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Passive immunotherapy for Middle East Respiratory Syndrome coronavirus infection with equine immunoglobulin or immunoglobulin fragments in a mouse model

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38
Abstract

39 Middle East Respiratory Syndrome (MERS) is a highly lethal pulmonary infection
40 caused by a coronavirus (CoV), MERS-CoV. With the continuing spread of MERS-
41 CoV, prophylactic and therapeutic treatments are urgently needed. In this study, we
42 prepared purified equine F(ab')$_2$ from horses immunized with MERS-CoV virus-like
43 particles (VLPs) expressing MERS-CoV S, M and E proteins. Both IgG and F(ab')$_2$
44 efficiently neutralized MERS-CoV replication in tissue culture. Passive transfer of
45 equine immune antibodies significantly reduced virus titers and accelerated virus
46 clearance from the lungs of MERS-CoV infected mice. Our data show that horses
47 immunized with MERS-CoV VLPs can serve as a primary source of protective
48 F(ab')$_2$ for potential use in the prophylactic or therapeutic treatment of exposed or
49 infected patients.

50

51 **Keywords:** Middle East Respiratory Syndrome coronavirus; equine immune serum;
52 immunoglobulin; F(ab')$_2$ fragment; animal model

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54 # 1. Introduction

55 Middle East Respiratory Syndrome (MERS)-CoV is an emerging pathogen that
56 causes severe pneumonia in humans in the Arabian Peninsula and in travelers from
57 this region (Assiri et al., 2013a; Zaki et al., 2012b; Zumla et al., 2015). Human-to-
58 human spread has been documented (Assiri et al., 2013b). While infections of
59 immunocompetent patients generally present with only mild symptoms, the elderly
60 and patients with pre-existing illnesses such as diabetes or renal failure are likely to
61 develop more severe disease (Assiri et al., 2013a). As of September 21, 2016, 1806
62 cases with 643 deaths (35.6% mortality) had been reported to the World Health
63 Organization, although the actual number of infections could be much larger since
64 mild, asymptomatic or undiagnosed cases are likely to be common (Drosten et al.,
65 2014).

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

71 As of now, the most promising treatment is the passive administration of anti-
72 MERS-CoV neutralizing antibodies. Several research groups have developed and
73 produced anti-MERS patient-derived or humanized monoclonal neutralizing
74 antibodies *in vitro* that were able to protect MERS-CoV infected mice (Corti et al.,
75 2015; Li et al., 2015; Zhao et al., 2014). However, since these antibodies react with a
76 single epitope on the MERS-CoV spike (S) protein and since coronaviruses are prone

77 to mutate, this approach has raised concerns about possible antibody escape (Corti et
78 al., 2015; Sabir et al., 2016).

79 Recently, we showed that sera from Middle East dromedary camels contained
80 high levels of anti-MERS-CoV neutralizing antibodies. Passive immunotherapy with
81 sera from these animals significantly reduced virus loads and accelerated virus
82 clearance from the lungs of MERS-CoV infected mice (Zhao et al., 2015). This
83 provides proof of concept that immune animal sera are potentially useful in the
84 treatment of patients with MERS (Hayden et al., 2014). Passive immunotherapy with
85 animal sera or antibodies has been successfully used to prevent rabies and to
86 neutralize snake venom (Both et al., 2012; Gutierrez et al., 2014). Convalescent
87 plasma used to treat patients with SARS has been found safe and has demonstrated
88 some efficacy in a study with a small number of patients (Mair-Jenkins et al., 2015).
89 However, neutralizing antibody titers in MERS patients are generally low and the
90 limited number of MERS survivors makes this approach impractical (Drosten et al.,
91 2013).

92 Here, we show that immunization of healthy horses with MERS-CoV virus-like
93 particles (VLPs) expressing MERS-CoV S, M and E proteins induces strong
94 polyclonal neutralizing antibodies against MERS-CoV. Since administration of whole
95 antibodies can induce allergic responses in some humans, we further tested $F(ab')_2$
96 fragments prepared by digestion of antibody with pepsin. Prophylactic or therapeutic
97 treatment of MERS-CoV infected mice with either IgG or $F(ab')_2$ significantly
98 decreased the virus load in their lungs.

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99 # 2. Materials and methods

100 **2.1 Antigen preparation.** MERS-CoV VLPs were produced and purified as
101 previously described (Wang C, 2016). In brief, army worm Sf9 cells were infected
102 with a single recombinant baculoviruses co-expressing MERS-CoV structural protein
103 genes S, M, and E, at a multiplicity of infection (MOI) of 0.5. Culture supernatants
104 were harvested at 96 h post-infection and centrifuged at 2,000 g for 30 min to remove
105 cell debris. Following centrifugation of the clarified supernatants at 100,000 g for 1 h
106 at 4℃ the resulting VLP pellets were resuspended in PBS and loaded onto a 30–40–
107 50% discontinuous sucrose gradient. After an additional centrifugation at 100,000 g
108 for 1.5 h at 4℃, bands between 30-40% sucrose containing MERS-CoV VLP were
109 collected.
110
111 **2.2 Animal immunization.** Four 4-year-old healthy horses received multi-point
112 intramuscular injections of 0.5, 1.5, 2, 3, and 5 mg MERS-CoV VLPs in 4ml PBS at
113 weeks 0, 2, 4, 6, and 8, respectively. Freund's complete adjuvant (Sigma) was
114 included in the first dose, and incomplete adjuvant in the remaining ones. Sera were
115 collected from the jugular vein 2 weeks after each injection, and stored at -20℃
116 before further analysis.
117
118 **2.3 MERS-CoV specific antibody measurement.** MERS-CoV specific antibodies in
119 the sera were measured by an indirect enzyme-linked immunosorbent assay (ELISA)
120 using purified MERS-CoV receptor-binding domain (RBD) protein (i.e., S protein
121 residues 358-662 cloned into the pET-30a expression vector and purified by Ni-NTA
122 affinity chromatograph column). Briefly, 96-well microtitration plates (Corning

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123 Costar, USA) were pre-coated with 100 μl purified RBD antigen diluted in 0.05 mol/L
124 carbonate sodium buffer (pH 9.6) to a final concentration of 1 μg/mL and incubated at
125 4℃ overnight. After blocking with skimmed milk for 2 h at 37℃, 100 μL twofold
126 serially diluted serum samples were added to the wells, and incubated at 37℃ for 1 h.
127 The plates were washed three times with PBS containing 0.05% Tween-20 (PBST),
128 before addition of 100 μL HRP-labeled rabbit antibody against horse IgG (Bioss,
129 China; 1:20,000) and incubation at 37℃ for 1 h. After washing with PBST, 100 μL 3,
130 3', 3, 5'-tetramethylbenzidine (TMB) (Sigma, USA) as substrate was added to each
131 well and incubated for 30 min. The reaction was stopped with 50 μL 2M H_2SO_4.
132 Optical densities at 450 nm were measured in an ELISA plate reader (Bio-Rad, USA).
133

134 **2.4 Immunoglobulin purification.** Horse antiserum was diluted with 2 volumes of
135 normal saline (0.9% NaCl) and a half volume of saturated ammonium sulfate was
136 then added and mixed gently at room temperature for 30 min before centrifugation at
137 5,000 g for 20 min. The resulting sediment was redissolved in saline and mixed with a
138 one-third volume of saturated ammonium sulfate. After incubation at ambient
139 temperature for 30 min and centrifugation at 5,000 g for 20 min, the second sediments
140 were dissolved in normal saline and dialyzed against normal saline to remove any
141 remaining ammonium salt.
142

143 **2.5 Immunoaffinity chromatography.** Immunoaffinity resins were prepared by
144 coupling 10 mg RBD protein to 0.02 M sodium periodate-activated Sepharose 4B (4
145 g), and then incubating with 150 μL sodium borohydride for 30 min. After reaction
146 with 1 M Tris (pH 7.5) for 30 min, a purified IgG sample was diluted 9-fold with PBS

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147 and incubated with the RBD resin overnight at 4℃ with constant rotation. The
148 flowthroughs (anti-RBD depleted) were collected, and then the flowthroughs were
149 tested against the RBD protein by ELISA to ensure RBD-specific IgG all bound with
150 the RBD Sepharose 4B. After washing with PBS, the bound antibodies (anti-RBD)
151 were eluted in 0.2 M glycine-HCl buffer (pH 2.7). The eluates were neutralized with 1
152 M Tris buffer (pH 9.0), and then dialyzed against PBS. All samples were adjusted to
153 the same protein concentration and sterilized by passage through microspin filters (0.2
154 μm pore size; Millipore). Neutralizing activity of the IgG, RBD-specific IgG, and
155 flowthroughs were tested.
156
157 **2.6 F(ab')$_2$ preparation.** The pH of the horse antiserum was adjusted to 3.3 with 1
158 mol/L HCl. Following incubation with pepsin (10000 IU/mL) at 30℃ for 2.5 h, the
159 reaction was stopped by adjusting the pH to 7.2 with 1 mol/L NaOH. The solution
160 was then applied to Protein-A and Protein-G columns sequentially to remove whole
161 immunoglobulins. The purity of the resulting F(ab')$_2$ protein was assessed by sodium
162 dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) followed by
163 Coomassie blue staining and the target fraction in the gel was analyzed in a thin layer
164 chromatography scanner (transmission, zigzag scan, dual wavelength, swing width:8
165 mm, delta Y: 0.1mm) (CS-9301, Shimadzu).
166
167 **2.7 Mice and virus.** Specific pathogen-free 6 week old BALB/c mice were purchased
168 from Charles River Laboratories International and maintained in the Animal Care
169 Facility, University of Iowa. Briefly, all mice were housed in Thoren individually
170 ventilated cages. Caging and bedding were autoclaved. Irradiated diet was fed.

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171 Filtered water (0.2 μm filter) was provided with Edstrom automatic watering system.
172 HEPA-filtered cage changing stations were used. All persons entering animal rooms
173 worn autoclaved gowns, gloves, hair bonnets, face masks, and shoe covers. All
174 protocols were approved by the University of Iowa Institutional Animal Care and Use
175 Committee. The EMC/2012 strain of MERS-CoV (passage 8, designated MERS-
176 CoV), kindly provided by Drs. Bart Haagmans and Ron Fouchier (Erasmus Medical
177 Center, Rotterdam, Holland), was passaged once in Vero 81 cells and titrated in the
178 same cell line. All work with MERS-CoV was conducted in the University of Iowa
179 Biosafety Level 3 (BSL-3) Laboratory.
180
181 **2.8 MERS-CoV plaque reduction neutralization assay.** Serum samples, purified
182 IgG or $F(ab')_2$ were serially diluted in DMEM and mixed with an equal volume of
183 MERS-CoV containing 80 PFU. Following incubation at 37℃ for 1 h, aliquots were
184 added to cultures of Vero 81 cells in 48 well plates and incubated at 37℃ in 5% CO_2
185 for 1 h with gentle rocking every 15 minutes. Plates were then overlaid with 1.2%
186 agarose/DMEM/2% calf serum. After further incubation for 3 days, agarose plugs
187 were removed using a small spatula, and the remaining plaques were visualized by
188 staining with 0.1% crystal violet.
189
190 **2.9 Antibody treatment and MERS-CoV infection of mice.** Six-week-old female
191 BALB/c mice were lightly anesthetized with isoflurane and transduced intranasally
192 with 2.5×10^8 PFU of Ad5-hDPP4 in 75 μL DMEM as described elsewhere (Zhao et
193 al., 2014). Five days post transduction, mice were infected intranasally with MERS-
194 CoV (1×10^5 PFU) in a total volume of 50 μL DMEM. Mice were monitored daily for

195 morbidity (weight loss) and mortality. All work with MERS-CoV was conducted in
196 the University of Iowa Biosafety Level 3 (BSL-3) Laboratory. Separate groups were
197 injected with 200 μL horse antiserum or 500 μg IgG or $F(ab')_2$ intraperitoneally (IP) 1
198 day before or after intranasal infection with 1×10^5 PFU MERS-CoV. Control mice
199 were given an equal volume of normal horse serum (Sigma).

200

201 **2.10 Virus titers**. To obtain virus titers, Lungs were harvested from subgroups of 3
202 animals at the indicated time points (see Results) and homogenized into 3 mL of
203 phosphate buffered saline (PBS), using a manual homogenizer. Lung homogenates
204 were aliquoted into micro tubes and kept in -80℃. Virus was titered on Vero 81 cells.
205 Cells were fixed with 10% formaldehyde and stained with crystal violet three days
206 post-infection (p.i.). Viral titers are expressed as PFU/g tissue for MERS-CoV (Zhao
207 et al., 2014).

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209 # 3. Results and Discussion

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

215

216 **3.1 Evaluation of equine antibodies.** RBD-specific IgG titers in the sera were all
217 above 1:20,480 after five immunizations (**Fig. 1**) as assessed by ELISA. RBD
218 contains the major neutralizing epitopes of the S protein, as shown by the observation
219 that absorption of SARS patient convalescent sera with SARS-CoV RBD removes the
220 majority of neutralizing antibodies (He et al., 2005). Independent research groups
221 have also shown more directly that the MERS-CoV RBD sequence contains the major
222 antigenic determinants for inducing neutralizing antibodies, and that neutralizing
223 epitopes within MERS-CoV S1 are also localized primarily in the RBD region (Du et
224 al., 2013; Mou et al., 2013). Here, we have demonstrated that anti-RBD antibodies
225 function as major components of neutralizing antibodies. We found that RBD-specific
226 IgG neutralized MERS-CoV infection with half maximal inhibitory concentration of
227 15.74 μg/mL, and 2.612×10^3 μg/mL for flowthroughs **(Fig. 2)**, suggesting that the
228 RBD of S protein act as an important neutralization determinant of MERS-CoV. Our
229 results demonstrate that equine antibodies are polyclonal and recognize more antigen
230 determinants in MERS-CoV S protein than single mAbs, which could potentially
231 prevent antibody escape.

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233 **3.2 Generation of IgG and F(ab')$_2$.** The integrity of IgG and F(ab')$_2$ fragments was
234 evaluated using an SDS-PAGE gel (**Fig. 3A**). The purity of the F(ab')$_2$ fragments after
235 Protein-A/G chromatography was >91% after gel electrophoresis (**Fig. 3B**). Passive
236 transfer of blood products from other humans poses a safety concern, with possible
237 contamination with agents of blood-borne diseases (e.g., HIV, hepatitis).
238 Heterologous antibody carries a potential risk of allergic reaction, but generation of
239 F(ab')$_2$ fragments, results in antibodies being less immunoreactive and safer for use in
240 humans.
241
242 **3.3 Equine antibodies neutralized MERS-CoV in cell culture.** While we
243 successfully generated equine antibodies against MERS-CoV VLPs, their protective
244 effect against authentic MERS-CoV infection remained untested. Using a plaque
245 reduction neutralizing assay, we confirmed that immune sera significantly neutralized
246 MERS-CoV infection *in vitro*, with a half effective maximal dilution of 1: 20,900
247 (**Fig. 4A, B**). Further, we found that equine IgG and F(ab')$_2$ also neutralized MERS-
248 CoV infection with half effective maximal concentrations (EC_{50}) of 2.16 μg/mL and
249 2.60 μg/mL for IgG and F(ab')$_2$, respectively (**Fig. 4C, D**). Collectively, these results
250 show that equine antibody products exhibit highly potent neutralizing activity against
251 MERS-CoV.
252
253 **3.4 Passive transfer of equine antibodies protected MERS-CoV infected mice.**
254 Next we asked if adoptive transfer of equine antibodies could protect mice from
255 MERS-CoV infection prophylactically and therapeutically. By using a mouse model
256 we previously generated (Zhao et al., 2014), we injected animals with immune serum

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257 (**Fig. 5A, B**), purified IgG (**Fig. 5C, D**) or F(ab')$_2$ (**Fig. 5E, F**) i.p. 1 day before (**Fig.**
258 **5A, C, E**) or after (**Fig. 5B, D, F**) MERS-CoV challenge. In both prophylactic and
259 therapeutic settings, passive transfer of equine immune antibodies resulted in a 2-4 log
260 reduction of virus titers in the lungs of MERS-CoV infected mice, and accelerated
261 virus clearance in the serum treated group (**Fig. 5A, B**). We did not observe any
262 difference in body weight loss and pathologic changes on the exterior surface of the
263 lungs in treated and untreated mice after infeciton, since in this model, mice only
264 develope mild lung disease. Rapid virus replication and inflammatory cell infiltration
265 in the infected lungs are the major parameters to measure (Zhao et al., 2014). Since
266 the half-life of F(ab')$_2$ in vivo is relatively short and MERS-CoV is cleared within 6
267 days in this model (Zhao et al., 2014), we did not inject F(ab')$_2$ antibodies before day -
268 1 or after day 1 p.i.

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

278 To date, there are several anti-MERS-CoV antibodies developed from
279 different origins. Each antibody contains its own advantages and disadvantages. For
280 monoclonal antibodies, mouse-derived monoclonal antibody needs to be humanized

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281 before human use (Li et al., 2015); a human neutralizing antibody derived from a
282 convalescent MERS patient can be produced in large amount from CHO cells (Corti
283 et al., 2015). However, the single clone antibody raises the concern of viral escape
284 mutant when applied to human. Administration of transchromosomic bovine human
285 immunoglobulins (Luke et al., 2016) or dromedary immune serum (Zhao et al., 2015)
286 resulted in rapidly viral clearance in infected mouse lungs. The disadvantage of these
287 antibodies is that these animals are not readily available. Compared to the antibodies
288 described above, the administration of equine IgG-derived $F(ab')_2$ fragment proved to
289 be a versatile and feasible method (Lu et al., 2006; Zhou et al., 2007). It provides a
290 useful platform to produce therapeutics against emerging infectious diseases.

291 In summary, by immunizing healthy horses with MERS-CoV VLPs, we have
292 successfully developed the first equine IgG-derived $F(ab')_2$ fragment that neutralizes
293 MERS-CoV *in vitro* and *in vivo*. Both prophylactic and therapeutic treatments
294 decreased virus loads and accelerated virus clearance in the lungs of MERS-CoV-
295 infected mice. Therefore, horses immunized with MERS-CoV VLPs can serve as a
296 useful initial source for developing protective $F(ab')_2$ fragments, for the purpose of
297 preparedness and to serve as a strategic reserve for a potential MERS epidemic and
298 other emergent pathogens.

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306

307 ## Potential conflicts of interest disclosure

308 The authors declare no competing interests.

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412 ## Figure legends

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

418

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

423

424 **Fig 3. Generation and purification of IgG and F(ab')$_2$.** Saturated ammonium
425 sulfate was added to serum to precipitate the IgG, and F(ab')$_2$ was generated by
426 digestion of the IgG with pepsin, followed by Protein-A/G chromatography. (**A**) SDS-
427 PAGE electrophoresis and Coomassie blue staining of IgG before dialysis, IgG and
428 F(ab')$_2$. (**B**) The purity of F(ab')$_2$ was 91.3%.

429

430 **Fig 4. Immune horse serum, purified IgG and F(ab')$_2$ neutralized MERS-CoV *in***
431 ***vitro***. (**A**) Serum or (**C**) antibody samples were serially diluted in DMEM and mixed
432 1:1 with 80 PFU MERS-CoV. After a 1 h incubation at 37°C, the mixture was added
433 to Vero 81 cell monolayers for an additional 1 hour. Following removal of the
434 supernatants, the cells were then overlaid with 1.2% agarose/containing DMEM/2%
435 calf serum. After a further incubation of 3 days, agarose plugs were removed for virus

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436 tiration. Plaques were visualized by staining with 0.1% crystal violet. (**B**) Dilutions or
437 (**D**) concentrations for 50% of maximal neutralizing effect are shown.
438
439 **Fig 5. Immune horse serum, purified IgG and F(ab')$_2$ protected MERS-CoV**
440 **infected mice.** Ad5-hDPP4 transduced BALB/c mice (6wks, female) were injected
441 intraperitoneally with 200 μL horse serum (**A, B**), 500 μg purified horse immune IgG
442 **(C, D)** or purified horse immune F(ab')$_2$ **(E, F)** 1 day before **(A, C, E)** or after **(B, D,**
443 **F)** intranasal infection with 1×10^5 PFU MERS-CoV. Virus titers in the lungs were
444 measured at the indicated time points. Titers are expressed as PFU/g tissue. n= 3
445 mice/group/time point. *P values of <0.05 as compared to control group.

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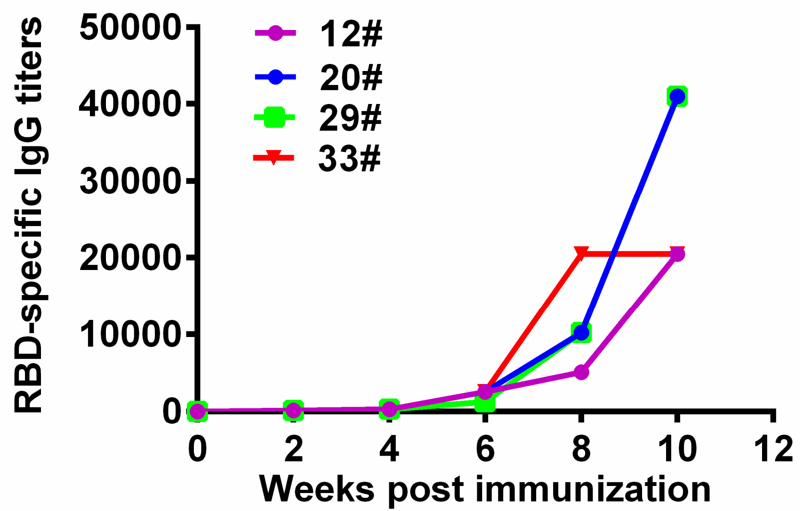

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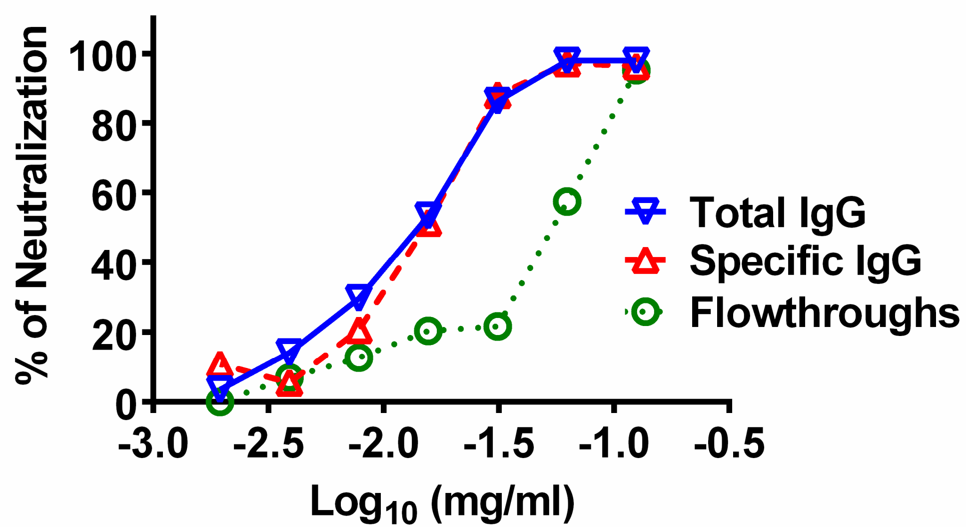

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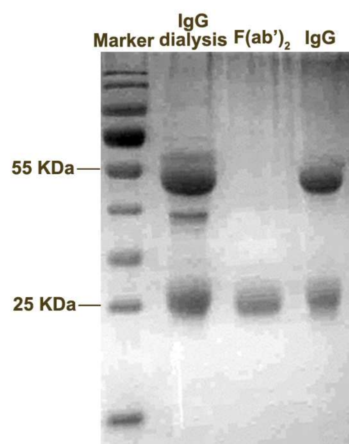
A



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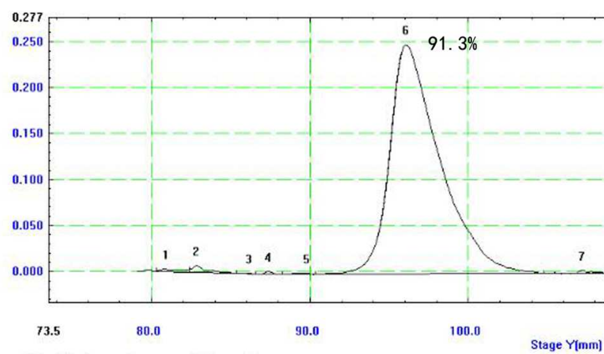

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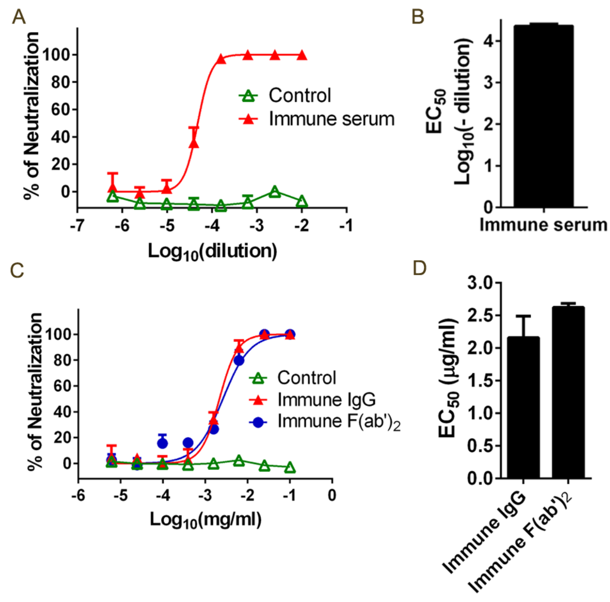

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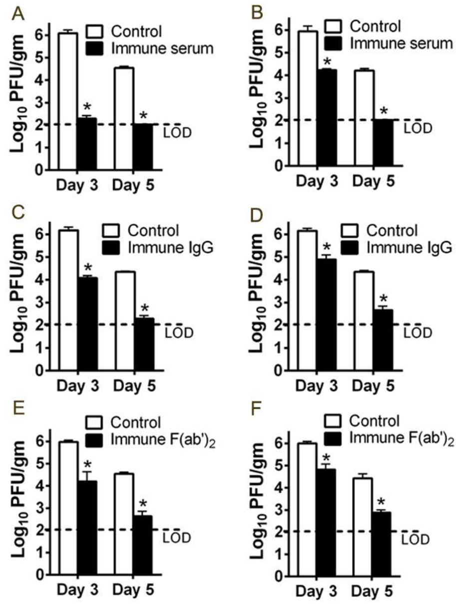

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Highlights

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(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.

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