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Protection from infection with influenza A H7N9 virus in a mouse model by equine neutralizing F(ab′)$_2$

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ABSTRACT

Influenza A H7N9 virus has demonstrated considerable pandemic potential in China ever since early spring 2013. Until now, there have been no specific medicines to treat influenza A H7N9 virus infected patients. Development of a safe and effective H7N9 therapeutic preparation is urgently needed. To this end, we prepared and evaluated the pepsin-digested F(ab′)$_2$ fragments of serum IgGs from the horses inoculated with a inactivated influenza A H7N9 whole virus antigens. The protective effects of the F(ab′)$_2$ fragments against H7N9 virus infection were determined in cultured MDCK cells by cytopathic effect (CPE) and evaluated in a BALB/c mouse model by observing death, weight loss and viral load. The in vitro results showed that the F(ab′)$_2$ fragments had an HI titer of 1:2048 and a neutralization titer of 1: 31,623. The in vivo assays suggested that 600U of the preparations could efficiently protect BALB/c mice from a lethal dose of A/Anhui/01/2013 (H7N9) infection even when administered two days post infection. Thus, this highly purified preparation should be a potential candidate for treating severe patients suffering from influenza A H7N9.

1. Introduction

In recent years, the emergence of highly pathogenic avian influenza viruses such as H7N9 and H5N1, their transmission from poultry to humans, and the increase in global travel frequency have created the potential of a new pandemic, which spread and severity should be limited by some strategies [1,2]. The prevention and treatment of highly pathogenic avian influenza (HPAI) virus include some traditional means such as segregation [3] and several contemporary preparations such as antiviral drugs, vaccines, and antibodies under development.

Currently, two classes of medicines are available with antiviral activity against influenza virus, which are the M2 inhibitors (Amantadine and Rimantadine) and the neuraminidase inhibitors (Oseltamivir and Zanamivir). Unfortunately, some currently circulating H7N9 strains are fully resistant to the neuraminidase inhibitors [4–6]. Resistance to M2 inhibitors has been clinically negligible so far, but which is likely to be detected during widespread use during a pandemic. Meanwhile, the neuraminidase inhibitors may improve the outcomes of HPAI infected patients if administered early, but the clinical evidence is not enough for proving the certain protective efficacy of treating severe HPAI infected patients.

Several strategies are being used to develop vaccines that protect humans from influenza virus infection, such as inactivated, subunit, and live attenuated vaccines [7–9]. Even developed successfully, large-scale innoculation of the vaccine may not be one of the best means of controlling the spread of the disease because of the low incidence of new cases of HPAI virus infection [10,11].

Passive immunity has long been used in the treatment of infectious diseases. The practice of administering polyclonal immunoglobulins from hyperimmune sera of animal or human origin has a century old history for controlling many viral infections, such as HIV [12], rabies [13] and hepatitis B virus [14]. In our previous studies, we have developed equine anti-SARS-CoV and anti-HPAI H5N1 virus F(ab′)2 fragments, which were shown to effectively neutralize SARS-CoV or H5N1 virus in vitro and in vivo [15,16].

In this study, we speculated that specific antibodies against HPAI H7N9 virus may be an alternative strategy for the treatment of severe HPAI H7N9 virus infected patients. So, the F(ab′)$_2$ fragments of the serum IgGs from the horses immunized with an inactivated influenza A H7N9 whole virus antigens were prepared. Thereafter, the protective efficacy of the F(ab′)$_2$ fragments against HPAI H7N9 virus was investigated in vitro and in vivo. These results reported herein will provide concrete experimental data for the further clinical studies.

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2. Materials and methods

2.1. Virus, cells and animals

Wild-type influenza H7N9 virus A/Anhui/01/2013 (Ah01/H7N9) was inoculated in the allantoic cavity of 10-day-old specific pathogen free (SPF) embryonated eggs. The allantoic fluid was harvested 3 days post inoculation and stored at −80 °C until use. MDCK cells were cultured in DMEM medium with 10% FBS in culture flask at 37 °C, 5% CO_2. 4 weekold BALB/c mice were approved for the experiments from the institutional animal welfare committee. All embryonated eggs and animals used in this study were provided by Laboratory Animal Center, Academy of Military Medical Science. All operations with live Ah01/H7N9 virus were performed in a bio-safety level 3 lab.

2.2. Preparation of inactivated whole virus influenza A H7N9 antigens

The purified inactivated H7N9 virus antigens were prepared according to the same standard techniques that are used for the production of seasonal influenza trivalent inactivated whole virus vaccine [8]. Briefly, Ah01/H7N9 was inoculated in the allantoic cavity of 10-day-old SPF embryonated eggs, harvested 3 days post inoculation and inactivated by formaldehyde in a bio-safety level 3 lab. Thereafter, the inactivated virus was concentrated, purified on a pilot scale using Good Manufacturing Practices in Hengye Biological Company, Shandong. The purity of the harvested virus was further analyzed with HPLC with TSKG4000SW (TosoHaas, Tokyo, Japan) as the molecular sieving matrix and stored at 4 °C until use.

2.3. Inoculating horses

The 4–6 year old, healthy brown horses (300–350 kg in weight) that had no detectable antibodies against influenza A H7N9 virus were provided by Chifeng Boen Pharmacy Co., LTD (Inner Mongolia, China) and inoculated subcutaneously with influenza A H7N9 antigens containing 1.0 mg, 1.5 mg, 2.0 mg and 3.0 mg HA content on days 0, 21, 42 and 63, respectively. For each inoculation, 5 ml of antigen suspension was mixed with an equal volume of Freund′s adjuvant according to the directions and injected into the several sites near the submandibular and inguinal lumph nodes. Two weeks post the last immunization, the horses were bled and the sera were detected for the titer of influenza A H7N9 virus specific antibody by hemagglutination inhibition (HI) assay as previously described [15].

2.4. Preparation of the F(ab′)$_2$ fragments of equine serum IgGs

The F(ab′)$_2$ fragments of equine serum IgGs with the HI titer of 1:1024 were prepared by Shanghai Serum Bio-tech Co., Ltd.(Shanghai, China) as previously described [15]. The final F(ab′)$_2$ products were determined by Lowry method, HPLC respectively and stored at 4 °C until use.

2.5. Determination of the neutralization titer of the F(ab′)$_2$ fragments (in vitro and in vivo)

The neutralization titer of the F(ab′)$_2$ fragments against influenza A H7N9 virus was determined in vitro as previously described [8]. In brief, the F(ab′)$_2$ fragments were diluted, initially to 2.5 μg/ml as the highest dose, and then by twofold serial dilution in culture medium. After mixture 1:1(*v*/*v*) with virus suspension containing 100$TCID_{50}$ of live Ah01/H7N9 virus and incubation at 37 °C for 1 h, the F(ab′)$_2$ and virus mixtures were added into fresh monolayer MDCK cells. The plates were incubated for 3 days at 37 °C in a 5% CO_2 atmosphere. The ND_{50} of equine F(ab′)$_2$ that gave 50% neutralization was calculated using the Reed and Muench method and expressed as the reciprocal of the highest F(ab′)$_2$ dilution. The bioactivity unit (U) of the F(ab′)$_2$ fragments against influenza A H7N9 virus was determined in a mouse model. The F(ab′)$_2$ fragments were diluted by twofold serial dilution in PBS, mixed 1:1(*v*/*v*) with virus suspension containing 10^8TCID_{50}/ml (about 10LD_{50}/10 μl in the mouse model) of live Ah01/H7N9 virus and incubated at 37 °C for 1 h, 20 μl of the F(ab′)$_2$ and virus mixtures were i.n. infected into four week old BALB/c mice on day 0. Mice were monitored for survival daily until day 14. The bioactivity unit (U) of the F(ab′)$_2$ fragments against influenza A H7N9 virus was expressed as the reciprocal of the highest dilution that can protect mice from 1 LD_{50} of live Ah01/H7N9 virus.

2.6. Therapeutic efficacy of equine F(ab′)$_2$ fragments against Ah01/H7N9 virus in mice (in vivo)

The therapeutic efficacy of equine F(ab′)$_2$ fragments against Ah01/H7N9 virus was investigated in a mouse model as previously described [15]. To investigate the dose–efficacy correlation, four week old BALB/c mice were i.n. infected with 10 LD_{50} live Ah01/H7N9 on day 0, followed by i.p. injection of the F(ab′)$_2$ fragments at four doses (120, 480, 600 and 720U) or normal horse F(ab′)$_2$ fragments (0U) as a negative control on day 2 post infection. To study the time–efficacy correlation, four week old BALB/c mice were i.n. infected with 10 LD_{50} live Ah01/H7N9 on day 0, followed by i.p. injection of the F(ab′)$_2$ fragments at 600U on different day post infection. Mice were monitored for clinical signs and body weight was measured daily until day 14. The viral load in the infected lung of 5 mice from each group was determined as previously described [15] after euthanasia on day 6.

2.7. Statistical analysis

All data were analyzed using the GraphPad Prism 5.0 software. Survival data were analyzed by Kaplan–Meier analysis. Antibody and virus titers were compared using the two-tailed *t*-test.

3. Results

3.1. Preparation of Ah01/H7N9 virus antigens and equine neutralizing F(ab′)$_2$ fragments

When run on a 12% SDS-PAGE gel (Fig. 1A), several viral major antigen bands of the Ah01/H7N9 virus antigens prepared by Hengye Biological Company were corresponding to the HA1, HA2, and NA protein. HPLC results (Fig. 1B) showed that the purity was 98.5%.

Two weeks after the last immunization, serum was harvested in Chifeng Boen Pharmacy Co., LTD when the HI titer of equine serum antibody against Ah01/H7N9 achieved over 1024 and the F(ab′)$_2$ fragments were prepared by Shanghai Serum Bio-tech Co., Ltd. The HI titers of the purified F(ab′)$_2$ fragments against Ah01/H7N9 were 2048 (Table 1). When analyzed in SDS-PAGE, the F(ab′)2 fragments migrated as one 100 kDa major band (Fig. 2A). The purity of the F(ab′)2 fragments reached over 85%, as measured by the folium scan method (Fig. 2B). Protein concentrations of equine crude serum and purified F(ab′)$_2$ fragments were determined by Lowry method. Compared with more than 65 mg/ml of equine crude serum, protein concentration of purified F(ab′)$_2$ fragments was about 1.5 mg/ml with the recovery rate about 84%.

3.2. Neutralization titers of F(ab′)$_2$ fragments against Ah01/H7N9 virus in vitro and in vivo

The results showed that the ND_{50} of the equine F(ab′)2 fragments against Ah01/H7N9 in cultured MDCK cells was 31,623 and the bioactivity units in the BALB/c model were 9000U/ml.

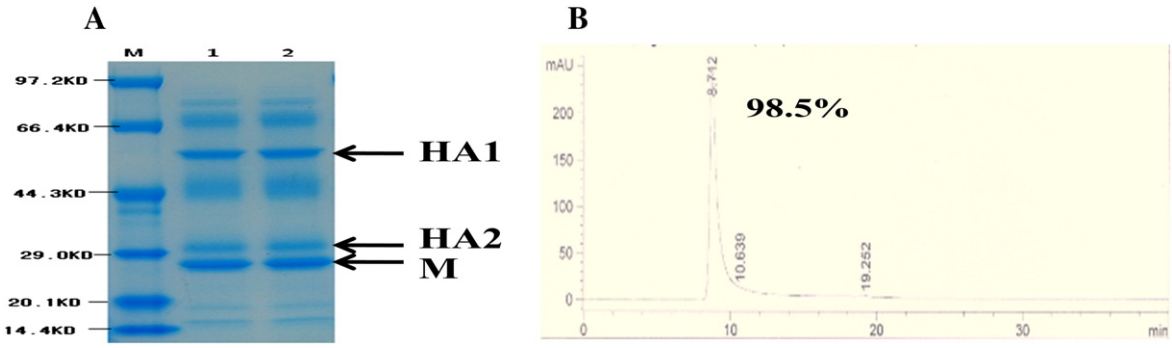

Fig. 1. Characterization of HPAI H7N9 virus antigen. (A) SDS-PAGE of HPAI H7N9 virus antigen. M, protein molecular weight ladder with indicated MW of each band. Lanes 1–2, HPAI H7N9 virus antigen, the HA1, HA2, and M protein bands were shown. (B) HPAI H7N9 virus antigen was identified by HPLC.

3.3. Therapeutic role of equine F(ab′)$_2$ against Ah01/H7N9 virus infection in BALB/c mice

To evaluate the therapeutic dose–efficacy correlation of equine F(ab′)2 fragments, mice received equine F(ab′)$_2$ fragments at the indicated doses on day 2 post lethal Ah01/H7N9 virus challenge. The data showed that 720U of equine F(ab′)2 fragments was required to confer complete protection, which could greatly relieve the clinical disease signs, with lower doses (120, 480U) showing partial protection (Fig. 3). The lower dosages of F(ab′)$_2$ fragments also showed increased clinical disease signs; however all of the mice that did not succumb to infection recovered almost the initial weight by day 14(Data not shown).

To further investigate the therapeutic time–efficacy correlation of equine F(ab′)$_2$ fragments, mice received 600U of equine F(ab′)$_2$ fragments on the indicated day post lethal Ah01/H7N9 virus challenge. The data showed that when 600U of equine F(ab′)$_2$ fragments was used, the sooner the preparation was injected, the better the protective efficacy showed (Fig. 4).

Our viral load assays further confirmed these results. For example, 600U of F(ab′)$_2$ fragments when used two days post lethal Ah01/H7N9 virus challenge could significantly decrease viral loads in infected mice lungs, even more than 1000 folds when compared to that administrated by normal equine F(ab′)$_2$ fragments control (data not shown).

4. Discussion

In this study, we confirmed that specific antibodies could improve the outcomes of severe HPAI H7N9 virus infected cases in a mouse model, which will allow the host's immune system to clear the remaining virus or provide the chance for the intervention of other strategies such as antiviral drugs and vaccines to ultimately eliminate the virus from the host.

Even though there are only sporadic cases infected with HPAI virus such as H5N1 and H7N9, HPAI virus currently has the character of high mortality, which could fatally jeopardize the already infected patient's life [2,3]. Many strategies for the prevention and treatment of HPAI virus infection are under development, but they almost all cease in clinical phase I because of low morbidity of these diseases and low commercial profits of these products [10]. Once HPAI virus suddenly causes a pandemic in humans by some uncertain factors, how can we arm ourselves against its threat? Especially, as the latent phase of HPAI virus infection is often short, and the early symptoms are usually hard to distinguish from those of the common cold, any delay in diagnosis and treatment could threat the patients' lives. The highly purified antibody fragments may be one of the best choices for controlling these diseases.

With more and more drug resistant HPAI H7N9 variant occurrence [4–6], it is urgent and ideal to develop antibody medicines. Compared with the longer time distance of any vaccines to elicit protective immunity in the healthy hosts, the highly purified F(ab′)$_2$ fragments against HPAI H7N9 virus could provide immediate protection regardless of the immune status of the host. Meanwhile, there is still a long way to go from bench to bedside for various vaccines against HPAI H7N9 virus under development [7,8].

Highly purified equine F(ab′)$_2$ fragments have been proved to be safe and used for adoptive humoral therapy for several diseases for many decades, which have many advantages, such as surmounting the bottleneck of antibody yield, possessing broader antigenic coverage and a lower likelihood of emergence of escape mutants compared with humanized monoclonal antibodies and without unavailability of

Table 1
Neutralization and HI titer of equine F(ab′)$_2$ against H7N9.

Purified equine F(ab′)$_2$	Virus (Ah01/H7N9)		
	Bioactivity units (BALB/c)[a]	ND50(MDCK)[b]	HI[c]
Negative F(ab′)$_2$ control	<10	<10	<10
Equine F(ab′)$_2$ bio-preparation	9000U/ml	31,623	2048

[a] Equine F(ab′)$_2$ was diluted by two-fold serial dilution in PBS, mixed 1:1(*v*/*v*) with virus suspension containing 10^8TCID$_{50}$/ml (about 10LD$_{50}$/10 μl in the mouse model) of live Ah01/H7N9 virus and incubated at 37 °C for 1 h, 20 μl of the F(ab′)$_2$ and virus mixtures were i.n. infected into four week old BALB/c mice on day 0. Mice were monitored for survival daily until day 14. The bioactivity unit (U) of the F(ab′)$_2$ fragments against influenza A H7N9 virus was expressed as the reciprocal of the highest dilution that can protect mice from 1 LD$_{50}$ of live Ah01/H7N9 virus.

[b] Equine F(ab′)$_2$ was diluted. The antibodies were mixed with 100 TCID$_{50}$ of Ah01/H7N9 virus and incubated at 37 °C for 1 h, and the mixture was added into monolayer of MDCK cells in triplicate wells. The plates were incubated for 3 days at 37 °C in a 5% CO_2 atmosphere. The ND$_{50}$ of equine F(ab′)$_2$ that gave 50% neutralization was calculated using the Reed and Muench method and expressed as the reciprocal of the highest F(ab′)$_2$ dilution.

[c] Equine F(ab′)$_2$ was tested for the titer of HI antibodies by standard methods using 4 HA unit of virus in v-bottom 96-well micro titer plates with 0.5% turkey erythrocytes and the HI titer was expressed as the reciprocal of the highest F(ab′)$_2$ dilution.

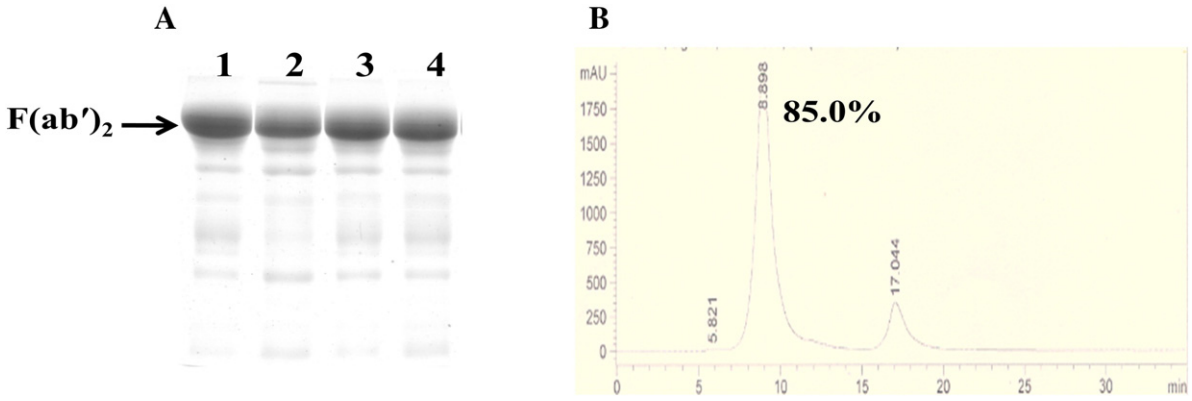

Fig. 2. Characterization of equine purified $F(ab')_2$. (A) SDS-PAGE of equine purified F(ab′)2. Lane 1–4, purified $F(ab')_2$. (B) HPLC of purified F(ab′)2 with the $F(ab')_2$ peak indicated.

convalescent or immunized human plasma [15–20]. Meanwhile, equine $F(ab')_2$ fragments are relatively economic and easily available upon request. Take HPAI H7N9 virus emergency for consideration; we prepared the $F(ab')_2$ fragments against HPAI H7N9 virus and investigated those protective efficacy in vitro and in vivo.

The quality of neutralization antibody can influence the treatment efficacy, which may depend on the quantity and quality of the innoculation antigens and F(ab′)2 fragments. According to our assays, the purity of the innoculation antigens and $F(ab')_2$ fragments was 98.5 and 85%, respectively, which meet the requests of the bio-preparation in china. The bioactivities of the $F(ab')_2$ bio-preparation were 31,623 in vitro and 9000U/ml in vivo, which satisfied the study.

Many studies of the humoral immune response of virus infected patients indicated that patients with a longer course of illness showed a lower neutralizing antibody response, a higher virus load and a longer virus discharge time than patients with a shorter illness duration, which suggested that neutralizing antibodies in patients play a pivotal role in virus clearance in vivo [18].

We don't know the dose–efficacy correlation of the $F(ab')_2$ bio-preparation against HPAI H7N9 virus infection. To address this question, we evaluated the therapeutic efficacy of different doses of $F(ab')_2$ fragments against Ah01/H7N9 virus in mice. The results showed that even 720U of the $F(ab')_2$ innoculated within two days post infection could completely protect mice from diseases upon lethal challenge.

To address the time–efficacy correlation of the $F(ab')_2$ bio-preparation against HPAI H7N9 virus infection, we investigated the therapeutic efficacy of 600U of $F(ab')_2$ fragments against Ah01/H7N9 virus in mice on different day post infection. The data indicated that when innoculated within three days post infection, 600U of the $F(ab')_2$ could greatly improve the outcomes of the diseases. However, when innoculated on day 4 post infection, 600U of the $F(ab')_2$ could not show any therapeutic efficacy. Higher dose of the $F(ab')_2$ could maybe treat the diseases in the later phase.

Accordingly, the level of protection observed (1000-fold decrease in viral load of the lung), although not complete, is remarkable, considering that the circulating antibodies can only neutralize the extracellular HPAI H7N9 virus. In addition, the protected animals showed lighter pathological changes in their lungs (data not shown). Our results also suggested that it might be necessary to combine passive antibody transfer with other methods such as antiviral drugs. It is reasonable that a saturating concentration of this antibody could not completely neutralize all viral particles.

In summary, equine $F(ab')_2$ fragments used in this study exhibited an excellent therapeutic effect against influenza A H7N9 virus, which provided strong experimental support for the further clinical large trials.

Disclosure statement

All authors declare no financial or commercial conflicts of interest.

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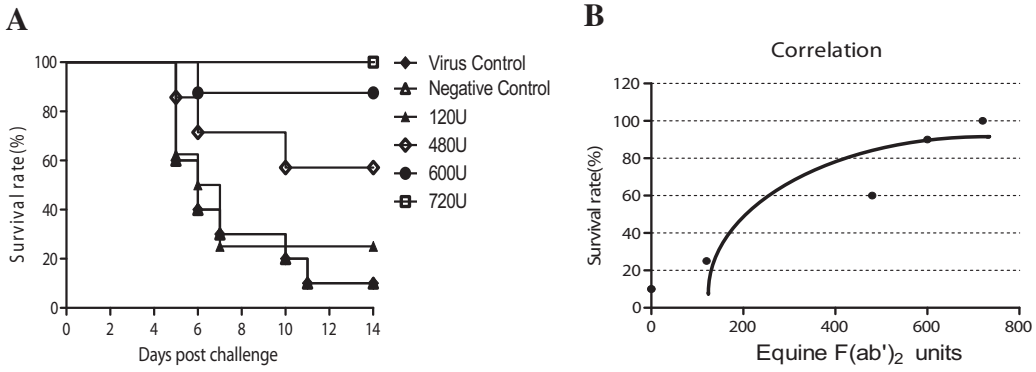

Fig. 3. The dose–efficacy correlation of equine $F(ab')_2$ fragments against Ah01/H7N9 virus in a mouse model. Four week old BALB/c mice were i.n. infected with 10 LD_{50} live Ah01/H7N9 on day 0, followed by i.p. injection of the $F(ab')_2$ fragments at four doses (120, 480, 600 and 720U) or normal horse $F(ab')_2$ fragments (0U) as a negative control on day 2 post infection. Mice were monitored for clinical signs until day 14. (A) Survival rates of mice (20 mice per group) after lethal dose Ah01/H7N9 virus challenge. (B) The dose–efficacy correlation of equine $F(ab')_2$ fragments against Ah01/H7N9 virus.

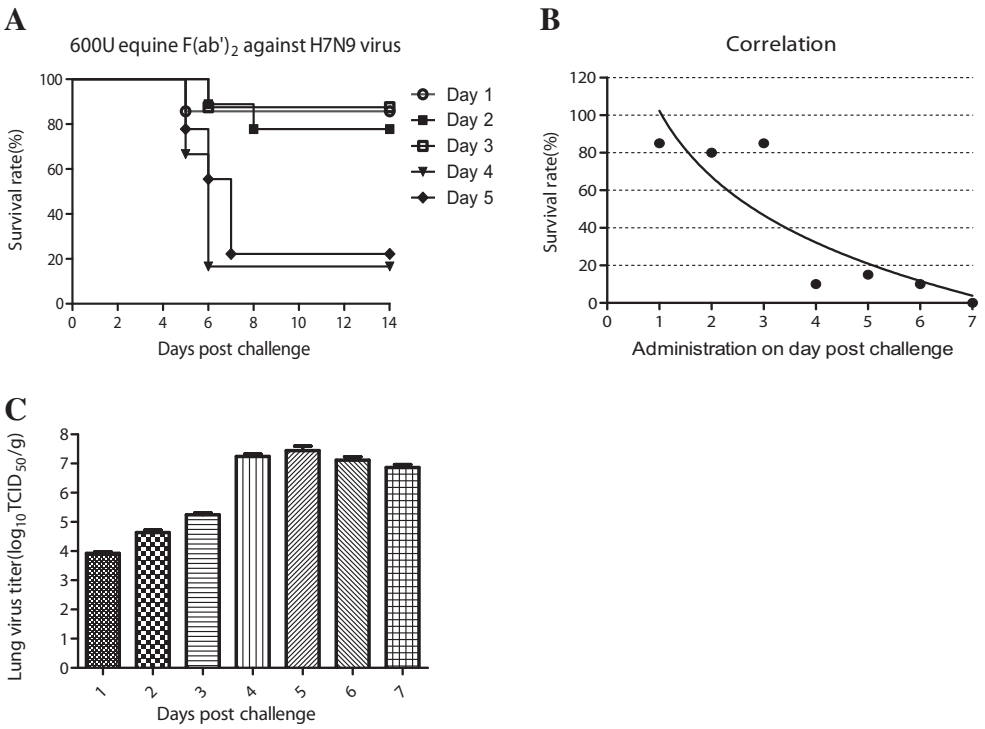

Fig. 4. The time–efficacy correlation of equine F(ab′)$_2$ fragments against Ah01/H7N9 virus in a mouse model. Four week old BALB/c mice were i.n. infected with 10 LD50 live Ah01/H7N9 on day 0, followed by i.p. injection of the F(ab′)$_2$ fragments at 600U (100 μg/injection) on different day post infection. Mice were monitored for clinical signs until day 14. (A) Survival rates of mice (20 mice per group) after lethal dose Ah01/H7N9 virus challenge. (B) The time–efficacy correlation of equine F(ab′)$_2$ fragments against Ah01/H7N9 virus. (C) Kinetic changes of the viral loads in the Ah01/H7N9 virus infected lungs.

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