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Adaptive evolution influences the infectious dose of MERS-CoV necessary to achieve severe respiratory disease

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ABSTRACT

We recently established a mouse model (288–330$^{+/+}$) that developed acute respiratory disease resembling human pathology following infection with a high dose (5×10^6 PFU) of mouse-adapted MERS-CoV (icMERSma1). Although this high dose conferred fatal respiratory disease in mice, achieving similar pathology at lower viral doses may more closely reflect naturally acquired infections. Through continued adaptive evolution of icMERSma1 we generated a novel mouse-adapted MERS-CoV (maM35c4) capable of achieving severe respiratory disease at doses between 10^3 and 10^5 PFU. Novel mutations were identified in the maM35c4 genome that may be responsible for eliciting etiologies of acute respiratory distress syndrome at 10–1000 fold lower viral doses. Importantly, comparative genetics of the two mouse-adapted MERS strains allowed us to identify specific mutations that remained fixed through an additional 20 cycles of adaptive evolution. Our data indicate that the extent of MERS-CoV adaptation determines the minimal infectious dose required to achieve severe respiratory disease.

1. Introduction

Middle East respiratory syndrome is an emerging viral respiratory infection caused by Middle East respiratory syndrome coronavirus (MERS-CoV), first identified in Saudi Arabia in 2012. This novel coronavirus is associated with severe pneumonia and acute respiratory distress syndrome (ARDS) that often results in mortality (~ 35%) (Alsaad et al., 2017; Arabi et al., 2017; Ng et al., 2016). Over 2100 cases and 733 deaths have been reported in twenty-seven countries through December 2017, all geographically linked to the Middle East (WHO, 2017). Though sporadic infections and small nosocomial outbreaks continue to occur across the Arabian Peninsula, the potential for global transmission was demonstrated by an outbreak in South Korea in 2015 (Arabi et al., 2017). Spread from a single infected individual returning from the Arabian Peninsula resulted in 186 confirmed cases with a ~ 20% mortality rate (Lee, 2015). Current treatment for MERS-CoV relies on supportive treatment of symptoms, with potential MERS-CoV therapeutic countermeasures gradually progressing toward clinical trials.

Developing therapeutic strategies has been hindered by a poor understanding of viral pathogenesis in humans, which has created challenges in developing applicable animal models that resemble the human disease outcomes. Only recently have there been two case reports describing the pathological consequences observed in MERS-CoV infected patients (Alsaad et al., 2017; Ng et al., 2016). Non-human primates (NHPs) offer advantages in preclinical therapeutic testing due to their physiological similarities to humans. Two NHP models (rhesus macaques and marmosets) were shown to support MERS-CoV infection; however, there are limitations with regard to the ability to detect replicating MERS-CoV in the lungs and the extent of respiratory disease appears to vary from mild-to-severe in a laboratory dependent manner (Chan et al., 2015; Falzarano et al., 2014; Johnson et al., 2016, 2015; Munster et al., 2013). Importantly, this may be a consequence of difficulty in establishing MERS-CoV infection in the lower respiratory tract, where the dipeptidyl peptidase 4 (DPP4) receptor is predominantly expressed in the human respiratory tract (Meyerholz et al., 2016), or potential species-specific differences in DPP4 signaling mechanisms may impact production of high titer MERS-CoV. Complexities in establishing infection in NHPs suggest that MERS-CoV may not be adapted to elicit the severe respiratory disease often associated with human infections. MERS-CoV adaptation by repeated passage within these NHP species may help to enhance virulence and replicate severe human disease reliably in NHPs.

Species adaptation has shown to be previously successful in establishing MERS-CoV infection that elicited lethal respiratory disease in two humanized mouse models (Cockrell et al., 2016; Li et al., 2017a). Cockrell et al. utilized CRISPR–Cas9 (clustered regularly interspaced

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short palindromic repeats and CRISPR-associated gene 9) technology to make amino acid substitutions at positions 288 and 330 of mDPP4 to generate a humanized mouse model (288–330 $^{+/+}$) that supported high levels of virus replication. Fifteen serial passages of virus in heterozygous 288–330$^{+/-}$ mice resulted in a mouse-adapted virus (icMERSma1) capable of achieving dramatic weight loss, severe lung pathology, and lethal respiratory disease in 288–330$^{+/+}$ mice. Importantly, minimizing changes to the mDPP4 precluded disruption of its innate role in glucose homeostasis and immune dysregulation (Ohnuma et al., 2008; Rohrborn et al., 2015). By replicating clinical disease, while also preserving host-specific DPP4 expression patterns and immune functionality, this humanized mouse model offers a useful tool to study pathogenesis and therapeutic testing.

A limitation of the icMERSma1 virus was the high dose (5×10^6 PFU) required to produce severe respiratory disease through the intranasal route of infection (Cockrell et al., 2016); whereas, other MERS-CoV mouse models demonstrated that disease could be achieved at lower infectious doses (Coleman et al., 2017; Li et al., 2017a). Evidence in MERS-CoV infected patients indicates that there may be an association between virus dose and the severity of respiratory disease (Oh et al., 2016). Initiating disease through natural acquisition of MERS-CoV may also be dose dependent. Therefore, to achieve a dose that more closely resembles a naturally acquired infection we continued the adaptive evolution of icMERSma1 in 288–330$^{+/-}$ mice. We generated a novel mouse-adapted MERS-CoV clone (maM35c4) that confers pathology indicative of severe respiratory disease at substantially lower doses in 288–330$^{+/+}$ mice. Mouse-adapted M35c4 causes pathology similar to icMERSma1, but exhibited dose-dependent effects at 10^3–10^5 PFU. We identified unique mutations in the maM35c4 clone that may promote severe respiratory disease at 10–1000 fold lower virus doses than previously observed. Most notably, comparative genetics of maM35c4 and icMERSma1 provided us a distinct advantage by allowing us to identify mutations that remained fixed after an additional 20 passages, implicating their importance in conferring virulence in mice.

2. Results

2.1. Evolving MERS-CoV in mouse lung maintains disease at lower infectious doses

MERS-CoV was mouse-adapted by serial passage through the lungs of the heterozygous, 288–330$^{+/-}$ mice as previously described (Cockrell et al., 2016). Fatal disease was routinely observed at the 5×10^6 PFU dose (Cockrell et al., 2016), a potential limitation that could be overcome through continued passaging through the lungs of 288–330$^{+/-}$ mice (Cockrell et al., 2016). Here, we demonstrate that continued passaging of the icMERSma1 virus through the lungs of 288–330$^{+/-}$ mice yielded mouse-adapted MERS strains that exhibited dramatic weight loss after 25 and 35 passages, respectively referred to as maMERS-25 and maMERS-35. The two heterogeneous mouse-adapted MERS-CoV populations (maMERS-25 and maMERS-35) were expanded in CCL81 Vero cells and tested at 5×10^4 and 5×10^5 PFU doses by intranasal inoculation of the 288–330$^{+/+}$ mice (Fig. 1). Significant weight loss was observed through day 8 post-infection (p.i.) at two different doses. Mouse-adapted maMERS-35 exhibited the most significant weight loss at both 5×10^4 PFU and 5×10^5 PFU with average weight loss of > 20% at both doses (Figs. 1a and 1b). Notably, all mice were euthanized as they approached our 30% weight exclusion limit. We anticipate that these mice would have succumbed to disease at later times post-infection. In contrast, the mice infected with maMERS-25 only reached 16–22% weight loss (Figs. 1a and 1b), indicating that maMERS-25 may be less virulent than maMERS-35. Despite the differences in disease severity, increased passaging resulted in two novel MERS-CoV heterogeneous virus populations that elicit weight loss, which is commonly associated with severe respiratory disease. These data indicate that novel virus genetic mutations were acquired during additional rounds of adaptation in the 288–330$^{+/-}$ mice. To gain insight into these genetic changes plaque purification was used to isolate a novel clone from the more virulent maMERS-35.

2.2. A clonal isolate of maMERS-35 induces fatal respiratory disease in 288–330$^{+/+}$ mice

A clonal isolate (maM35c4) was derived from a plaque purified virus of maMERS-35 on CCL81 Vero cells. 288–330$^{+/+}$ mice were intranasally infected with three different doses of maM35c4 (5×10^3, 5×10^4, and 5×10^5 PFU) (Fig. 2). Severe weight loss (> 20%) was associated with 5×10^4 and 5×10^5 PFU by day 7 p.i., with little weight loss at the 5×10^3 PFU dose (Fig. 2a). Significant mortality (50% succumbed to disease during the course of the experiment) was observed at the 5×10^5 PFU dose, whereas all animals survived through day 7 p.i. at the 5×10^4 and 5×10^3 PFU doses (Fig. 2b). Graphing survival at 20% or 30% weight loss demonstrates decreased survival at 5×10^3 and 5×10^4 PFU doses (Supplementary Fig. 1). However, all mice were euthanized as mice receiving 5×10^4 and 5×10^5 PFU doses approached our 30% weight exclusion limit. Based on these results we would suggest an LD_{50} of ~ 1–2 $\times 10^4$ PFU. Dramatic weight loss and mortality correlated well with severe respiratory disease as quantified by gross investigation of hemorrhaging in the lungs at days 3 and 7 post-infection (Fig. 2c). Lungs were scored for hemorrhaging on a scale of 0 (no hemorrhaging) to 4 (severe hemorrhaging) throughout all lung lobes. Early after infection (day 3 p.i.), hemorrhage scores correlated with the dose of virus administered (Fig. 2c). Compared to minor hemorrhaging at 5×10^3 PFU, both the 5×10^4 and 5×10^5 infectious doses elicited significant hemorrhaging at day 3 p.i. (Fig. 2c). By day 7 p.i. 5×10^4 and 5×10^5 PFU doses invoked dramatic hemorrhaging across all lung lobes (Fig. 2c). Interestingly, although minimal weight loss occurred in mice infected with 5×10^3 PFU dose, substantial lung hemorrhaging was observed by day 7 p.i., indicating that the 5×10^3 dose of maM35c4 causes significant lung disease (Fig. 2c). The viral loads in the lungs are consistent with the disease elicited by the different doses (Fig. 2d). At day 3 p.i. maMERS35c4 replicated to high titers (10^8–10^9 PFU/ml/gram lung tissue) in the lungs at all doses, with mean lung titers increasing with increased dose (Fig. 2d). Titers at day 7 p.i. exhibited a dose-dependent effect on clearance of maM35c4 from the lungs (Fig. 2d). Mice infected with 5×10^3 PFU dose show little to no detection of virus at day 7 p.i., while higher titers persist in mice infected at both 5×10^4 and 5×10^5 PFU doses (Fig. 2d). Weight loss, mortality, hemorrhaging and viral load in the lungs are consistent with a dose-dependent effect on MERS-CoV-induced disease. Lung disease by gross hemorrhaging was observed at doses as low as 5×10^3 PFU, indicating that infection elicited dramatic pathological changes in the lung architecture.

2.3. maM35c4 causes widespread infection of the lungs and pathology characteristic of acute respiratory distress syndrome (ARDS)

MERS-CoV viral loads in the lungs of infected humans exhibit a phased correlation with the development of respiratory disease. Viral loads peak early after infection and as viral loads begin to decrease, symptoms associated with respiratory disease increase (Oh et al., 2016). A similar correlation was observed following infection of 288–330$^{+/+}$ mice with maM35c4. Mouse-adapted M35c4 reached peak viral loads shortly after infection (3 days p.i.), and began to resolve at late times post-infection (7 days p.i.) (Fig. 2d and Fig. 3). MERS-CoV nucleocapsid antigen staining by immunohistochemistry revealed widespread infection of the lungs at day 3 p.i. at all three doses and substantial clearance by day 7.p.i., affecting both the parenchyma and airway epithelium (Fig. 3 and Table 1). This corresponds well with the dose-dependent effect of viral loads in the lungs (Fig. 2d). Consistent with viral replication peaking early by day 3 p.i. the initial signs of lung pathology

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