de Wit E, Rasmussen A, Falzarano D, Bushmaker T, Feldmann F, Brining D, et al. Middle East respiratory syndrome coronavirus (MERS-CoV) causes transient lower respiratory tract infection in rhesus macaques. Proceedings of the National Academy of Sciences of the United States of America. 2013;110(41):16598-603.
55. Munster V, de Wit E, Feldmann H. Pneumonia from human coronavirus in a macaque model. The New England journal of medicine. 2013;368(16):1560-2.
56. Yao Y, Bao L, Deng W, Xu L, Li F, Lv Q, et al. An Animal Model of MERS Produced by Infection of Rhesus Macaques With MERS Coronavirus. The Journal of infectious diseases. 2013.

57. Falzarano D, de Wit E, Feldmann F, Rasmussen AL, Okumura A, Peng X, et al. Infection with MERS-CoV causes lethal pneumonia in the common marmoset. PLoS Pathog. 2014 Aug;10(8):e1004250.
58. Johnson RF, Via LE, Kumar MR, Cornish JP, Yellayi S, Huzella L, et al. Intratracheal exposure of common marmosets to MERS-CoV Jordan-n3/2012 or MERS-CoV EMC/2012 isolates does not result in lethal disease. Virology. 2015 Nov;485:422-30.
59. Haagmans BL, van den Brand JM, Provacia LB, Raj VS, Stittelaar KJ, Getu S, et al. Asymptomatic Middle East respiratory syndrome coronavirus infection in rabbits. Journal of virology. 2015 Jun;89(11):6131-5.
60. Falzarano D, de Wit E, Rasmussen AL, Feldmann F, Okumura A, Scott DP, et al. Treatment with interferon-alpha2b and ribavirin improves outcome in MERS-CoV-infected rhesus macaques. Nat Med. 2013 Oct;19(10):1313-7.
61. Hart B, Dyall J, Postnikova E, Zhou H, Kindrachuk J, Johnson R, et al. Interferon-beta and mycophenolic acid are potent inhibitors of Middle East respiratory syndrome coronavirus in cell-based assays. The Journal of general virology. 2013.
62. Omrani AS, Saad MM, Baig K, Bahloul A, Abdul-Matin M, Alaidaroos AY, et al. Ribavirin and interferon alfa-2a for severe Middle East respiratory syndrome coronavirus infection: a retrospective cohort study. The Lancet Infectious diseases. 2014 Nov;14(11):1090-5.
63. Khalid M, Khan B, Al Rabiah F, Alismaili R, Saleemi S, Rehan-Khaliq AM, et al. Middle Eastern Respiratory Syndrome Corona Virus (MERS CoV): case reports from a tertiary care hospital in Saudi Arabia. Annals of Saudi medicine. 2014 Sep-Oct;34(5):396-400.
64. Al-Tawfiq JA, Momattin H, Dib J, Memish ZA. Ribavirin and interferon therapy in patients infected with the Middle East respiratory syndrome coronavirus: an observational study. International journal of infectious diseases : IJID : official publication of the International Society for Infectious Diseases. 2014 Mar;20:42-6.
65. Lau EH, Cowling BJ, Muller MP, Ho LM, Tsang T, Lo SV, et al. Effectiveness of ribavirin and corticosteroids for severe acute respiratory syndrome. The American journal of medicine. 2009 Dec;122(12):1150 e11-21.
66. Adedeji AO, Singh K, Kassim A, Coleman CM, Elliott R, Weiss SR, et al. Evaluation of SSYA10-001 as a replication inhibitor of severe acute respiratory syndrome, mouse hepatitis, and Middle East respiratory syndrome coronaviruses. Antimicrobial agents and chemotherapy. 2014 Aug;58(8):4894-8.
67. Luke T, Wu H, Zhao J, Channappanavar R, Coleman CM, Jiao JA, et al. Human polyclonal immunoglobulin G from transchromosomic bovines inhibits MERS-CoV in vivo. Science translational medicine. 2016 Feb 17;8(326):326ra21.
68. Middleton D, Pallister J, Klein R, Feng YR, Haining J, Arkinstall R, et al. Hendra virus vaccine, a one health approach to protecting horse, human, and environmental health. Emerging infectious diseases. 2014 Mar;20(3):372-9.
69. Mou H, Raj V, van Kuppeveld F, Rottier P, Haagmans B, Bosch B. The receptor binding domain of the new Middle East respiratory syndrome coronavirus maps to a 231-residue region in the spike protein that efficiently elicits neutralizing antibodies. Journal of virology. 2013;87(16):9379-83.
70. Ma C, Li Y, Wang L, Zhao G, Tao X, Tseng CT, et al. Intranasal vaccination with recombinant receptor-binding domain of MERS-CoV spike protein induces much stronger

local mucosal immune responses than subcutaneous immunization: Implication for designing novel mucosal MERS vaccines. Vaccine. 2014 Apr 11;32(18):2100-8.
71. Ma C, Wang L, Tao X, Zhang N, Yang Y, Tseng CT, et al. Searching for an ideal vaccine candidate among different MERS coronavirus receptor-binding fragments--the importance of immunofocusing in subunit vaccine design. Vaccine. 2014 Oct 21;32(46):6170-6.
72. Du L, Zhao G, Kou Z, Ma C, Sun S, Poon V, et al. Identification of a receptor-binding domain in the s protein of the novel human coronavirus middle East respiratory syndrome coronavirus as an essential target for vaccine development. Journal of virology. 2013;87(17):9939-42.
73. Lan J, Yao Y, Deng Y, Chen H, Lu G, Wang W, et al. Recombinant Receptor Binding Domain Protein Induces Partial Protective Immunity in Rhesus Macaques Against Middle East Respiratory Syndrome Coronavirus Challenge. EBioMedicine. 2015 Oct;2(10):1438-46.
74. Yang Y, Deng Y, Wen B, Wang H, Meng X, Lan J, et al. The amino acids 736-761 of the MERS-CoV spike protein induce neutralizing antibodies: implications for the development of vaccines and antiviral agents. Viral immunology. 2014 Dec;27(10):543-50.
75. Jiang S, Lu L, Du L, Debnath AK. A predicted receptor-binding and critical neutralizing domain in S protein of the novel human coronavirus HCoV-EMC. J Infect. 2013 May;66(5):464-6.
76. Du L, Zhao G, Yang Y, Qiu H, Wang L, Kou Z, et al. A conformation-dependent neutralizing monoclonal antibody specifically targeting receptor-binding domain in Middle East respiratory syndrome coronavirus spike protein. Journal of virology. 2014 Jun;88(12):7045-53.
77. Coleman CM, Liu YV, Mu H, Taylor JK, Massare M, Flyer DC, et al. Purified coronavirus spike protein nanoparticles induce coronavirus neutralizing antibodies in mice. Vaccine. 2014 May 30;32(26):3169-74.
78. Scobey T, Yount B, Sims A, Donaldson E, Agnihothram S, Menachery V, et al. Reverse genetics with a full-length infectious cDNA of the Middle East respiratory syndrome coronavirus. Proceedings of the National Academy of Sciences of the United States of America. 2013;110(40):16157-62.
79. Almazán F, Dediego M, Sola I, Zuñiga S, Nieto-Torres J, Marquez-Jurado S, et al. Engineering a replication-competent, propagation-defective middle East respiratory syndrome coronavirus as a vaccine candidate. mBio. 2013;4(5).
80. Song F, Fux R, Provacia L, Volz A, Eickmann M, Becker S, et al. Middle East Respiratory Syndrome Coronavirus Spike Protein Delivered by Modified Vaccinia Virus Ankara Efficiently Induces Virus-Neutralizing Antibodies. Journal of virology. 2013;87(21):11950-4.
81. Haagmans BL, van den Brand JM, Raj VS, Volz A, Wohlsein P, Smits SL, et al. An orthopoxvirus-based vaccine reduces virus excretion after MERS-CoV infection in dromedary camels. Science (New York, NY). 2015 Dec 17.
82. Guo X, Deng Y, Chen H, Lan J, Wang W, Zou X, et al. Systemic and mucosal immunity in mice elicited by a single immunisation with human adenovirus type 5 or 41 vector-based vaccines carrying the spike protein of Middle East respiratory syndrome coronavirus (MERS-CoV). Immunology. 2015 Mar 12.

83. Kim E, Okada K, Kenniston T, Raj VS, AlHajri MM, Farag EA, et al. Immunogenicity of an adenoviral-based Middle East Respiratory Syndrome coronavirus vaccine in BALB/c mice. Vaccine. 2014 Oct 14;32(45):5975-82.
84. Graham RL, Donaldson EF, Baric RS. A decade after SARS: strategies for controlling emerging coronaviruses. Nature reviews Microbiology. 2013 Dec;11(12):836-48.
85. Zhao J, Zhao J, Perlman S. T cell responses are required for protection from clinical disease and for virus clearance in severe acute respiratory syndrome coronavirus-infected mice. Journal of virology. 2010 Sep;84(18):9318-25.
86. Tseng C-T, Sbrana E, Iwata-Yoshikawa N, Newman P, Garron T, Atmar R, et al. Immunization with SARS coronavirus vaccines leads to pulmonary immunopathology on challenge with the SARS virus. PloS one. 2012;7(4).
87. Moratelli R, Calisher CH. Bats and zoonotic viruses: can we confidently link bats with emerging deadly viruses? Memorias do Instituto Oswaldo Cruz. 2015 Feb;110(1):1-22.
88. Calisher CH, Childs JE, Field HE, Holmes KV, Schountz T. Bats: important reservoir hosts of emerging viruses. Clinical microbiology reviews. 2006 Jul;19(3):531-45.
89. Guan Y, Zheng BJ, He YQ, Liu XL, Zhuang ZX, Cheung CL, et al. Isolation and characterization of viruses related to the SARS coronavirus from animals in southern China. Science (New York, NY). 2003 Oct 10;302(5643):276-8.
90. Lau SK, Woo PC, Li KS, Huang Y, Tsoi HW, Wong BH, et al. Severe acute respiratory syndrome coronavirus-like virus in Chinese horseshoe bats. Proceedings of the National Academy of Sciences of the United States of America. 2005 Sep 27;102(39):14040-5.
91. Wang M, Yan M, Xu H, Liang W, Kan B, Zheng B, et al. SARS-CoV Infection in a Restaurant from Palm Civet. Emerging infectious diseases. 2005 Dec;11(12):1860-5.
92. Ithete NL, Stoffberg S, Corman VM, Cottontail VM, Richards LR, Schoeman MC, et al. Close relative of human Middle East respiratory syndrome coronavirus in bat, South Africa. Emerging infectious diseases. 2013 Oct;19(10):1697-9.
93. Wang Q, Qi J, Yuan Y, Xuan Y, Han P, Wan Y, et al. Bat origins of MERS-CoV supported by bat coronavirus HKU4 usage of human receptor CD26. Cell host & microbe. 2014 Sep 10;16(3):328-37.
94. Corman VM, Ithete NL, Richards LR, Schoeman MC, Preiser W, Drosten C, et al. Rooting the phylogenetic tree of middle East respiratory syndrome coronavirus by characterization of a conspecific virus from an African bat. Journal of virology. 2014 Oct;88(19):11297-303.
95. Corman VM, Kallies R, Philipps H, Gopner G, Muller MA, Eckerle I, et al. Characterization of a novel betacoronavirus related to MERS-CoV in European hedgehogs. Journal of virology. 2013 Oct 16.
96. Cai Y, Yu SQ, Postnikova EN, Mazur S, Bernbaum JG, Burk R, et al. CD26/DPP4 cell-surface expression in bat cells correlates with bat cell susceptibility to Middle East respiratory syndrome coronavirus (MERS-CoV) infection and evolution of persistent infection. PloS one. 2014;9(11):e112060.
97. Memish Z, Mishra N, Olival K, Fagbo S, Kapoor V, Epstein J, et al. Middle East respiratory syndrome coronavirus in bats, saudi arabia. Emerging infectious diseases. 2013;19(11).
98. Munster VJ, Adney DR, van Doremalen N, Brown VR, Miazgowicz KL, Milne-Price S, et al. Replication and shedding of MERS-CoV in Jamaican fruit bats (Artibeus jamaicensis). Scientific reports. 2016;6:21878.

99. Hemida MG, Elmoslemany A, Al-Hizab F, Alnaeem A, Almathen F, Faye B, et al. Dromedary Camels and the Transmission of Middle East Respiratory Syndrome Coronavirus (MERS-CoV). Transboundary and emerging diseases. 2015 Aug 10.
100. Yagil R. Camels and camel milk. Animal Production and Health Paper. 1982;26:1-69.
101. Faye B. The camel, new challenges for a sustainable development. Tropical animal health and production. 2016 Apr;48(4):689-92.
102. Yagil R. Camels and camel milk: FAO, Roma (Italia). 1982.
103. Guanche Garcell H, Guilarte Garcia E, Vazquez Pueyo P, Rodriguez Martin I, Villanueva Arias A, Alfonso Serrano RN. Outbreaks of brucellosis related to the consumption of unpasteurized camel milk. Journal of infection and public health. 2016 Jan 12.
104. Albarrak A, Stephens G, Hewson R, Memish Z. Recovery from severe novel coronavirus infection. Saudi medical journal. 2012;33(12):1265-9.
105. Drosten C, Seilmaier M, Corman V, Hartmann W, Scheible G, Sack S, et al. Clinical features and virological analysis of a case of Middle East respiratory syndrome coronavirus infection. The Lancet infectious diseases. 2013;13(9):745-51.
106. Buchholz U, Müller M, Nitsche A, Sanewski A, Wevering N, Bauer-Balci T, et al. Contact investigation of a case of human novel coronavirus infection treated in a German hospital, October-November 2012. Euro surveillance : bulletin Europeen sur les maladies transmissibles = European communicable disease bulletin. 2013;18(8).
107. Reusken C, Haagmans B, Müller M, Gutierrez C, Godeke G-J, Meyer B, et al. Middle East respiratory syndrome coronavirus neutralising serum antibodies in dromedary camels: a comparative serological study. The Lancet infectious diseases. 2013;13(10):859-66.
108. Gutiérrez C T-JM, González M, Lattwein E, Renneker S. Presence of antibodies but no evidence for circulation of MERS-CoV in dromedaries on the Canary Islands, 2015. Euro surveillance : bulletin Europeen sur les maladies transmissibles = European communicable disease bulletin. 2015;20(37):1-4.
109. Alagaili AN, Briese T, Mishra N, Kapoor V, Sameroff SC, de Wit E, et al. Middle East Respiratory Syndrome Coronavirus Infection in Dromedary Camels in Saudi Arabia. Mbio. 2014 Mar-Apr;5(2).
110. Hemida MG, Chu DK, Poon LL, Perera RA, Alhammadi MA, Ng HY, et al. MERS Coronavirus in Dromedary Camel Herd, Saudi Arabia. Emerging infectious diseases. 2014 Jul;20(7).
111. Briese T, Mishra N, Jain K, Zalmout IS, Jabado OJ, Karesh WB, et al. Middle East respiratory syndrome coronavirus quasispecies that include homologues of human isolates revealed through whole-genome analysis and virus cultured from dromedary camels in Saudi Arabia. MBio. 2014;5(3):e01146-14.
112. Hemida M, Perera RA, Wang P, Alhammadi MA, Siu LY, Li M, et al. Middle East Respiratory Syndrome (MERS) coronavirus seroprevalence in domestic livestock in Saudi Arabia, 2010 to 2013. Eurosurveillance 2013;18(50).
113. Yusof MF, Eltahir YM, Serhan WS, Hashem FM, Elsayed EA, Marzoug BA, et al. Prevalence of Middle East respiratory syndrome coronavirus (MERS-CoV) in dromedary camels in Abu Dhabi Emirate, United Arab Emirates. Virus genes. 2015 Jun;50(3):509-13.

114. Meyer B, Muller MA, Corman VM, Reusken CB, Ritz D, Godeke GJ, et al. Antibodies against MERS coronavirus in dromedary camels, United Arab Emirates, 2003 and 2013. Emerging infectious diseases. 2014 Apr;20(4):552-9.
115. Alexandersen S, Kobinger GP, Soule G, Wernery U. Middle East Respiratory Syndrome Coronavirus Antibody Reactors Among Camels in Dubai, United Arab Emirates, in 2005. Transboundary and emerging diseases. 2014 Apr;61(2):105-8.
116. Reusken C, Farag E, Jonges M, Godeke G, El-Sayed A, Pas S, et al. Middle East respiratory syndrome coronavirus (MERS-CoV) RNA and neutralising antibodies in milk collected according to local customs from dromedary camels, Qatar, April 2014. Euro surveillance : bulletin Europeen sur les maladies transmissibles = European communicable disease bulletin. 2014;19(23).
117. Raj VS, Farag EA, Reusken CB, Lamers MM, Pas SD, Voermans J, et al. Isolation of MERS coronavirus from a dromedary camel, Qatar, 2014. Emerging infectious diseases. 2014 Aug;20(8):1339-42.
118. Khalafalla AI, Lu X, Al-Mubarak AI, Dalab AH, Al-Busadah KA, Erdman DD. MERS-CoV in Upper Respiratory Tract and Lungs of Dromedary Camels, Saudi Arabia, 2013-2014. Emerging infectious diseases. 2015 Jul;21(7):1153-8.
119. Nowotny N, Kolodziejek J. Middle East respiratory syndrome coronavirus (MERS-CoV) in dromedary camels, Oman, 2013. Euro surveillance : bulletin Europeen sur les maladies transmissibles = European communicable disease bulletin. 2014;19(16):20781.
120. Reusken C, Ababneh M, Raj VS, Meyer B, Eljarah A, Abutarbush S, et al. Middle East Respiratory Syndrome coronavirus (MERS-CoV) serology in major livestock species in an affected region in Jordan, June to September 2013. Eurosurveillance 2013;18(50).
121. Limited NO. Managing the impacts of feral camel across remote Australia: Final report of the Australian Feral Camel Management Project. 2013.
122. Miguel E, Perera RA, Baubekova A, Chevalier V, Faye B, Akhmetsadykov N, et al. Absence of Middle East Respiratory Syndrome Coronavirus in Camelids, Kazakhstan, 2015. Emerging infectious diseases. 2016 Mar;22(3).
123. Chan SM, Damdinjav B, Perera RA, Chu DK, Khishgee B, Enkhbold B, et al. Absence of MERS-Coronavirus in Bactrian Camels, Southern Mongolia, November 2014. Emerging infectious diseases. 2015 Jul;21(7):1269-71.
124. Shirato K, Azumano A, Nakao T, Hagihara D, Ishida M, Tamai K, et al. Middle East respiratory syndrome coronavirus infection not found in camels in Japan. Japanese journal of infectious diseases. 2015;68(3):256-8.
125. Chan R, Chan M, Agnihothram S, Chan L, Kuok D, Fong J, et al. Tropism of and innate immune responses to the novel human betacoronavirus lineage C virus in human ex vivo respiratory organ cultures. Journal of virology. 2013;87(12):6604-14.
126. van Doremalen N, Bushmaker T, Munster V. Stability of Middle East respiratory syndrome coronavirus (MERS-CoV) under different environmental conditions. Euro surveillance : bulletin Europeen sur les maladies transmissibles = European communicable disease bulletin. 2013;18(38).
127. Azhar EI, Hashem AM, El-Kafrawy SA, Sohrab SS, Aburizaiza AS, Farraj SA, et al. Detection of the Middle East Respiratory Syndrome Coronavirus Genome in an Air Sample Originating from a Camel Barn Owned by an Infected Patient. MBio. 2014;5(4).

128. Hemida MG, Al-Naeem A, Perera RA, Chin AW, Poon LL, Peiris M. Lack of middle East respiratory syndrome coronavirus transmission from infected camels. Emerging infectious diseases. 2015 Apr;21(4):699-701.
129. Memish ZA, Alsahly A, Masri MA, Heil GL, Anderson BD, Peiris M, et al. Sparse evidence of MERS-CoV infection among animal workers living in Southern Saudi Arabia during 2012. Influenza and other respiratory viruses. 2015 Mar;9(2):64-7.
130. Reusken CB, Farag EA, Haagmans BL, Mohran KA, Godeke GJt, Raj S, et al. Occupational Exposure to Dromedaries and Risk for MERS-CoV Infection, Qatar, 2013-2014. Emerging infectious diseases. 2015 Aug;21(8):1422-5.
131. Farag EA, Reusken CB, Haagmans BL, Mohran KA, Stalin Raj V, Pas SD, et al. High proportion of MERS-CoV shedding dromedaries at slaughterhouse with a potential epidemiological link to human cases, Qatar 2014. Infection ecology & epidemiology. 2015;5:28305.
132. Memish ZA, Cotten M, Meyer B, Watson SJ, Alsahafi AJ, Al Rabeeah AA, et al. Human infection with MERS coronavirus after exposure to infected camels, Saudi Arabia, 2013. Emerging infectious diseases. 2014 Jun;20(6):1012-5.
133. Azhar EI, El-Kafrawy SA, Farraj SA, Hassan AM, Al-Saeed MS, Hashem AM, et al. Evidence for camel-to-human transmission of MERS coronavirus. N Engl J Med. 2014 Jun 26;370(26):2499-505.
134. Muller MA, Corman VM, Jores J, Meyer B, Younan M, Liljander A, et al. MERS coronavirus neutralizing antibodies in camels, Eastern Africa, 1983-1997. Emerging infectious diseases. 2014 Dec;20(12):2093-5.
135. Reusken CB, Messadi L, Feyisa A, Ularamu H, Godeke GJ, Danmarwa A, et al. Geographic distribution of MERS coronavirus among dromedary camels, Africa. Emerging infectious diseases. 2014 Aug;20(8):1370-4.
136. Liljander A, Meyer B, Jores J, Muller MA, Lattwein E, Njeru I, et al. MERS-CoV Antibodies in Humans, Africa, 2013-2014. Emerging infectious diseases. 2016 Jun 15;22(6).
137. Alexandersen S, Kobinger GP, Soule G, Wernery U. Middle East respiratory syndrome coronavirus antibody reactors among camels in Dubai, United Arab Emirates, in 2005. Transboundary and emerging diseases. 2014 Apr;61(2):105-8.
138. Perera RA, Wang P, Gomaa MR, El-Shesheny R, Kandeil A, Bagato O, et al. Seroepidemiology for MERS coronavirus using microneutralisation and pseudoparticle virus neutralisation assays reveal a high prevalence of antibody in dromedary camels in Egypt, June 2013. Eurosurveillance. 2013 Sep 5;18(36):8-14.
139. Reusken CB, Schilp C, Raj VS, De Bruin E, Kohl RH, Farag EA, et al. MERS-CoV Infection of Alpaca in a Region Where MERS-CoV is Endemic. Emerging infectious diseases. 2016 Jun 15;22(6).
140. Zaki AM, van Boheemen S, Bestebroer TM, Osterhaus AD, Fouchier RA. Isolation of a novel coronavirus from a man with pneumonia in Saudi Arabia. N Engl J Med. 2012 Nov 8;367(19):1814-20.
141. Response WGaa. Middle East respiratory syndrome coronavirus (MERS-CoV) – update. 2014 [cited; Available from: http://www.who.int/csr/don/2014_06_16_mers/en/
142. Guery B, Poissy J, el Mansouf L, Sejourne C, Ettahar N, Lemaire X, et al. Clinical features and viral diagnosis of two cases of infection with Middle East Respiratory Syndrome coronavirus: a report of nosocomial transmission. Lancet (London, England). 2013 Jun 29;381(9885):2265-72.

143. Omrani AS, Matin MA, Haddad Q, Al-Nakhli D, Memish ZA, Albarrak AM. A family cluster of Middle East Respiratory Syndrome Coronavirus infections related to a likely unrecognized asymptomatic or mild case. International journal of infectious diseases : IJID : official publication of the International Society for Infectious Diseases. 2013 Sep;17(9):e668-72.
144. Memish ZA, Al-Tawfiq JA, Assiri A, Alrabiah FA, Hajjar SA, Albarrak A, et al. Middle East Respiratory Syndrome Coronavirus Disease in Children. Pediatr Infect Dis J. 2014 Apr 23.
145. Al-Tawfiq JA, Assiri A, Memish ZA. Middle East respiratory syndrome novel corona MERS-CoV infection. Epidemiology and outcome update. Saudi Med J. 2013 Oct;34(10):991-4.
146. Milne-Price S, Miazgowicz KL, Munster VJ. The emergence of the Middle East Respiratory Syndrome coronavirus. Pathog Dis. 2014 Jul;71(2):119-34.
147. Puzelli S, Azzi A, Santini MG, Di Martino A, Facchini M, Castrucci MR, et al. Investigation of an imported case of Middle East Respiratory Syndrome Coronavirus (MERS-CoV) infection in Florence, Italy, May to June 2013. Euro surveillance : bulletin Europeen sur les maladies transmissibles = European communicable disease bulletin. 2013;18(34).
148. Assiri A, McGeer A, Perl TM, Price CS, Al Rabeeah AA, Cummings DA, et al. Hospital outbreak of Middle East respiratory syndrome coronavirus. N Engl J Med. 2013 Aug 1;369(5):407-16.
149. Memish ZA, Al-Tawfiq JA, Assiri A. Hospital-associated Middle East respiratory syndrome coronavirus infections. N Engl J Med. 2013 Oct 31;369(18):1761-2.
150. van Boheemen S, de Graaf M, Lauber C, Bestebroer TM, Raj VS, Zaki AM, et al. Genomic Characterization of a Newly Discovered Coronavirus Associated with Acute Respiratory Distress Syndrome in Humans. Mbio. 2012 Nov-Dec;3(6).
151. Memish ZA, Mishra N, Olival KJ, Fagbo SF, Kapoor V, Epstein JH, et al. Middle East respiratory syndrome coronavirus in bats, Saudi Arabia. Emerging infectious diseases. 2013 Nov;19(11):1819-23.
152. Albarrak AM, Stephens GM, Hewson R, Memish ZA. Recovery from severe novel coronavirus infection. Saudi medical journal. 2012 Dec;33(12):1265-9.
153. Buchholz U, Muller MA, Nitsche A, Sanewski A, Wevering N, Bauer-Balci T, et al. Contact investigation of a case of human novel coronavirus infection treated in a German hospital, October-November 2012. Euro surveillance : bulletin Europeen sur les maladies transmissibles = European communicable disease bulletin. 2013;18(8).
154. Drosten C, Seilmaier M, Corman VM, Hartmann W, Scheible G, Sack S, et al. Clinical features and virological analysis of a case of Middle East respiratory syndrome coronavirus infection. Lancet Infectious Diseases. 2013 Sep;13(9):745-51.
155. Reusken CBEM, Haagmans BL, Muller MA, Gutierrez C, Godeke GJ, Meyer B, et al. Middle East respiratory syndrome coronavirus neutralising serum antibodies in dromedary camels: a comparative serological study. Lancet Infectious Diseases. 2013 Oct;13(10):859-66.
156. Haagmans BL, Al Dhahiry SHS, Reusken CBEM, Raj VS, Galiano M, Myers R, et al. Middle East respiratory syndrome coronavirus in dromedary camels: an outbreak investigation. Lancet Infectious Diseases. 2014 Feb;14(2):140-5.

157. Reusken CB, Ababneh M, Raj VS, Meyer B, Eljarah A, Abutarbush S, et al. Middle East Respiratory Syndrome coronavirus (MERS-CoV) serology in major livestock species in an affected region in Jordan, June to September 2013. Euro surveillance : bulletin Europeen sur les maladies transmissibles = European communicable disease bulletin. 2013;18(50):20662.
158. Hemida MG, Perera RA, Wang P, Alhammadi MA, Siu LY, Li M, et al. Middle East Respiratory Syndrome (MERS) coronavirus seroprevalence in domestic livestock in Saudi Arabia, 2010 to 2013. Euro surveillance : bulletin Europeen sur les maladies transmissibles = European communicable disease bulletin. 2013;18(50):20659.
159. Samara EM, Abdoun KA. Concerns about Misinterpretation of Recent Scientific Data Implicating Dromedary Camels in Epidemiology of Middle East Respiratory Syndrome (MERS). MBio. 2014;5(4).
160. Alagaili AN, Briese T, Karesh WB, Daszak P, Lipkin WI. Reply to "Concerns About Misinterpretation of Recent Scientific Data Implicating Dromedary Camels in Epidemiology of Middle East Respiratory Syndrome (MERS)". MBio. 2014;5(4).
161. Corman VM, Muller MA, Costabel U, Timm J, Binger T, Meyer B, et al. Assays for laboratory confirmation of novel human coronavirus (hCoV-EMC) infections. Euro surveillance : bulletin Europeen sur les maladies transmissibles = European communicable disease bulletin. 2012;17(49).
162. van Doremalen N, Bushmaker T, Munster VJ. Stability of Middle East respiratory syndrome coronavirus (MERS-CoV) under different environmental conditions. Euro surveillance : bulletin Europeen sur les maladies transmissibles = European communicable disease bulletin. 2013;18(38).
163. Memish ZA, Zumla AI, Al-Hakeem RF, Al-Rabeeah AA, Stephens GM. Family cluster of Middle East respiratory syndrome coronavirus infections. N Engl J Med. 2013 Jun 27;368(26):2487-94.
164. Memish ZA, Al-Tawfiq JA, Makhdoom HQ, Assiri A, Alhakeem RF, Albarrak A, et al. Respiratory Tract Samples, Viral Load, and Genome Fraction Yield in Patients With Middle East Respiratory Syndrome. The Journal of infectious diseases. 2014 May 15.
165. de Wit E, Rasmussen AL, Falzarano D, Bushmaker T, Feldmann F, Brining DL, et al. Middle East respiratory syndrome coronavirus (MERS-CoV) causes transient lower respiratory tract infection in rhesus macaques. Proceedings of the National Academy of Sciences of the United States of America. 2013 Oct 8;110(41):16598-603.
166. Chan RW, Chan MC, Agnihothram S, Chan LL, Kuok DI, Fong JH, et al. Tropism of and innate immune responses to the novel human betacoronavirus lineage C virus in human ex vivo respiratory organ cultures. Journal of virology. 2013 Jun;87(12):6604-14.
167. Haagmans BL, Al Dhahiry SH, Reusken CB, Raj VS, Galiano M, Myers R, et al. Middle East respiratory syndrome coronavirus in dromedary camels: an outbreak investigation. The Lancet Infectious diseases. 2014 Feb;14(2):140-5.
168. Chu DK, Oladipo JO, Perera RA, Kuranga SA, Chan SM, Poon LL, et al. Middle East respiratory syndrome coronavirus (MERS-CoV) in dromedary camels in Nigeria, 2015. Euro surveillance : bulletin Europeen sur les maladies transmissibles = European communicable disease bulletin. 2015 Dec 10;20(49).
169. Chu DK, Poon LL, Gomaa MM, Shehata MM, Perera RA, Abu Zeid D, et al. MERS coronaviruses in dromedary camels, Egypt. Emerging infectious diseases. 2014 Jun;20(6):1049-53.

170. Corman VM, Jores J, Meyer B, Younan M, Liljander A, Said MY, et al. Antibodies against MERS coronavirus in dromedary camels, Kenya, 1992-2013. Emerging infectious diseases. 2014 Aug;20(8):1319-22.
171. Adney DR, van Doremalen N, Brown VR, Bushmaker T, Scott D, de Wit E, et al. Replication and shedding of MERS-CoV in upper respiratory tract of inoculated dromedary camels. Emerging infectious diseases. 2014 Dec;20(12):1999-2005.
172. Nemeth NM, Bowen RA. Dynamics of passive immunity to West Nile virus in domestic chickens (Gallus gallus domesticus). Am J Trop Med Hyg. 2007 Feb;76(2):310-7.
173. Wernery U, Corman VM, Wong EY, Tsang AK, Muth D, Lau SK, et al. Acute middle East respiratory syndrome coronavirus infection in livestock Dromedaries, Dubai, 2014. Emerging infectious diseases. 2015 Jun;21(6):1019-22.
174. Flowler ME. Medicine and Surgery of Camelids: Third Edition Third ed. Ames, Iowa: Wiley-Blackwell 2010.
175. Hemida MG, Perera RA, Al Jassim RA, Kayali G, Siu LY, Wang P, et al. Seroepidemiology of Middle East respiratory syndrome (MERS) coronavirus in Saudi Arabia (1993) and Australia (2014) and characterisation of assay specificity. Euro surveillance : bulletin Europeen sur les maladies transmissibles = European communicable disease bulletin. 2014;19(23).
176. Perera R, Wang P, Gomaa M, El-Shesheny R, Kandeil A, Bagato O, et al. Seroepidemiology for MERS coronavirus using microneutralisation and pseudoparticle virus neutralisation assays reveal a high prevalence of antibody in dromedary camels in Egypt, June 2013. Euro surveillance : bulletin Europeen sur les maladies transmissibles = European communicable disease bulletin. 2013;18(36).
177. Muller MA, Meyer B, Corman VM, Al-Masri M, Turkestani A, Ritz D, et al. Presence of Middle East respiratory syndrome coronavirus antibodies in Saudi Arabia: a nationwide, cross-sectional, serological study. The Lancet Infectious diseases. 2015 Apr 8.
178. Muthumani K, Falzarano D, Reuschel EL, Tingey C, Flingai S, Villarreal DO, et al. A synthetic consensus anti-spike protein DNA vaccine induces protective immunity against Middle East respiratory syndrome coronavirus in nonhuman primates. Science translational medicine. 2015 Aug 19;7(301):301ra132.
179. Wang L, Shi W, Joyce MG, Modjarrad K, Zhang Y, Leung K, et al. Evaluation of candidate vaccine approaches for MERS-CoV. Nature communications. 2015;6:7712.
180. Adney DR B-OH, Hartwig AE, Bowen RA. Infection, replication, and transmission of Middle East respiratory syndrome coronavirus in alpacas Emerging infectious diseases. 2016.