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Abstract

Middle East Respiratory Syndrome (MERS) is a highly lethal pulmonary infection caused by a coronavirus (CoV), MERS-CoV. With the continuing spread of MERS-CoV, prophylactic and therapeutic treatments are urgently needed. In this study, we prepared purified equine F(\text{ab}')\text{2} from horses immunized with MERS-CoV virus-like particles (VLPs) expressing MERS-CoV S, M and E proteins. Both IgG and F(\text{ab}')\text{2} efficiently neutralized MERS-CoV replication in tissue culture. Passive transfer of equine immune antibodies significantly reduced virus titers and accelerated virus clearance from the lungs of MERS-CoV infected mice. Our data show that horses immunized with MERS-CoV VLPs can serve as a primary source of protective F(\text{ab}')\text{2} for potential use in the prophylactic or therapeutic treatment of exposed or infected patients.

Keywords: Middle East Respiratory Syndrome coronavirus; equine immune serum; immunoglobulin; F(\text{ab}')\text{2} fragment; animal model
1. Introduction

Middle East Respiratory Syndrome (MERS)-CoV is an emerging pathogen that causes severe pneumonia in humans in the Arabian Peninsula and in travelers from this region (Assiri et al., 2013a; Zaki et al., 2012b; Zumla et al., 2015). Human-to-human spread has been documented (Assiri et al., 2013b). While infections of immunocompetent patients generally present with only mild symptoms, the elderly and patients with pre-existing illnesses such as diabetes or renal failure are likely to develop more severe disease (Assiri et al., 2013a). As of September 21, 2016, 1806 cases with 643 deaths (35.6% mortality) had been reported to the World Health Organization, although the actual number of infections could be much larger since mild, asymptomatic or undiagnosed cases are likely to be common (Drosten et al., 2014).

As yet there are neither licensed vaccines nor any prophylactic or therapeutic treatments effective against MERS-CoV. Given the ability of coronaviruses to rapidly adapt to new hosts, a major public health concern is that MERS-CoV will further adapt to replication in humans, triggering a global severe acute respiratory syndrome (SARS)-like pandemic (Peiris et al., 2004; Zaki et al., 2012a).

As of now, the most promising treatment is the passive administration of anti-MERS-CoV neutralizing antibodies. Several research groups have developed and produced anti-MERS patient-derived or humanized monoclonal neutralizing antibodies \textit{in vitro} that were able to protect MERS-CoV infected mice (Corti et al., 2015; Li et al., 2015; Zhao et al., 2014). However, since these antibodies react with a single epitope on the MERS-CoV spike (S) protein and since coronaviruses are prone
to mutate, this approach has raised concerns about possible antibody escape (Corti et al., 2015; Sabir et al., 2016).

Recently, we showed that sera from Middle East dromedary camels contained high levels of anti-MERS-CoV neutralizing antibodies. Passive immunotherapy with sera from these animals significantly reduced virus loads and accelerated virus clearance from the lungs of MERS-CoV infected mice (Zhao et al., 2015). This provides proof of concept that immune animal sera are potentially useful in the treatment of patients with MERS (Hayden et al., 2014). Passive immunotherapy with animal sera or antibodies has been successfully used to prevent rabies and to neutralize snake venom (Both et al., 2012; Gutierrez et al., 2014). Convalescent plasma used to treat patients with SARS has been found safe and has demonstrated some efficacy in a study with a small number of patients (Mair-Jenkins et al., 2015). However, neutralizing antibody titers in MERS patients are generally low and the limited number of MERS survivors makes this approach impractical (Drosten et al., 2013).

Here, we show that immunization of healthy horses with MERS-CoV virus-like particles (VLPs) expressing MERS-CoV S, M and E proteins induces strong polyclonal neutralizing antibodies against MERS-CoV. Since administration of whole antibodies can induce allergic responses in some humans, we further tested F(ab′)2 fragments prepared by digestion of antibody with pepsin. Prophylactic or therapeutic treatment of MERS-CoV infected mice with either IgG or F(ab′)2 significantly decreased the virus load in their lungs.
2. Materials and methods

2.1 Antigen preparation. MERS-CoV VLPs were produced and purified as previously described (Wang C, 2016). In brief, army worm Sf9 cells were infected with a single recombinant baculoviruses co-expressing MERS-CoV structural protein genes S, M, and E, at a multiplicity of infection (MOI) of 0.5. Culture supernatants were harvested at 96 h post-infection and centrifuged at 2,000 g for 30 min to remove cell debris. Following centrifugation of the clarified supernatants at 100,000 g for 1 h at 4°C the resulting VLP pellets were resuspended in PBS and loaded onto a 30–40–50% discontinuous sucrose gradient. After an additional centrifugation at 100,000 g for 1.5 h at 4°C, bands between 30-40% sucrose containing MERS-CoV VLP were collected.

2.2 Animal immunization. Four 4-year-old healthy horses received multi-point intramuscular injections of 0.5, 1.5, 2, 3, and 5 mg MERS-CoV VLPs in 4ml PBS at weeks 0, 2, 4, 6, and 8, respectively. Freund’s complete adjuvant (Sigma) was included in the first dose, and incomplete adjuvant in the remaining ones. Sera were collected from the jugular vein 2 weeks after each injection, and stored at -20°C before further analysis.

2.3 MERS-CoV specific antibody measurement. MERS-CoV specific antibodies in the sera were measured by an indirect enzyme-linked immunosorbent assay (ELISA) using purified MERS-CoV receptor-binding domain (RBD) protein (i.e., S protein residues 358-662 cloned into the pET-30a expression vector and purified by Ni-NTA affinity chromatograph column). Briefly, 96-well microtitration plates (Corning
Costar, USA) were pre-coated with 100 µl purified RBD antigen diluted in 0.05 mol/L carbonate sodium buffer (pH 9.6) to a final concentration of 1 µg/mL and incubated at 4°C overnight. After blocking with skimmed milk for 2 h at 37°C, 100 µL twofold serially diluted serum samples were added to the wells, and incubated at 37°C for 1 h. The plates were washed three times with PBS containing 0.05% Tween-20 (PBST), before addition of 100 µL HRP-labeled rabbit antibody against horse IgG (Bioss, China; 1:20,000) and incubation at 37°C for 1 h. After washing with PBST, 100 µL 3', 3', 5'-tetramethylbenzidine (TMB) (Sigma, USA) as substrate was added to each well and incubated for 30 min. The reaction was stopped with 50 µL 2M H2SO4. Optical densities at 450 nm were measured in an ELISA plate reader (Bio-Rad, USA).

2.4 Immunoglobulin purification. Horse antiserum was diluted with 2 volumes of normal saline (0.9% NaCl) and a half volume of saturated ammonium sulfate was then added and mixed gently at room temperature for 30 min before centrifugation at 5,000 g for 20 min. The resulting sediment was redissolved in saline and mixed with a one-third volume of saturated ammonium sulfate. After incubation at ambient temperature for 30 min and centrifugation at 5,000 g for 20 min, the second sediments were dissolved in normal saline and dialyzed against normal saline to remove any remaining ammonium salt.

2.5 Immunoaffinity chromatography. Immunoaffinity resins were prepared by coupling 10 mg RBD protein to 0.02 M sodium periodate-activated Sepharose 4B (4 g), and then incubating with 150 µL sodium borohydride for 30 min. After reaction with 1 M Tris (pH 7.5) for 30 min, a purified IgG sample was diluted 9-fold with PBS
and incubated with the RBD resin overnight at 4°C with constant rotation. The flowthroughs (anti-RBD depleted) were collected, and then the flowthroughs were tested against the RBD protein by ELISA to ensure RBD-specific IgG all bound with the RBD Sepharose 4B. After washing with PBS, the bound antibodies (anti-RBD) were eluted in 0.2 M glycine-HCl buffer (pH 2.7). The eluates were neutralized with 1 M Tris buffer (pH 9.0), and then dialyzed against PBS. All samples were adjusted to the same protein concentration and sterilized by passage through microspin filters (0.2 µm pore size; Millipore). Neutralizing activity of the IgG, RBD-specific IgG, and flowthroughs were tested.

2.6 F(\text{ab}’)\text{2} preparation. The pH of the horse antiserum was adjusted to 3.3 with 1 mol/L HCl. Following incubation with pepsin (10000 IU/mL) at 30°C for 2.5 h, the reaction was stopped by adjusting the pH to 7.2 with 1 mol/L NaOH. The solution was then applied to Protein-A and Protein-G columns sequentially to remove whole immunoglobulins. The purity of the resulting F(\text{ab}’)\text{2} protein was assessed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) followed by Coomassie blue staining and the target fraction in the gel was analyzed in a thin layer chromatography scanner (transmission, zigzag scan, dual wavelength, swing width:8 mm, delta Y: 0.1mm) (CS-9301, Shimadzu).

2.7 Mice and virus. Specific pathogen-free 6 week old BALB/c mice were purchased from Charles River Laboratories International and maintained in the Animal Care Facility, University of Iowa. Briefly, all mice were housed in Thoren individually ventilated cages. Caging and bedding were autoclaved. Irradiated diet was fed.
Filtered water (0.2 µm filter) was provided with Edstrom automatic watering system. HEPA-filtered cage changing stations were used. All persons entering animal rooms worn autoclaved gowns, gloves, hair bonnets, face masks, and shoe covers. All protocols were approved by the University of Iowa Institutional Animal Care and Use Committee. The EMC/2012 strain of MERS-CoV (passage 8, designated MERS-CoV), kindly provided by Drs. Bart Haagmans and Ron Fouchier (Erasmus Medical Center, Rotterdam, Holland), was passaged once in Vero 81 cells and titrated in the same cell line. All work with MERS-CoV was conducted in the University of Iowa Biosafety Level 3 (BSL-3) Laboratory.

2.8 MERS-CoV plaque reduction neutralization assay. Serum samples, purified IgG or F(ab’)2 were serially diluted in DMEM and mixed with an equal volume of MERS-CoV containing 80 PFU. Following incubation at 37°C for 1 h, aliquots were added to cultures of Vero 81 cells in 48 well plates and incubated at 37°C in 5% CO2 for 1 h with gentle rocking every 15 minutes. Plates were then overlaid with 1.2% agarose/DMEM/2% calf serum. After further incubation for 3 days, agarose plugs were removed using a small spatula, and the remaining plaques were visualized by staining with 0.1% crystal violet.

2.9 Antibody treatment and MERS-CoV infection of mice. Six-week-old female BALB/c mice were lightly anesthetized with isoflurane and transduced intranasally with 2.5×10^8 PFU of Ad5-hDPP4 in 75 µL DMEM as described elsewhere (Zhao et al., 2014). Five days post transduction, mice were infected intranasally with MERS-CoV (1×10^5 PFU) in a total volume of 50 µL DMEM. Mice were monitored daily for
morbidity (weight loss) and mortality. All work with MERS-CoV was conducted in the University of Iowa Biosafety Level 3 (BSL-3) Laboratory. Separate groups were injected with 200 µL horse antiserum or 500 µg IgG or F(ab')2 intraperitoneally (IP) 1 day before or after intranasal infection with 1×10^5 PFU MERS-CoV. Control mice were given an equal volume of normal horse serum (Sigma).

2.10 Virus titers. To obtain virus titers, Lungs were harvested from subgroups of 3 animals at the indicated time points (see Results) and homogenized into 3 mL of phosphate buffered saline (PBS), using a manual homogenizer. Lung homogenates were aliquoted into micro tubes and kept in -80°C. Virus was titered on Vero 81 cells. Cells were fixed with 10% formaldehyde and stained with crystal violet three days post-infection (p.i.). Viral titers are expressed as PFU/g tissue for MERS-CoV (Zhao et al., 2014).
3. Results and Discussion

Due to the biosafety risk, MERS-CoV must be handled in a BSL-3 laboratory, whereas VLPs can be rapidly generated under BSL-2 conditions as an immunogen inducing high antibody titers. In addition, the horse provides little risk to humans and produces high antibody yields, making these animals an effective source for production of hyperimmune sera (Zheng et al., 2016).

3.1 Evaluation of equine antibodies. RBD-specific IgG titers in the sera were all above 1:20,480 after five immunizations (Fig. 1) as assessed by ELISA. RBD contains the major neutralizing epitopes of the S protein, as shown by the observation that absorption of SARS patient convalescent sera with SARS-CoV RBD removes the majority of neutralizing antibodies (He et al., 2005). Independent research groups have also shown more directly that the MERS-CoV RBD sequence contains the major antigenic determinants for inducing neutralizing antibodies, and that neutralizing epitopes within MERS-CoV S1 are also localized primarily in the RBD region (Du et al., 2013; Mou et al., 2013). Here, we have demonstrated that anti-RBD antibodies function as major components of neutralizing antibodies. We found that RBD-specific IgG neutralized MERS-CoV infection with half maximal inhibitory concentration of 15.74 µg/mL, and 2.612 \times 10^3 µg/mL for flowthroughs (Fig. 2), suggesting that the RBD of S protein act as an important neutralization determinant of MERS-CoV. Our results demonstrate that equine antibodies are polyclonal and recognize more antigen determinants in MERS-CoV S protein than single mAbs, which could potentially prevent antibody escape.
3.2 Generation of IgG and F(ab’)2. The integrity of IgG and F(ab’)2 fragments was evaluated using an SDS-PAGE gel (Fig. 3A). The purity of the F(ab’)2 fragments after Protein-A/G chromatography was >91% after gel electrophoresis (Fig. 3B). Passive transfer of blood products from other humans poses a safety concern, with possible contamination with agents of blood-borne diseases (e.g., HIV, hepatitis). Heterologous antibody carries a potential risk of allergic reaction, but generation of F(ab’)2 fragments, results in antibodies being less immunoreactive and safer for use in humans.

3.3 Equine antibodies neutralized MERS-CoV in cell culture. While we successfully generated equine antibodies against MERS-CoV VLPs, their protective effect against authentic MERS-CoV infection remained untested. Using a plaque reduction neutralizing assay, we confirmed that immune sera significantly neutralized MERS-CoV infection *in vitro*, with a half effective maximal dilution of 1: 20,900 (Fig. 4A, B). Further, we found that equine IgG and F(ab’)2 also neutralized MERS-CoV infection with half effective maximal concentrations (EC50) of 2.16 µg/mL and 2.60 µg/mL for IgG and F(ab’)2, respectively (Fig. 4C, D). Collectively, these results show that equine antibody products exhibit highly potent neutralizing activity against MERS-CoV.

3.4 Passive transfer of equine antibodies protected MERS-CoV infected mice. Next we asked if adoptive transfer of equine antibodies could protect mice from MERS-CoV infection prophylactically and therapeutically. By using a mouse model we previously generated (Zhao et al., 2014), we injected animals with immune serum
(Fig. 5A, B), purified IgG (Fig. 5C, D) or F(ab’)2 (Fig. 5E, F) i.p. 1 day before (Fig. 5A, C, E) or after (Fig. 5B, D, F) MERS-CoV challenge. In both prophylactic and therapeutic settings, passive transfer of equine immune antibodies resulted in a 2-4 log reduction of virus titers in the lungs of MERS-CoV infected mice, and accelerated virus clearance in the serum treated group (Fig. 5A, B). We did not observe any difference in body weight loss and pathologic changes on the exterior surface of the lungs in treated and untreated mice after infection, since in this model, mice only develop mild lung disease. Rapid virus replication and inflammatory cell infiltration in the infected lungs are the major parameters to measure (Zhao et al., 2014). Since the half-life of F(ab’)2 in vivo is relatively short and MERS-CoV is cleared within 6 days in this model (Zhao et al., 2014), we did not inject F(ab’)2 antibodies before day -1 or after day 1 p.i.

Of note, the purified IgG seemed to have lower protective potency than that of the immune serum in vivo (Fig. 5). The concentration of IgG in serum is >10 mg/ml. We used 200 µl of immune serum (equal to 2 mg IgG) per mouse which is much higher than the immune IgG we used (500 µg/mice). The other reason could be we purified immune IgG using saturated ammonium sulfate precipitation method, which needed to be performed under room temperature. We speculated that some IgGs were degraded or misfolded, and unable to bind to MERS-CoV spike protein under this circumstance. While, immune sera were properly stored at -20°C and contained high concentration of BSA and other proteins, which made the antiserum more stable.

To date, there are several anti-MERS-CoV antibodies developed from different origins. Each antibody contains its own advantages and disadvantages. For monoclonal antibodies, mouse-derived monoclonal antibody needs to be humanized
before human use (Li et al., 2015); a human neutralizing antibody derived from a convalescent MERS patient can be produced in large amount from CHO cells (Corti et al., 2015). However, the single clone antibody raises the concern of viral escape mutant when applied to human. Administration of transchromosomic bovine human immunoglobulins (Luke et al., 2016) or dromedary immune serum (Zhao et al., 2015) resulted in rapidly viral clearance in infected mouse lungs. The disadvantage of these antibodies is that these animals are not readily available. Compared to the antibodies described above, the administration of equine IgG-derived F(ab’)\textsubscript{2} fragment proved to be a versatile and feasible method (Lu et al., 2006; Zhou et al., 2007). It provides a useful platform to produce therapeutics against emerging infectious diseases.

In summary, by immunizing healthy horses with MERS-CoV VLPs, we have successfully developed the first equine IgG-derived F(ab’)\textsubscript{2} fragment that neutralizes MERS-CoV \textit{in vitro} and \textit{in vivo}. Both prophylactic and therapeutic treatments decreased virus loads and accelerated virus clearance in the lungs of MERS-CoV-infected mice. Therefore, horses immunized with MERS-CoV VLPs can serve as a useful initial source for developing protective F(\textit{ab’})\textsubscript{2} fragments, for the purpose of preparedness and to serve as a strategic reserve for a potential MERS epidemic and other emergent pathogens.
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Potential conflicts of interest disclosure

The authors declare no competing interests.
Reference


caused by severe acute respiratory syndrome-associated coronavirus by equine neutralizing antibody in aged mice. Int Immunopharmacol 7, 392-400.

Figure legends

**Fig 1. Robust MERS-CoV RBD-specific antibody in immunized horse sera.**
Horses (n=4) were injected intramuscularly with MERS-CoV VLPs and boosted every two weeks an additional 4 times. Sera were collected 2 weeks after each immunization. RBD-specific antibodies in immunized horse sera were detected using ELISA.

**Fig 2. Neutralizing activity of the RBD-specific antibodies in IgG.** *In vitro* neutralization tests of total IgG, RBD-specific IgG, and flowthroughs, were determined in a series of 2-fold dilutions and 50% neutralization was calculated using Graphpad Prism.

**Fig 3. Generation and purification of IgG and F(ab’)2.** Saturated ammonium sulfate was added to serum to precipitate the IgG, and F(ab’)2 was generated by digestion of the IgG with pepsin, followed by Protein-A/G chromatography. (A) SDS-PAGE electrophoresis and Coomassie blue staining of IgG before dialysis, IgG and F(ab’)2. (B) The purity of F(ab’)2 was 91.3%.

**Fig 4. Immune horse serum, purified IgG and F(ab’)2 neutralized MERS-CoV in vitro.** (A) Serum or (C) antibody samples were serially diluted in DMEM and mixed 1:1 with 80 PFU MERS-CoV. After a 1 h incubation at 37°C, the mixture was added to Vero 81 cell monolayers for an additional 1 hour. Following removal of the supernatants, the cells were then overlaid with 1.2% agarose/containing DMEM/2% calf serum. After a further incubation of 3 days, agarose plugs were removed for virus
tiration. Plaques were visualized by staining with 0.1% crystal violet. (B) Dilutions or (D) concentrations for 50% of maximal neutralizing effect are shown.

**Fig 5. Immune horse serum, purified IgG and F(ab’)2 protected MERS-CoV infected mice.** Ad5-hDPP4 transduced BALB/c mice (6wks, female) were injected intraperitoneally with 200 µL horse serum (A, B), 500 µg purified horse immune IgG (C, D) or purified horse immune F(ab’)2 (E, F) 1 day before (A, C, E) or after (B, D, F) intranasal infection with 1×10^5 PFU MERS-CoV. Virus titers in the lungs were measured at the indicated time points. Titers are expressed as PFU/g tissue. n= 3 mice/group/time point. *P values of <0.05 as compared to control group.
Highlights

1. Healthy horses immunized with MERS-CoV virus-like particles rapidly generate high titers of virus neutralizing antibodies.

2. Passive transfer of equine immune antibodies significantly reduced virus titers from the lungs of MERS-CoV infected mice.

3. F(\text{ab}')_2 fragments prepared by digestion of antibody with pepsin to reduce possible allergic responses.

4. Equine immune antibodies are polyclonal and recognize more antigen determinants in MERS-CoV spike protein.