Small Particle Aerosol Exposure of African Green Monkeys to MERS-CoV as a Model for Highly Pathogenic Coronavirus Infection

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Research Article

Keywords: coronavirus, CoV, Middle East respiratory syndrome, MERS, MERS-CoV, primate model, African green monkey, respiratory, aerosol, infectious disease, animal model, medical countermeasure, small particle aerosol

Posted Date: March 19th, 2020

DOI: <https://doi.org/10.21203/rs.3.rs-17952/v1>

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Version of Record: A version of this preprint was published at Emerging Infectious Diseases on December 1st, 2020. See the published version at <https://doi.org/10.3201/eid2612.201664>.

Abstract

Emerging highly pathogenic coronaviruses (CoV) are a global public health threat due to the potential for person-to-person transmission and higher mortality rates than common seasonal respiratory pathogens. Middle East respiratory syndrome coronavirus (MERS-CoV) emerged in 2012, causing lethal respiratory disease in approximately 35% of human cases. Primate models of highly pathogenic coronavirus infection are needed to support development of therapeutics or vaccines, but few models exist that recapitulate severe disease signs. For initial development of a MERS-CoV primate model, twelve African green monkeys (AGMs) were exposed to 103, 104, or 105 PFU target doses of aerosolized MERS-CoV. We observed a dose-dependent increase of respiratory disease signs and viral titers in serum and throat swabs between the 103 PFU and the 105 PFU dose groups, although all AGMs survived for the 28 day duration of the study. This study is the first to describe dose-dependent effects of highly pathogenic coronavirus infection of primates and uses a route of infection (small particle aerosol) with potential relevance to MERS-CoV transmission in humans. Aerosol exposure of AGMs may provide a platform for the development of primate models of novel coronavirus disease, with potential utility in therapeutic development and viral pathogenesis studies.

Introduction

Since 2002, three novel coronaviruses (nCoVs) have emerged into human populations causing severe respiratory disease: severe acute respiratory syndrome coronavirus (SARS-CoV, 2002–2004), Middle East respiratory syndrome coronavirus (MERS-CoV, 2012-present) and most recently a coronavirus disease in 2019 (COVID–19, SARS-CoV–2, 2019-present)^{1–3}. All three of these highly pathogenic coronaviruses have the capacity to cause lethal respiratory disease characterized by acute atypical pneumonia. Subclinical or asymptomatic infection has been reported for both MERS-CoV and SARS-CoV–2, but the actual number of asymptomatic infections and the pathogenesis of mild cases are not well understood^{4,5}. Onset of clinical disease from highly pathogenic coronaviruses typically follows an incubation period of 2–14 days, beginning as mild and nonspecific influenza-like illness including fever, fatigue, rhinorrhea, or dry cough. Many patients progress to symptoms of dyspnea and atypical pneumonia, often requiring hospitalization or supportive medical intervention including ventilation.

Severe cases of highly pathogenic coronavirus infections require intensive care, and clinically present as Acute Respiratory Distress Syndrome (ARDS), the most severe form of acute lung injury (Figure 1)⁶. Patients that succumb to ARDS from coronavirus infection have fatal conditions such as respiratory failure, end-stage lung disease, diffuse alveolar damage or multi-organ dysfunction syndrome (MODS). While the case fatality rate of COVID–19 is currently 2.3% (outcomes are not yet known for the majority of patients), SARS was fatal in approximately 10% of confirmed cases and MERS is fatal in approximately 35% of confirmed cases^{7–9}. In addition, risk factors for more severe coronavirus disease and mortality have been identified in patients over the age of 65 or possessing comorbidities such as

heart disease, hypertension, renal disease, and diabetes^{10,11}. Survivors of severe coronavirus disease may suffer from long-term lung dysfunction following recovery from infection^{12–14}.

Coronaviridae is a family of positive-sense, single-stranded RNA genome enveloped viruses that includes the genera alphacoronavirus, betacoronavirus, gammacoronavirus, and deltacoronavirus. While all of the coronavirus genera contain viruses that infect animals, only the alphacoronavirus and betacoronavirus contain viruses known to infect humans. The known human coronaviruses include OC43, 229E, NL63, and HKU1 which produce only mild common cold type illness seasonally, as well as highly pathogenic viruses SARS-CoV, MERS-CoV, and SARS-CoV-2¹⁵. Highly pathogenic coronaviruses including SARS-CoV, MERS-CoV, and SARS-CoV-2 (all betacoronaviruses) likely emerged from bats, which are a diverse reservoir of both alphacoronaviruses and betacoronaviruses^{16–19}. Cross-species transmission of MERS-CoV or similar zoonotic precursor viruses from bats to camels established an intermediate reservoir of MERS-CoV in dromedary camels²⁰. MERS-CoV replicates in the upper respiratory tract of camels, but camels demonstrate only mild disease signs, and a high percentage of camels are seropositive for antibodies against MERS-CoV^{21,22}.

The MERS-CoV enzootic cycle within dromedary camels likely facilitates continued emergence in humans, where animal workers and healthcare workers are at risk of occupational exposure to MERS-CoV transmission^{23,24}. Sporadic MERS cases on the Arabian Peninsula continue to seed outbreaks primarily in Saudi Arabia with the potential for exported MERS cases by travelers to other regions. A major outbreak of MERS occurred in 2015 in South Korea, where a single traveler case returning from Saudi Arabia resulted in 186 cases with an additional 16,000 contacts traced to prevent viral spread²⁵. Outbreaks of MERS-CoV since 2012 have resulted in more than 2500 cases of MERS, while 8096 cases were identified in the SARS epidemic and more than 80,000 cases of COVID-19 have been confirmed as of February 27, 2020^{8,26,27}. Transmission of highly pathogenic coronaviruses is likely complex and thus difficult to characterize. MERS-CoV in humans is thought to result from direct and indirect exposure to infected camels, consumption of contaminated camel products, or close contact with infected MERS patients. It is likely that respiratory droplets facilitate transmission of highly pathogenic coronaviruses, including MERS-CoV²⁸. However, unlike with SARS-CoV and SARS-CoV-2, MERS-CoV person-to-person transmission is somewhat limited and not often observed outside of households or healthcare settings. Within healthcare settings, aerosol generating procedures are associated with increased risk of transmission of coronaviruses from infected patient to healthcare workers^{29,30}.

No proven antiviral therapies or vaccines exist for highly pathogenic coronaviruses. Current treatment regimens for MERS include supportive care and administration of general antivirals. However, most medical countermeasures for MERS lack conclusive anti-coronavirus activity supported by robust *in vitro* and *in vivo* models of MERS-CoV infection. Although the ability to test therapeutic targets in primary lung cell models and small animal models of MERS-CoV has been developed, current nonhuman primate (NHP) models of MERS are not adequate for testing of antivirals or vaccines targeting MERS-CoV. NHP models of SARS-CoV were initially pursued but were never characterized to the extent necessary to

support therapeutic evaluation^{31–34}. In particular, platform approaches to developing animal models of highly pathogenic coronavirus infection have significant value in that they could be rapidly applied to novel emerging viruses where medical countermeasures are needed.

Prior development of NHP models of MERS-CoV has been reported in the common marmoset (*Callithrix jacchus*) model and rhesus macaque (*Macaca mulatta*) model. Rhesus macaques experienced only mild, transient respiratory symptoms when infected with 10^6 – 10^8 PFU of MERS-CoV by either intratracheal route (IT) or multiple route (MR: IT, intranasal, oral, and ocular concurrently)^{35,36}. Common marmosets developed more severe MERS disease signs using similar doses and routes of exposure, but discrepancies have been reported in the marmoset model dependent on route of infection^{37–39}. In rhesus macaque and marmoset models of MERS-CoV infection endpoints for therapeutic testing are not well-defined. Lack of robust primate models that replicate severe MERS disease observed in humans is a major obstacle to evaluation of medical countermeasure against MERS-CoV. Therefore, in this study we hypothesized that exposing African green monkeys (*Chlorocebus aethiops*, AGM) to aerosolized MERS-CoV would result in an NHP model that recapitulates severe MERS disease signs to establish a platform that is useful for medical countermeasure development.

Methods

Animal Study Design

Twelve healthy, adult AGMs of both sexes (M = 6, F = 6) were exposed to low (10^3 PFU, n = 4), medium (10^4 PFU, n = 4) and high (10^5 PFU, n = 4) target doses of aerosolized MERS-CoV strain EMC/2012. Animals were experimentally-naïve and were in good physical condition, lacked physical malformations and were free of clinical signs of infectious, contagious, or communicable diseases or parasites. All animals exposed to MERS-CoV were handled in a Biosafety Level 3 containment laboratory at USAMRIID and were examined and evaluated twice per day by study personnel. Early endpoint criteria, as specified by the score parameters within the “Animal observations” section of these methods, were used to determine when animals should be humanely euthanized.

Ethics Statement

All AGMs were obtained from USAMRIID approved commercial vendors. Research was conducted in compliance with the Animal Welfare Act and other federal statutes and regulations relating to animals and experiments involving animals, and adhered principles stated in the Guide for the Care and Use of Laboratory Animals, National Research Council, 2011. The facility where this research was conducted (USAMRIID) is fully accredited by the Association for the Assessment and Accreditation of Laboratory Animal Care International. Research was conducted under a protocol approved by the Institutional Animal Care and Use Committee (IACUC) at USAMRIID.

Virus and Cells

MERS-CoV was obtained through BEI Resources, NIAID, NIH: Middle East Respiratory Syndrome Coronavirus, EMC/2012, #NR-44260. MERS-CoV was amplified on Vero E6 cells, supernatants from infected cells were collected and clarified by centrifugation. A passage 9 stock was used for aerosol exposures of primates. Plaque assay in Vero (CCL-81) cells was used to titrate the amount of virus in samples as has been previously described⁴⁰. Neutral red was used to visualize plaques at 2–3 days after inoculation.

Aerosol Exposures

Each AGM was anesthetized by intramuscular (i.m.) injection of ketamine (8–12 mg/kg) and challenged by aerosol as previously described⁴¹. Briefly, the respiratory function of each of the NHPs was measured using whole-body plethysmography (Buxco Systems, Sharon, CT) before aerosol challenge. Aerosol procedures were conducted using a 16-liter, airtight Lexan chamber assembled in a head-only configuration for individual NHP exposures in a class III biological safety cabinet located inside a BSL-3 suite. All airflows, environmental monitoring, and system balancing were configured, controlled, and recorded through the institute standard automated bioaerosol exposure system (ABES). Integrated air samples were obtained for each individual exposure using an all-glass impinger (AGI) drawing from a port centered on the chamber and opposite to the animal's head. Aerosols were generated with a three-jet Collison nebulizer running at 7.5 liter/min. The amounts of live virus in AGI samples were analyzed on the same day as the aerosol exposure by performing a plaque assay to determine the plaque forming units (PFU)/ml. Actual doses were calculated by incorporating plethysmography data, and viral titration of AGIs and starting material (plaque assays completed in triplicate).

Animal Observation and Endpoint Criteria

AGMs were observed at least twice a day after aerosol exposure and scored for clinical signs of disease prior to, and while under anesthesia. The scoring parameters were: Responsiveness: 0 = active, 1 = decreased activity; 2 = mildly unresponsive (becomes active when approached), occasional prostration; 3 = moderate unresponsiveness (may require prodding to respond), weakness; 4 = moderate to severe unresponsiveness requires prodding, moderate prostration; 5 = moribund, severe unresponsiveness, pronounced prostration; Respiration: 0 = normal breathing; 1 = mildly labored; 2 = moderately labored; 3 = agonal breathing. Any observable disease signs including dehydration, lymphadenopathy, and respiratory signs were recorded during physical examinations. The early endpoint criteria for humane euthanasia were > 4 responsiveness score, $\geq 4^{\circ}\text{C}$ decrease from baseline body temperature without anesthesia, and agonal breathing.

Telemetry

AGMs were implanted subcutaneously with a radiotelemetry device [Data Sciences International (DSI), St. Paul, MN] at least 14 days before aerosol exposure. Body temperatures were recorded every 15 minutes by the DataQuest A. R. T. 4.1 system (DSI). Fever was pre-defined as two standard deviations above individual baseline temperature as determined by ARIMA modeling. Baselines for each animal were calculated by averaging the recorded 15 minute temperature intervals for three days prior to challenge.

Sample Collection

Blood samples and throat swabs were collected at indicated days following aerosol exposure to MERS-CoV (Figure 2). Blood samples were collected from the femoral vein of AGMs anesthetized with 3 mg/kg intramuscular Ketamine. Samples collected seven days prior to exposure served as a reference baseline for each animal. Blood chemistry values were analyzed with VITROS 250 chemistry analyzers.

Necropsy and Histology

At the conclusion of this study (Day 28 post infection), each AGM was euthanized with an overdose of pentobarbital and then submitted for necropsy. The necropsies were performed under BSL-3 biocontainment by a pathologist certified by the American College of Veterinary Pathologists. During the necropsies, samples of the following organs were collected from each monkey for histologic evaluation: liver, gallbladder, spleen, both kidneys, and thoracic pluck (consisting of lungs, trachea, aorta, esophagus, thymus, mediastinal lymph nodes, and tracheobronchial lymph nodes). The samples for histology were fixed by immersion in containers containing 10% neutral-buffered formalin. These containers were held for at least 30 days under BSL-3 biocontainment before being transferred to the USAMRIID histology laboratory. The formalin-fixed tissues were then trimmed, processed, and embedded in paraffin according to standard operating procedures. The paraffin-embedded tissues were cut into sections 5 μ thick which were placed onto glass microscope slides, stained with hematoxylin and eosin, and coverslipped prior to histologic evaluation by the study pathologist.

Results

Clinical Disease Signs Observed in AGMs Exposed to Aerosolized MERS-CoV

To enable comparison of aerosol exposure to previously published NHP model development studies, AGMs were exposed to aerosolized MERS-CoV strain EMC/2012, which is the same strain used previously by other research groups^{35,37,38}. The expected range of doses was 10^3 PFU– 10^5 PFU. The actual range of the infection was $10^{2.88}$ – $10^{4.57}$ PFU (Table 1). All of the AGMs survived the MERS-CoV exposure and subsequent manipulations to the conclusion of the study at 28 days postinfection (Figure 2). Notably, the highest dose group of AGMs exposed to aerosolized MERS-CoV in this study were estimated to have

received considerably lower doses than in prior published NHP models using MR ($5-7 \times 10^6$ TCID₅₀) or IT only ($5 \times 10^6-5 \times 10^7$ PFU) routes of infection³⁵⁻³⁸.

To determine if exposure of AGMs to aerosolized MERS-CoV results in observable disease signs that recapitulate MERS disease observed in human cases, physical observations of AGMs were performed including calculation of respiratory rates (Figure 3A) and clinical scores (Figure 3B). Clinical scores included observation of disease signs including responsiveness, lymphadenopathy, dehydration signs, and respiratory signs by physical examination. Clinical disease signs that recapitulated human cases of MERS were observed in all groups, but were most pronounced in the group that received the highest dose of MERS-CoV (Figure 3B). All groups had increased clinical scores over the course of infection compared to pre-infection clinical scoring. No statistically significant changes in weight of AGMs were observed over the course of the study or between groups (data not shown). The 10^5 PFU group had significantly higher respiratory rates than the 10^3 PFU group, beginning at 3 days post-infection (dpi), and continuing through 10 dpi (Figure 3A). Respiratory rates were not significantly different between the 10^4 PFU dose group and 10^3 PFU dose group. Significantly higher clinical scores were observed in the 10^5 PFU group of NHPs than the 10^4 PFU or 10^3 PFU group. Onset of respiratory disease signs occurred on 6 dpi and persisted in some animals through 16 dpi. All of the AGMs in the 10^5 PFU group displayed observable respiratory disease signs including chest congestion, rales, or wheezing. Baseline temperatures were measured over a 24 hour period prior to infection with aerosolized MERS-CoV. Individual AGMs in 10^4 and 10^5 dose group had elevated temperature following exposure to MERS-CoV aerosols between day 1-3 (2 out of 4 AGMS in each group, data not shown).

Viral Replication and Tissue Damage Resulting from MERS-CoV Infection of AGMs

To determine if respiratory disease signs observed in AGMs were the result of robust MERS-CoV viral replication, viral loads were titrated from throat swab and serum samples. Virus was detected by plaque assay in throat swabs collected 6 dpi from all AGMs following exposure to aerosolized MERS-CoV. Significantly higher viral titers were observed in the throat swabs collected from the highest dose group (10^5 PFU MERS-CoV) of AGMs than the lower two dose groups between 6 dpi and 12 dpi (Figure 4A). Onset of respiratory symptoms recorded during physical evaluations of AGMs was concurrent with detection of virus in throat swab samples. Significantly higher viral titers in the serum were observed in the 10^5 PFU group and 10^4 PFU group compared to the 10^3 PFU group in the serum on 6 dpi (Figure 4B). Elevated blood chemistry values were observed in AGMs infected with the highest dose of MERS-CoV (Figure S1 A-D). No statistically significant differences were observed in blood urea nitrogen (BUN) or creatinine (CRE) levels (Figure S1 A-B). Significantly elevated levels of enzymes indicative of liver damage, aspartate aminotransferase (AST) and Gamma-glutamyl transferase (GGT), were observed in the 10^5 PFU group at 8 and 10 days post-infection (Figure S1 C-D).

Although the AGMs survived the infection, all of the AGMs exposed to aerosolized MERS-CoV showed mild or minimal lung lesions at 28 days postinfection (Table 2, Figure 5A-C). At the point in the disease course when lung samples were collected, all of the AGMs were recovering from the infection, although some were still experiencing very mild disease signs correlated by the clinical disease score (Figure 3B). The most common histopathological observation was multifocal interstitial pneumonia, which was likely attributable to the MERS-CoV aerosol exposure. The location, severity, and type of lung lesion observed appeared to be independent of the dose of aerosolized MERS-CoV. Another common finding was lymphoid hyperplasia in the mediastinal and/or tracheobronchial lymph nodes which can be attributed to antigenic stimulation due to the viral infection (data not shown). These were not clinically significant and, because these lymph nodes receive lymphatic drainage from the lungs, this was not a surprising finding following an aerosol exposure to a virus. Tissues from other major organs were surveyed for pathological disease signs, but no other findings were consistent with MERS-CoV disease (data not shown).

Discussion

Currently, China is experiencing an outbreak of a novel coronavirus (SARS-CoV-2) with more cases in the first month since identification than all of the previous SARS-CoV and MERS-CoV epidemics combined. Novel CoVs from the *Betacoronavirus* genus have emerged into the human population three separate times in less than 20 years: SARS-CoV (2002), MERS-CoV (2012) and SARS-CoV-2 (2019). In each instance, the novel CoVs most likely emerged from viruses originally circulating in bats^{16,18,19}. During the emergence of SARS-CoV and MERS-CoV, spillover into human populations was driven by an intermediate animal reservoir in closer proximity to humans (civets or raccoon dogs for SARS-CoV and dromedary camel for MERS-CoV)^{20,42}. Currently, it is not known if SARS-CoV-2 emergence resulted directly transmission from bats to humans or via another intermediate animal host. Human disease from emergent CoVs presented as a respiratory syndrome with the hallmarks of atypical pneumonia progressing to more severe lung dysfunction accompanied by varying degrees of morbidity (asymptomatic illness to long-term lung dysfunction) and mortality (~10% mortality rate from SARS, ~35% from MERS, yet to be determined from COVID-19) reviewed in⁴³. Forewarned by the knowledge that coronaviruses circulating in animals have the capability of causing severe disease in humans, it is necessary to develop platform approaches for the generation of animal models to allow evaluation of medical countermeasures against coronaviruses.

Currently, mouse models of SARS-CoV and MERS-CoV are well-developed, demonstrating reproducible severe disease by the intranasal (IN) route⁴⁴⁻⁴⁶. Mouse models of SARS were initially developed by serial passage of SARS-CoV in the lungs of mice, adapting the virus to the host⁴⁴. Mouse models of MERS-CoV required an additional step of modifying the host receptor to allow viral replication, followed by additional viral passage in the lungs of mice to cause severe disease relevant to MERS^{45,46}. However, the serial adaptation of SARS-CoV and MERS-CoV in these mouse models compressed the disease course from several weeks in humans to just days in mice. In the evaluation of medical countermeasures to be given after onset of symptoms, this rapid disease course can be a disadvantage because the window to deliver

the drug has been shortened. In addition, serial adaptation of both SARS-CoV and MERS-CoV resulted in adaptive mutations in the spike glycoprotein, one of the main targets of both vaccines and monoclonal antibodies, which could potentially complicate evaluation of these countermeasures. In some cases, as with MERS-CoV, host receptor modifications may need to be made to rodent models to allow viral replication would increase the time needed to bring small animal platforms online for emerging novel coronaviruses. The need for serial passage to acquire pathogenicity in mice means that different clinical isolates of emerging coronaviruses cannot be rapidly compared in these mouse models of SARS-CoV or MERS-CoV. Mice do not transmit SARS-CoV or MERS-CoV, so evaluation of transmission properties in these models is not possible. Therefore, the development of well-characterized NHP models of CoV infection could provide a complementary approach to animal models of pathogenesis, transmission, and countermeasure development.

In this study, we initiated development of an AGM model of highly pathogenic coronavirus infection by aerosol as a first step to establishing a platform for medical countermeasure testing against highly pathogenic coronaviruses. A prior study in NHP models of SARS-CoV delivered by combined IN/IT (intranasal/intratracheal) route compared disease signs in multiple NHP species: rhesus macaques, cynomolgus macaques, and African green monkeys³². Although none of the NHPs developed severe disease signs from SARS-CoV infection, AGMs had higher titers of virus recovered from nasal swabs and throat lavage than rhesus or cynomolgus macaques, particularly at later times postinfection (days 4–10). Building on this observation, we evaluated three different target doses of MERS-CoV delivered via aerosol to determine if AGMs have a dose-dependent disease response to MERS-CoV infection. Although we did not observe lethal or severe MERS disease signs, all of the AGMs in the highest dose group had clinical disease signs and elevated respiratory rates.

Route of infection in respiratory viruses can be absolutely critical in development of disease signs and severity in animal models of highly pathogenic viral infection. In NHP studies of Nipah virus infection, IT and small particle inoculation of virus were observed to have difference in the disease severity of the lungs of AGMs, while larger particle aerosol delivery increased neurological disease signs, an important hallmark of human Nipah cases^{47,48}. In comparing routes of infection in NHP models of H5N1 influenza virus, cynomolgus macaques infected with a commercial nebulizer had a wider distribution of infection in the lung but similar clinical disease signs to the multiple route (MR: oral, ocular, IN/IT)) of exposure⁴⁹. Conversely, cynomolgus macaques infected by small particle aerosol exposure rapidly developed ARDS and succumbed to infection from H5N1 influenza virus⁵⁰. It is likely that the route of infection as well as particle size targets virus to different tissues, resulting in variability of pathogenesis in NHP models of respiratory virus infection, but this is an area that is not well understood and requires additional study.

Previous studies of MERS-CoV in NHPs focused on infection of either rhesus macaques or marmosets. The approaches of different research groups typically used very high titer inoculum delivered by either MR or IT only routes of infection. Rhesus macaques were observed to have only very mild disease signs (if any) following MERS-CoV infection by either MR or IT route^{35,36}. A study of a small particle aerosol

exposure of rhesus macaques to MERS-CoV yielded subclinical infection with no disease signs⁵¹. Viral replication of MERS-CoV in rhesus macaques is limited and difficult to detect, indicating limitations for the use of this model for evaluation of medical countermeasures to MERS-CoV. Marmosets infected with MERS-CoV were observed to have more severe disease signs by MR but not IT^{35–38}. Marmosets infected by MR had high viral loads in serum and throat swabs, as was observed in this study with AGMs infected by the aerosol route³⁷. Although the marmoset model of MERS-CoV infection by MR yields severe respiratory disease, marmosets are a smaller NHP species that may present challenges when evaluating medical countermeasures due to sampling limitations. NHP model development in rhesus and marmoset models utilized a higher titer inoculum of MERS-CoV than was used in this study of aerosol exposure of AGMs to MERS-CoV, and did not compare dose-response. The response of animal models to differing doses of coronavirus infection may play an important role in the evaluation of viral pathogenesis of subclinical disease compared to more severe disease.

Additional refinements to our AGM model of MERS-CoV infection are needed for medical countermeasure testing and evaluation. Although the AGMs had observable disease signs including elevated respiratory rates and other respiratory disease signs, our model did not recapitulate severe disease or lethality seen in MERS patients. However, we are encouraged by the dose dependence of the clinical disease signs and response to MERS-CoV infection that we observed, indicating the potential for increased severity of disease in future iterations of this study with higher titers aerosol exposure. In addition, other NHP studies of MERS-CoV model development used early endpoint euthanasia to explore pathological outcomes at acute times during viral infection that we would like to compare in future studies. The presence of pneumonia in all of the AGMs at the end of the study indicates that it is likely that pathological findings related to MERS disease may be present during the acute phase of infection when respiratory disease signs are observed. In addition, the presence of histological lesions even after AGMs have mostly recovered from MERS-CoV infection may be of use as a model of long-term lung dysfunction seen in MERS and SARS survivors.

In conclusion, animal models of viral infection must couple methods with desired outcomes: routes and doses of infection must be sufficiently reproducible but they must also target the viral inoculum to the appropriate tissues to model respiratory disease. Animal model development, particularly where it applies to generating a model that is appropriate for medical countermeasure evaluation has been understudied in coronavirus research. We anticipate that the approach we have defined in this study can provide a starting point for additional model development in AGMs or other NHPs using the aerosol route of infection for MERS-CoV, SARS-CoV, SARS-CoV-2 or other novel highly pathogenic coronaviruses.

Declarations

Acknowledgements

We would like to thank Dr. Melek Sunay of the Countermeasures division at USAMRIID for careful reading of the manuscript and many helpful scientific discussions. We would also like to thank Dr. Simon Long of

the Pathology Division of USAMRIID for the collection of additional pathology images.

This research was also supported in part by an appointment to the Postgraduate Research Participation Program at the U.S. Army Medical Research Institute of Infectious Diseases administered by the Oak Ridge Institute for Science and Education through an interagency agreement between the U.S. Department of Energy and USAMRMC. Opinions, interpretations, conclusions and recommendations are those of the authors and are not necessarily endorsed by the United States Army.

References

- 1 Ksiazek, T. G. *et al.* A Novel Coronavirus Associated with Severe Acute Respiratory Syndrome. *New England Journal of Medicine* **348**, 1953-1966, doi:10.1056/NEJMoa030781 (2003).
- 2 Zaki, A. M., van Boheemen, S., Bestebroer, T. M., Osterhaus, A. D. M. E. & Fouchier, R. A. M. Isolation of a Novel Coronavirus from a Man with Pneumonia in Saudi Arabia. *New England Journal of Medicine* **367**, 1814-1820, doi:10.1056/NEJMoa1211721 (2012).
- 3 Zhu, N. *et al.* A Novel Coronavirus from Patients with Pneumonia in China, 2019. *New England Journal of Medicine*, doi:10.1056/NEJMoa2001017 (2020).
- 4 Chan, J. F.-W. *et al.* A familial cluster of pneumonia associated with the 2019 novel coronavirus indicating person-to-person transmission: a study of a family cluster. *The Lancet*, doi:10.1016/S0140-6736(20)30154-9.
- 5 Omrani, A. S. *et al.* A family cluster of Middle East Respiratory Syndrome Coronavirus infections related to a likely unrecognized asymptomatic or mild case. *International Journal of Infectious Diseases* **17**, e668-e672, doi:10.1016/j.ijid.2013.07.001 (2013).
- 6 Shah, R. D. & Wunderink, R. G. Viral Pneumonia and Acute Respiratory Distress Syndrome. *Clinics in Chest Medicine* **38**, 113-125, doi:<https://doi.org/10.1016/j.ccm.2016.11.013> (2017).
- 7 World Health Organization: SARS case fatality ratio, incubation period, <http://www.who.int/csr/sarsarchive/2003_05_07a/en/> (2003).
- 8 World Health Organization: MERS Situation Update, December 2019, <<http://www.emro.who.int/pandemic-epidemic-diseases/mers-cov/mers-situation-update-december-2019.html>> (2019).
- 9 Wu, Z. & McGoogan, J. M. Characteristics of and Important Lessons From the Coronavirus Disease 2019 (COVID-19) Outbreak in China: Summary of a Report of 72 314 Cases From the Chinese Center for Disease Control and Prevention. *JAMA*, doi:10.1001/jama.2020.2648 (2020).
- 10 Lew, T. W. K. *et al.* Acute Respiratory Distress Syndrome in Critically Ill Patients With Severe Acute Respiratory Syndrome. *JAMA* **290**, 374-380, doi:10.1001/jama.290.3.374 (2003).

- 11 Wu, P. *et al.* Real-time tentative assessment of the epidemiological characteristics of novel coronavirus infections in Wuhan, China, as at 22 January 2020. *Eurosurveillance* **25**, 2000044, doi:doi:<https://doi.org/10.2807/1560-7917.ES.2020.25.3.2000044> (2020).
- 12 Xie, L. *et al.* Follow-up Study on Pulmonary Function and Lung Radiographic Changes in Rehabilitating Severe Acute Respiratory Syndrome Patients After Discharge. *CHEST* **127**, 2119-2124, doi:10.1378/chest.127.6.2119 (2005).
- 13 Park, W. B. *et al.* Correlation between Pneumonia Severity and Pulmonary Complications in Middle East Respiratory Syndrome. *J Korean Med Sci* **33**, e169-e169, doi:10.3346/jkms.2018.33.e169 (2018).
- 14 Wu, Q. *et al.* Altered Lipid Metabolism in Recovered SARS Patients Twelve Years after Infection. *Scientific reports* **7**, 9110-9110, doi:10.1038/s41598-017-09536-z (2017).
- 15 Corman, V. M., Muth, D., Niemeyer, D. & Drosten, C. in *Advances in Virus Research* Vol. 100 (eds Margaret Kielian, Thomas C. Mettenleiter, & Marilyn J. Roossinck) 163-188 (Academic Press, 2018).
- 16 Lau, S. K. P. *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* **102**, 14040-14045, doi:10.1073/pnas.0506735102 (2005).
- 17 Li, W. *et al.* Bats Are Natural Reservoirs of SARS-Like Coronaviruses. *Science* **310**, 676-679, doi:10.1126/science.1118391 (2005).
- 18 Memish, Z. A. *et al.* Middle East respiratory syndrome coronavirus in bats, Saudi Arabia. *Emerging infectious diseases* **19**, 1819-1823, doi:10.3201/eid1911.131172 (2013).
- 19 Zhou, P. *et al.* Discovery of a novel coronavirus associated with the recent pneumonia outbreak in humans and its potential bat origin. *bioRxiv*, 2020.2001.2022.914952, doi:10.1101/2020.01.22.914952 (2020).
- 20 Alagaili, A. N. *et al.* Middle East Respiratory Syndrome Coronavirus Infection in Dromedary Camels in Saudi Arabia. *mBio* **5**, doi:10.1128/mBio.00884-14 (2014).
- 21 Adney, D. R. *et al.* Replication and Shedding of MERS-CoV in Upper Respiratory Tract of Inoculated Dromedary Camels. *Emerging Infectious Disease journal* **20**, 1999, doi:10.3201/eid2012.141280 (2014).
- 22 Sikkema, R. S. *et al.* Global status of Middle East respiratory syndrome coronavirus in dromedary camels: a systematic review. *Epidemiology and infection* **147**, e84-e84, doi:10.1017/S095026881800345X (2019).

- 23 Reusken, C. B. E. M. *et al.* Occupational Exposure to Dromedaries and Risk for MERS-CoV Infection, Qatar, 2013–2014. *Emerging Infectious Diseases* **21**, 1422-1425, doi:10.3201/eid2108.150481 (2015).
- 24 Prevention, K. C. f. D. C. Middle East Respiratory Syndrome Coronavirus Outbreak in the Republic of Korea, 2015. *Osong Public Health and Research Perspectives* **6**, 269-278, doi:<https://doi.org/10.1016/j.phrp.2015.08.006> (2015).
- 25 Korea Centers for Disease, C., & Prevention. Middle East Respiratory Syndrome Coronavirus Outbreak in the Republic of Korea, 2015. *Osong Public Health and Research Perspectives* **6**, 269-278, doi:10.1016/j.phrp.2015.08.006 (2015).
- 26 WHO. *Summary of probable SARS cases with onset of illness from 1 November 2002 to 31 July 2003*, <http://www.who.int/csr/sars/country/table2004_04_21/en/index.html> (
- 27 World Health Organization: Coronavirus Disease 2019 (COVID-19) Situation Report - 38. (2020).
- 28 Jones, R. M. & Brosseau, L. M. Aerosol Transmission of Infectious Disease. *Journal of Occupational and Environmental Medicine* **57**, 501-508, doi:10.1097/jom.0000000000000448 (2015).
- 29 Tran, K., Cimon, K., Severn, M., Pessoa-Silva, C. L. & Conly, J. Aerosol generating procedures and risk of transmission of acute respiratory infections to healthcare workers: a systematic review. *PloS one* **7**, e35797-e35797, doi:10.1371/journal.pone.0035797 (2012).
- 30 Nam, H.-S., Yeon, M.-Y., Park, J. W., Hong, J.-Y. & Son, J. W. Healthcare worker infected with Middle East Respiratory Syndrome during cardiopulmonary resuscitation in Korea, 2015. *Epidemiol Health* **39**, e2017052-e2017052, doi:10.4178/epih.e2017052 (2017).
- 31 Haagmans, B. L. *et al.* Pegylated interferon- α protects type 1 pneumocytes against SARS coronavirus infection in macaques. *Nature Medicine* **10**, 290-293, doi:10.1038/nm1001 (2004).
- 32 McAuliffe, J. *et al.* Replication of SARS coronavirus administered into the respiratory tract of African Green, rhesus and cynomolgus monkeys. *Virology* **330**, 8-15 (2004).
- 33 Greenough, T. C. *et al.* Pneumonitis and Multi-Organ System Disease in Common Marmosets (*Callithrix jacchus*) Infected with the Severe Acute Respiratory Syndrome-Associated Coronavirus. *The American Journal of Pathology* **167**, 455-463, doi:10.1016/S0002-9440(10)62989-6 (2005).
- 34 Smits, S. L. *et al.* Distinct Severe Acute Respiratory Syndrome Coronavirus-Induced Acute Lung Injury Pathways in Two Different Nonhuman Primate Species. *Journal of Virology* **85**, 4234-4245, doi:10.1128/jvi.02395-10 (2011).

- 35 de Wit, E. *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* **110**, 16598-16603, doi:10.1073/pnas.1310744110 (2013).
- 36 Johnson, R. F. *et al.* 3B11-N, a monoclonal antibody against MERS-CoV, reduces lung pathology in rhesus monkeys following intratracheal inoculation of MERS-CoV Jordan-n3/2012. *Virology* **490**, 49-58, doi:10.1016/j.virol.2016.01.004 (2016).
- 37 Falzarano, D. *et al.* Infection with MERS-CoV Causes Lethal Pneumonia in the Common Marmoset. *PLoS Pathog* **10**, e1004250, doi:10.1371/journal.ppat.1004250 (2014).
- 38 Johnson, R. F. *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* **485**, 422-430, doi:10.1016/j.virol.2015.07.013 (2015).
- 39 Chan, J. F.-W. *et al.* Treatment With Lopinavir/Ritonavir or Interferon- β 1b Improves Outcome of MERS-CoV Infection in a Nonhuman Primate Model of Common Marmoset. *Journal of Infectious Diseases* **212**, 1904-1913, doi:10.1093/infdis/jiv392 (2015).
- 40 Scobey, T. *et al.* Reverse genetics with a full-length infectious cDNA of the Middle East respiratory syndrome coronavirus. *Proceedings of the National Academy of Sciences* **110**, 16157-16162, doi:10.1073/pnas.1311542110 (2013).
- 41 Nalca, A. *et al.* Experimental infection of cynomolgus macaques (*Macaca fascicularis*) with aerosolized monkeypox virus. *PloS one* **5**, e12880, doi:10.1371/journal.pone.0012880 (2010).
- 42 Guan, Y. *et al.* Isolation and Characterization of Viruses Related to the SARS Coronavirus from Animals in Southern China. *Science* **302**, 276-278, doi:10.1126/science.1087139 (2003).
- 43 Gralinski, L. E. & Baric, R. S. Molecular pathology of emerging coronavirus infections. *The Journal of Pathology* **235**, 185-195, doi:10.1002/path.4454 (2015).
- 44 Roberts, A. *et al.* A Mouse-Adapted SARS-Coronavirus Causes Disease and Mortality in BALB/c Mice. *PLoS Pathog* **3**, e5 (2007).
- 45 Li, K. *et al.* Mouse-adapted MERS coronavirus causes lethal lung disease in human DPP4 knockin mice. **114**, E3119-E3128, doi:10.1073/pnas.1619109114 %J Proceedings of the National Academy of Sciences (2017).
- 46 Cockrell, A. S. *et al.* A mouse model for MERS coronavirus-induced acute respiratory distress syndrome. *Nature Microbiology* **2**, 16226, doi:10.1038/nmicrobiol.2016.226 (2016).
- 47 Cong, Y. *et al.* Loss in lung volume and changes in the immune response demonstrate disease progression in African green monkeys infected by small-particle aerosol and intratracheal exposure to

Nipah virus. *PLOS Neglected Tropical Diseases* **11**, e0005532, doi:10.1371/journal.pntd.0005532 (2017).

48 Hammoud, D. A. *et al.* Aerosol exposure to intermediate size Nipah virus particles induces neurological disease in African green monkeys. *PLOS Neglected Tropical Diseases* **12**, e0006978, doi:10.1371/journal.pntd.0006978 (2018).

49 Watanabe, T. *et al.* Experimental infection of Cynomolgus Macaques with highly pathogenic H5N1 influenza virus through the aerosol route. *Scientific Reports* **8**, 4801, doi:10.1038/s41598-018-23022-0 (2018).

50 Wonderlich, E. R. *et al.* Widespread Virus Replication in Alveoli Drives Acute Respiratory Distress Syndrome in Aerosolized H5N1 Influenza Infection of Macaques. *The Journal of Immunology* **198**, 1616 (2017).

51 Chefer, S. *et al.* Modeling [18F]-FDG lymphoid tissue kinetics to characterize nonhuman primate immune response to Middle East respiratory syndrome-coronavirus aerosol challenge. *EJNMMI Research* **5**, 65, doi:10.1186/s13550-015-0143-x (2015).

Tables

Table 1 Inhaled Doses of African Green Monkeys Exposed to MERS-CoV

Animal ID	M/F	Target Dose (10 ^x PFU)	Actual Dose (PFU)	Actual Dose (10 ^x PFU)
1	F	3	7.60 x 10 ²	2.88
2	M	3	1.17 x 10 ³	3.07
3	M	3	8.70 x 10 ²	2.94
4	F	3	1.11 x 10 ³	3.04
5	F	4	6.31 x 10 ³	3.80
6	M	4	7.79 x 10 ³	3.89
7	M	4	7.25 x 10 ³	3.86
8	F	4	4.65 x 10 ³	3.67
9	F	5	2.78 x 10 ⁴	4.44
10	M	5	3.75 x 10 ⁴	4.57
11	M	5	2.83 x 10 ⁴	4.45
12	F	5	2.27 x 10 ⁴	4.36

Table 2. Summary of Histological Findings from African Green Monkeys Exposed to MERS-CoV

Animal ID	M/F	Target Dose (10 ^x PFU)	Type of Lung Lesion	Location and Severity of Lung Lesion			
				Left Superior	Right Superior	Left Inferior	Right Inferior
1	F	3	Multifocal Interstitial Pneumonia	minimal	minimal	minimal	minimal
2	M	3	Multifocal Interstitial Pneumonia	minimal	minimal	NLP	NLP
3	M	3	Multifocal Broncho-Interstitial Pneumonia	mild	mild	minimal	minimal
4	F	3	Multifocal Interstitial Pneumonia	minimal	minimal	minimal	minimal
5	F	4	Multifocal Broncho-interstitial Pneumonia	minimal	minimal	NLP	NLP
6	M	4	Multifocal Foreign-Body Bronchiolitis	NLP	NLP	mild	mild
7	M	4	Multifocal Broncho-interstitial Pneumonia	minimal	minimal	NLP	NLP
8	F	4	Multifocal Interstitial Pneumonia	minimal	minimal	mild	mild
9	F	5	Multifocal Interstitial Pneumonia	NLP	NLP	minimal	minimal
10	M	5	Multifocal Interstitial Pneumonia	mild	mild	minimal	minimal
11	M	5	Multifocal Interstitial Pneumonia	minimal	minimal	minimal	minimal
12	F	5	Multifocal Interstitial Fibrosis	NLP	NLP	minimal	NLP

NLP- No Lesion Present

Figures

Figure 1

Figure 1. Disease Progression of Novel Coronaviruses Infection of viral particles from mucosal membrane exposure results in viral replication of cells along the airway. Here, ciliated cells are depicted as a cell where novel coronaviruses are replicating, although cell tropism is at least partially dependent on host receptor expression, and receptor usage may vary between novel coronaviruses (nCoV). Following initial phases of viral replication, onset of symptoms includes nonspecific influenza-like illness that progresses in many patients to acute atypical pneumonia. In patients with severe disease from novel coronavirus infection, pneumonia may progress to Acute Respiratory Distress Syndrome (ARDS), the most severe form of acute lung injury. In particular, highly pathogenic coronaviruses may result in the infiltration of inflammatory cells, deposition of fibrin, and diffuse alveolar damage. Survivors of highly pathogenic coronaviruses who progress to ARDS may experience long term lung dysfunction as a result of the infection.

Figure 2

Figure 2. Study Schedule for Small Particle Aerosol Infection of African Green Monkeys with MERS-CoV. Three groups of AGMs (n=4) were exposed to three different target doses of MERS-CoV EMC/2012 strain by small particle aerosol exposure. AGMs were observed at indicated days postinfection for metrics indicating recapitulation of MERS disease in human cases.

Figure 3

Figure 3. Clinical Disease Signs in AGMs Following Small Particle Aerosol MERS-CoV Infection
Respiratory rates were observed twice daily for all groups and recorded (A). Significant differences on the graph reflects analysis comparing respiratory rates between the 10^3 and 10^5 dose groups. Clinical scores incorporated MERS disease signs including responsiveness, respiratory observation (other than respiratory rate), lymphadenopathy, and dehydration (B). Differences between respiratory rate and clinical

score groups were determined by one way ANOVA (Tukey's multiple comparison test, * $p<0.05$, ** $p<0.01$, *** $p<0.001$).

Figure 4

Figure 4. Detection of MERS-CoV Viral Loads in Throat Swabs and Serum from AGMs Infectious MERS-CoV was titrated on Vero cells from throat swab (A) and serum (B) samples collected at indicated days following MERS-CoV aerosol exposure of AGMs. Plaque Assays were completed for each sample in triplicate. Differences between groups were evaluated by one way ANOVA (Tukey's multiple comparison test, * $p<0.05$, ** $p<0.01$, *** $p<0.001$).

Figure 5

Figure 5. Interstitial Pneumonia Observed in Lung Samples from AGMs H&E stained lung samples sections were collected AGMs exposed to aerosolized MERS-CoV at 28 days postinfection. Images shown reflect a sample of lung disease observed in the 103 (A, 20X objective), 104 (B, 20X objective) and 105 (C, 10X objective) PFU dose groups. Samples were evaluated by a trained pathologist and scored for lesions associated with severe respiratory disease.

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