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Intratracheal exposure of common marmosets to MERS-CoV Jordan-n3/2012 or MERS-CoV EMC/2012 isolates does not result in lethal disease

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ABSTRACT

Middle East Respiratory Syndrome Coronavirus (MERS-CoV) continues to be a threat to human health in the Middle East. Development of countermeasures is ongoing; however, an animal model that faithfully recapitulates human disease has yet to be defined. A recent study indicated that inoculation of common marmosets resulted in inconsistent lethality. Based on these data we sought to compare two isolates of MERS-CoV. We followed disease progression in common marmosets after intratracheal exposure with: MERS-CoV-EMC/2012, MERS-CoV-Jordan-n3/2012, media, or inactivated virus. Our data suggest that common marmosets developed a mild to moderate non-lethal respiratory disease, which was quantifiable by computed tomography (CT), with limited other clinical signs. Based on CT data, clinical data, and virological data, MERS-CoV inoculation of common marmosets results in mild to moderate clinical signs of disease that are likely due to manipulations of the marmoset rather than as a result of robust viral replication.

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Introduction

Infection with Middle East Respiratory Syndrome Coronavirus (MERS-CoV) has been associated with Middle East Respiratory Syndrome commonly known as MERS, a respiratory syndrome with acute severe hypoxic respiratory failure often accompanied by renal failure (Arabi et al., 2014; Assiri et al., 2013; Zaki et al., 2012). As of March, 2015 there have been approximately 1000 laboratory confirmed cases reported with a 36% case fatality rate (http://www.who.int/csr/disease/coronavirus_infections/). Considering the geographical location of MERS and the Hajj pilgrimage which draws an estimated 2.5 million visitors, roughly 1.8 million of whom travel internationally (http://www.cdsi.gov.sa/english/index.php?option=com_docman&Itemid=173) MERS-CoV represents a global health risk. Common signs and symptoms of MERS include fever, cough, shortness of breath, and myalgia. Gastrointestinal signs are also frequently observed which include vomiting, diarrhea, and abdominal pain (Assiri et al., 2013). Often, MERS patients also have underlying comorbidities such as diabetes, hypertension, and chronic cardiac or renal disease (Assiri et al., 2013).

To guide development of MERS countermeasures, an appropriate animal model must be identified and characterized. Ideally, a laboratory animal model would demonstrate clinical signs consistent with all aspects of human disease. As with most infectious diseases, mice have been evaluated as a potential MERS model for pathogenesis and countermeasure screening. Balb/c and STAT-1 knockout mice did not develop signs of disease, such as weight loss, nor could infectious virus be recovered from lung homogenates (Coleman et al., 2014). Zhao et al. developed a murine model for MERS by transduction of the respiratory tract with the putative MERS-CoV receptor, human dipeptidyl peptidase 4 (DPP4 or CD26), using an adenovirus construct (Zhao et al., 2014). Infected mice developed limited clinical signs including a

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small degree of weight loss. Histopathological analysis found peribronchial and perivascular lymphoid infiltrates which later progressed to an interstitial pneumonia. Nearly 6 $\log_{10}$ plaque forming units (PFU)/g of infectious virus could be detected in the lungs of infected DPP4 transduced mice (Zhao et al., 2014). The DPP4 transduced mouse model has been used to evaluate countermeasures and pathogenesis (Channappanavar et al., 2014a, 2014b).

Nonhuman primate (NHP) models are considered to be essential to understanding pathogenesis and evaluating countermeasures. Results from several challenge studies of MERS-CoV in rhesus monkeys (*Macaca Mulatta*) have varied between laboratories. The first published NHP model used rhesus monkeys inoculated via multiple routes and evaluated for virological, immunological, and histopathological changes up to 6 days post-inoculation (de Wit et al., 2013). NHPs demonstrated signs of pneumonia and virus could be detected in tissues and mucous membranes by quantitative reverse transcriptase polymerase chain reaction (qRT-PCR), but attempts to determine the load of infectious virus was not reported. A follow up study demonstrated that administration of interferon-alpha2b and ribavirin reduced viral burden and lessened disease (Falzarano et al., 2013). Results from a natural history study of MERS-CoV-infected rhesus monkeys indicated that intratracheal inoculation induced a non-lethal disease with limited pathology observed in recovering animals at 28 days post-inoculation and infectious virus could be recovered from lung but not other tissues assayed (Yao et al., 2014). Standard radiological examination revealed lung infiltrates at days 3 and 5 post-inoculation, suggesting virus-induced lung disease. More recently, Falzarano et al. described multiple route inoculation of the common marmoset (*Callithrix jacchus*) in which transcriptional changes indicating induction of immune, inflammatory, and repair pathways were cataloged and partial lethality was observed (Falzarano et al., 2014).

Here we characterize intratracheal (IT) inoculation of MERS-CoV into common marmosets as a model for MERS and to determine differences between two common isolates of MERS-CoV, MERS-CoV-Jordan-n3/2012 virus and MERS-CoV-EMC/2012 virus. We inoculated 4 groups of marmosets and followed disease progression by periodic physical exams that included computed tomography (CT). Previously, positron emission tomography with computed tomography (PET)/CT has been used to evaluate tuberculosis therapies in common marmosets (Via et al., 2013), disease progression in monkeypox-virus-inoculated cynomolgus monkeys (Dyall et al., 2011), and influenza-A-virus-inoculated ferrets (Jonsson et al., 2012). CT has two distinct advantages when compared to standard x-ray radiography 1) CT provides three dimensional data, and 2) CT data can be quantified, which allows unbiased comparisons (Elke et al., 2013; Li et al., 2013; Romanova et al., 2014).

Results

An ex-vivo experiment with marmoset lung and kidney primary cultures found that these tissues could support MERS-CoV replication; this suggested that common marmosets might be developed as a suitable animal model for MERS (Fig. 1). Therefore, we sought to determine if intratracheal inoculation of marmosets would result in disease presentation similar to human disease. The experimental design is shown in Fig. 2. Two pre-inoculation baseline CT's were performed to establish normal lung volumes and any pre-existing anomalies.

MERS-CoV-Jordan-n3/2012 virus (MERS-JOR, Genbank KC776174) and MERS-CoV-EMC/2012 virus (MERS-EMC, Genbank JX869059) were obtained and propagated as described in Materials and Methods. To ensure that no gross cross-contamination of

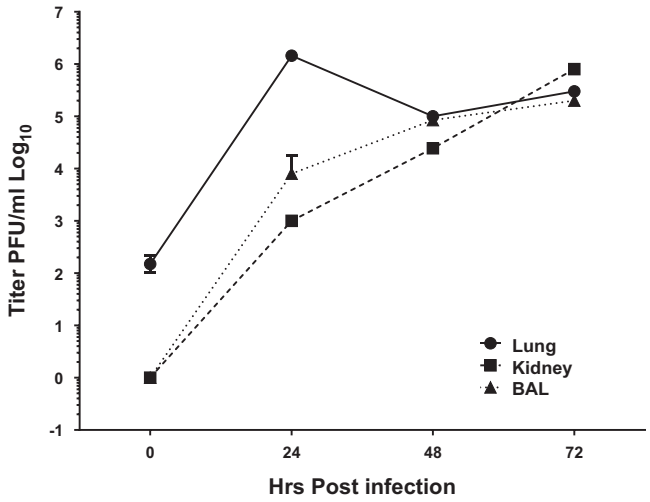

Fig. 1. Ex-vivo analysis of primary cells. Cells were isolated from lung, kidney, and bronchoalveolar lavage (BAL), and one-step growth kinetics were performed as described in Materials and Methods. Lung, Kidney and BAL demonstrate that MERS-CoV is able to replicate to at least 4 $\log_{10}$ PFU/mL.

the MERS-CoV stocks used for the marmoset experiments occurred during preparation, the spike protein of each stock was sequenced and compared to reference sequences (Cotten et al., 2013; Frey et al., 2014). The spike region was chosen for comparison due to high diversity associated with viral glycoproteins. Strain-specific differences found in MERS-JOR and MERS-EMC reference sequences were maintained in our stocks (Table 1), indicating that no gross cross-contamination occurred. Three single nucleotide polymorphisms (SNPs) were seen in our stocks, two in MERS-EMC and one in MERS-JOR. BLASTX alignments indicate that two of these SNPs lead to changes in the S2 protein sequence. The MERS-JOR stock had a T to C change at position 2636 which induced an I839T change in S2. Two changes were observed in MERS-EMC C to T at position 2604 that did not alter amino acid sequence and a C to A change at 3044 which resulted in a N1016T change in S2. Changes in the S2 region seen here are likely a result of serial passage in cell culture (Frey et al., 2014).

Clinical signs and hematology indicate mild disease

Body temperature, peripheral oxygen saturation, respiratory rate, and overall condition were evaluated at each physical exam. No increases in body temperatures above normal ranges were observed; subjects maintained peripheral oxygenation throughout the study, and respiratory rates increased above normal range sporadically throughout the study (Fig. 3A). Tremors were noted on daily observations including and between days 3 and 9 post-inoculation, but were not consistently observed (data not shown).

Subjects underwent blood withdrawal on days 0, 4 or 5, and at necropsy to determine if hematological parameters indicated changes consistent with disease. Day 4 or 5 was chosen based on our data from MERS-JOR inoculated rhesus monkeys which demonstrated a peak in lung disease at day 5 post-inoculation by CT (manuscript in preparation). Subjects did not develop clinically significant changes in total white blood cell count, lymphocyte number, monocyte number, or neutrophil number on day 4 or 5 post-inoculation and remained within the normal range, as indicated by shaded gray area (Fig. 3B). Together, these data indicate that subjects did not develop systemic clinical disease.

To determine if virus was disseminating or shedding, whole blood, oropharyngeal, rectal swabs, and fecal samples from mock and inactivated virus subjects, 2 subjects per group, were collected on days −21, −8, 1, 3, 5, 7, 14 or 15, and 25. MERS Jordan subjects