Vaccine 37 (2019) 5341–5349

Contents lists available at ScienceDirect

Vaccine

journal homepage: www.elsevier.com/locate/vaccine

An adult gerbil model for evaluating potential coxsackievirus A16 vaccine candidates

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ARTICLE INFO

Article history:
Received 19 March 2019
Received in revised form 19 June 2019
Accepted 11 July 2019
Available online 24 July 2019

Keywords:
Coxsackievirus A16
Animal model
Gerbils
Inactivated vaccine

ABSTRACT

A suitable animal model of CVA16 infection is crucial in order to understand its pathogenesis and to help develop antiviral vaccines or screen therapeutic drugs. The neonatal mouse model has a short sensitivity period to CA16 infection, which is a major limitation. In this study, we demonstrate that adult (60-day-old) gerbils are susceptible to CVA16 infection at high doses ($10^{8.0}$ $TCID_{50}$). A clinical isolate strain of CVA16 was inoculated intraperitoneally into adult gerbils, which subsequently developed significant clinical symptoms, including hind limb weakness, paralysis of one or both hind limbs, tremors, and eventual death from neurological disorders. Real-time RT-PCR revealed that viral loads in the spinal cord and brainstem were higher than those in other organs/tissues. Histopathological changes, such as neuronal degeneration, neuronal loss, and neuronophagia, were observed in the spinal cord, brainstem, and heart muscle, along with necrotizing myositis. Gerbils receiving both prime and boost immunizations of alum adjuvant inactivated vaccine exhibited no clinical signs of disease or mortality following challenge by CVA16, whereas 80% of control animals showed obvious clinical signs, including slowness, paralysis of one or both hind limbs, and eventual death, suggesting that the CVA16 vaccine can fully protect gerbils against CVA16 challenge. These results demonstrate that an adult gerbil model provides us with a useful tool for studying the pathogenesis and evaluating antiviral reagents of CVA16 infection. The development of this animal model would also be conducive to screening promising CVA16 vaccine candidates as well as further vaccination evaluation.

1. Introduction

Coxsackievirus A16 (CVA16) belongs to the human enterovirus A (HEV-A) species of the enterovirus genus in the Picornaviridae family. CVA16 is one of the major pathogens associated with hand, foot, and mouth disease (HFMD). HFMD is a viral infection that is prevalent worldwide, especially in the Asia-Pacific region [1–11]. CVA16 is a small, nonenveloped, single-stranded, positive-sense RNA virus and it was first isolated in South Africa in 1951 [12–14]. Clinical data show that CVA16 infection generally induces mild and self-limiting symptoms, such as vesicular maculopapular rash, blisters/ulcers, and pharyngitis, and only occasionally causes severe neurological manifestations, such as meningitis, encephalitis, myelitis, myocarditis, and pneumonia [15–17]. Fatal cases of CVA16 infection are sporadically reported in various countries [18–21]. CVA16 mostly affects infants and children under the age of five. CVA16 infection can also occur in adolescents and adults. Recently, a fatal CVA16 infection associated with pneumonitis in an adult was reported [20]. To date, the specific clinical characteristics and unknown pathogenesis of CVA16 infection have impeded the development of antiviral agents.

An appropriate infection animal model would provide an important tool for insight into the pathogenesis of CVA16 disease and vaccine development. Most studies of CVA16, however, have been conducted solely in neonatal mice. Sickles et al. discovered

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https://doi.org/10.1016/j.vaccine.2019.07.046
0264-410X/

in 1951 that coxsackievirus A generally has strong pathogenicity in suckling mice, inducing pronounced flaccid paralysis but no significant lesions in the central nervous system (CNS) [13]. Mao et al. used the clinically isolated BJCA08/CVA16 strain to develop the first CVA16 neonatal mouse model for the evaluation of the protective efficacy of vaccines [22].

Nevertheless, the greatest limitation of these models was the immaturity of their immune systems. Their brief susceptibility period to disease limits their usefulness for vaccine efficacy studies. An adult animal model would provide necessary data on the pathogenesis and protective mechanisms of immunity in adults infected with CVA16. A host-virus system in adult mice would provide an excellent model in which to study the mechanisms of active immunity against CVA16 and the pathological characteristics of CVA16-infected adult mice. Moreover, the infection model would provide an important tool for evaluating CVA16 vaccines. Thus far, no adult animal model has been established to investigate the pathogenesis of CVA16 disease and to evaluate the efficacy of vaccines. Therefore, an appropriate adult animal model is urgently needed.

In this paper, we first demonstrate that adult (60-day-old) gerbils can be infected by CVA16 at relatively high virus titer ($10^{8.0}$ $TCID_{50}$). Some of the infected gerbils developed neurological lesion-related symptoms. Severe necrosis was observed in the spinal cord, brainstem, and heart muscle, eventually resulting in death. Adult gerbils immunized with inactivated CVA16 whole-virus vaccine were protected from lethal challenges, and no pathological changes were observed in various tissues. The neutralizing antibody responses elicited by CVA16 vaccine prime-boost immunization could be sustained for 12 weeks. Collectively, the CVA16 vaccine was found to protect gerbils from infection. The CVA16-infected adult gerbil model is a promising tool for the study of pathogenesis and vaccine development of CVA16 infection.

2. Materials and methods

2.1. Ethics statement

All of the animal care and use protocols were performed in accordance with the Regulations for the Administration of Affairs Concerning Experimental Animals of the People's Republic of China. All of the animal experiments were approved by the Institutional Animal Care and Use Committee of the Zhejiang Provincial Center for Disease Control and Prevention. All of the study procedures were carried out in accordance with the World Medical Association's Declaration of Helsinki. Written informed consent was obtained from the parents of each patient. The study protocol was approved by the Hangzhou Sixth People's Hospital Ethics Committee.

2.2. Viruses and cells

Vero (African green monkey kidney) and MRC-5 (human diploid cell) cells were maintained in Modified Essential Medium (MEM) (Life Technologies Corporation) and supplemented with 10% heat-inactivated fetal bovine serum (FBS) as well as 100 units/mL of penicillin and 100 μg/mL of streptomycin. All of the cells were cultured in a 37 °C incubator with 5% CO_2. The CVA16 isolate, CVA16-393 (GenBank ID: KY014077), was obtained from a 15-month-old patient in the Hangzhou Sixth People's Hospital, Hangzhou China who suffered from mild HFMD. This virus strain was selected for the preparation of a CVA16 candidate vaccine due to its high immunogenicity and high yield in MRC-5 cell culture. The CVA16-194 (GenBank ID: KX056216) used has been previously described [23]. Two stock viruses were prepared from infected Vero, which displayed 80% cytopathic effect (CPE) 2–3 days after inoculation. Two freeze–thaw cycles were completed, followed by centrifugation at 2000*g* for 15 min at 4 °C to remove cellular debris. Viral supernatants were then harvested and stored at −80 °C. The 50% tissue culture infective dose ($TCID_{50}$) was determined in the Vero cells using the Reed and Muench formula. The titers of CVA16-393 and CVA16-194 were $10^{7.5}$ and $10^{8.5}$ $TCID_{50}$, respectively.

2.3. CVA16 infection experiments

Healthy gerbils were purchased from the Animal Center of the Zhejiang Academy of Medical Sciences, Hangzhou, China. To determine the susceptibility of adult gerbils to CVA16 infection, 3 groups of 60-day-old gerbils (n = 10 for each group, 5 males and 5 females) were inoculated intraperitoneally with CVA16-194 at $TCID_{50}$ of $10^{6.0}$, $10^{7.0}$, and $10^{8.0}$. All of the gerbils were monitored twice daily for 20 days for clinical symptoms, weight changes, and mortality. The grade of clinical disease was scored as follows: 0, healthy; 1, ruffled hair, hunchbacked, or reduced mobility; 2, limb weakness; 3, paralysis in one hind limb; 4, paralysis in both hind limbs or deep lethargy; 5, death. The disease symptoms were recorded twice daily after infection. Control animals were inoculated with phosphate-buffered saline (PBS).

2.4. Histopathology and immunohistochemistry

The 60-day-old gerbils were inoculated intraperitoneally with CVA16-194 at a $TCID_{50}$ of $10^{8.0}$. When severe clinical symptoms were observed 5 days after infection, the gerbils were killed and subjected to histopathological and immunohistochemical examination. Various organs/tissues (brain, brainstem, spinal cord, skeletal muscle, heart, lung, kidney, spleen, stomach, and intestine) were collected for histological analysis. The tissues were washed in PBS and fixed immediately in 10% formalin for at least 48 h. Central nervous system (CNS) tissue was embedded in paraffin, cut into 4–5 μm-thick sections, and stained with hematoxylin and eosin (H&E) or Nissl stain for morphological examination. Immunohistochemical was performed as described previously. Briefly, sections of spinal cord, brainstem, and heart were dewaxed and incubated with 0.3% H_2O_2 in PBS to inhibit endogenous peroxidase activity. CVA16 antigen was detected using polyclonal rabbit anti-CVA16 primary antibody prepared by vaccinating a rabbit with the entire inactive vaccine derived from CVA16-194. The sections were then incubated with goat anti-rabbit IgG-peroxidase as a secondary antibody (F9887, Sigma-Aldrich). The specific antigen signals were developed using DAB substrate (Dako, Glostrup, Denmark), followed by hematoxylin counterstaining (Merck, Darmstadt, Germany). Control sections were incubated in normal rabbit serum instead of polyclonal rabbit anti-CVA16 antibody.

2.5. Quantitative detection of CVA16 RNA using RT-PCR

To examine the viral loads of CVA16 in the tissues of different age groups, the 60-day-old gerbils (n = 3 per age group) were inoculated with CVA16-194 at a $TCID_{50}$ of $10^{8.0}$, 14- and 21-day-old gerbils were inoculated with CVA16-194 at $10^{5.5}$ $TCID_{50}$. Gerbil brain, brainstem, spinal cord, skeletal muscle, heart, lung, kidney, spleen, stomach, and intestine samples were collected. Total RNA of the tissues was extracted using the RNeasy extraction kit (Qiagen, USA), according to the manufacturer's instructions. Briefly, 25 mg of tissue was added to 600 μL of lysis buffer and homogenized by ultrasonic amplitude in ice. The viral RNA was finally eluted in 30 μL nuclease-free water and stored at −80 °C. The viral loads in each tissue sample were determined with a real-time reverse transcriptase PCR (RT-PCR) assay using the Applied Biosystems 7500 system. Reactions were performed with a one-step

RT-PCR kit (Liveriver, ZJBio-Tech Co., Ltd. Shanghai). Each assay was performed in duplicate. Standard curves were generated from 10-fold serial dilutions of stock CVA16-194 ($10^{8.0}$ $TCID_{50}$/mL).

2.6. Humoral immune response to CVA16 infection

To investigate the dynamic changes of neutralizing antibody response to CVA16 in gerbils after infection, 28-, 42-, and 60-day-old gerbils (n = 6 for each age group) were inoculated intraperitoneally with CVA16-194 at a $TCID_{50}$ of $10^{5.0}$. Blood samples were collected 21, 28, 35, 42, 49, and 84 days post-infection. The timeline of neutralizing antibody detection was shown in Fig. 1. CVA16-neutralizing antibodies were analyzed using a standard protocol. Briefly, two-fold dilutions of heat-inactivated sera were mixed with 50 μL CVA16-containing solution at a dose of $10^{2.0}$ $TCID_{50}$ per well in a 96-well plate, and incubated for 2 h at 37 °C. After incubation, the mixtures were added onto a monolayer of Vero cells and the cells were inspected daily for cytopathic effect (CPE) for up to 4 days. Neutralizing antibody titers were taken to be the highest dilution of serum that inhibited viral growth.

2.7. Preparation of CVA16 vaccines

Virus CVA16-393 was cultured in MRC-5 for 3 days, until the monolayer cells showed obvious CPE, and then harvested. Supernatant mixture was subjected to 3 freeze–thaw cycles, followed by a 20-min centrifugation at 2000*g* (Beckman) at 4 °C. The clarified supernatant was inactivated with 1:2500 formalin (Sigma) at 37 °C for 5–6 days. After inactivation, the concentration of the inactivated virus suspension was detected by ELISA. Briefly, 100 μL of two-fold serial dilutions of the virus suspension dissolved in bicarbonate buffer, pH 9.6 (15 mM Na_2CO_3 and 35 mM NaHCO3) was coated onto a 96-well microtiter plate (Corning) overnight at 4 °C. Then 100 μL of polyclonal CVA16 rabbit antiserum (1:800) was added to all wells, and the plates were then incubated at 37 °C for 60 min. Positive antigen-antibody reactions were visualized using horseradish peroxidase conjugated anti-rabbit antibodies (1:5000; Sigma-Aldrich). Optical densities (ODs) were measured at 450 nm in a microtiter plate reader (Bio-Rad), with values over 0.1 considered to be positive. Since an end-point dilution of two-fold serial dilutions of the virus suspension that gave a positive signal was 1:640, the concentration of this solution was therefore defined as 6400 U/mL in order to facilitate further calculations. The dosage of the final vaccine formulation was adjusted with PBS and adsorbed onto 0.5 mg/mL aluminum hydroxide (Rehydragel LV, General Chemical, LLC) at the final concentration. The vaccine with aluminum hydroxide was shaken at 200 rpm for 1 h and stored at 4 °C until use.

2.8. Humoral immunity induced by the CVA16 vaccine

To investigate the immunogenicity of the vaccine derived from the CVA16-393 strain in gerbils, 2 groups of 10 30-day-old gerbils were immunized intraperitoneally with 1 ml alum-absorbed CVA16 vaccine (3200 U/gerbil). The immunized gerbils were bled 14 days after the inoculation, and the serum was collected and used to detect the neutralizing antibodies.

2.9. Immunization and challenge assay

To investigate the protective effects of the CVA16 vaccine produced by the CVA16-393 strain in gerbils, 46-day-old gerbils (n = 50) were divided equally into 5 groups, and immunized intraperitoneally at 5 different doses (approximately 320 U/gerbil, 80 U/gerbil, 20 U/gerbil, 5 U/gerbil and 1.25 U/gerbil) of CVA16 vaccine mixed with aluminum hydroxide adjuvant, boosted 7 days later in all 5 groups. A negative control group of gerbils (n = 10) received aluminum hydroxide adjuvant alone. Finally, 14 days after first immunization, all 60-day-old gerbils were challenged with CVA16-194 using $10^{8.0}$ $TCID_{50}$ injected intraperitoneally. Following the challenge, all of the gerbils were monitored daily for survival and clinical symptoms for 20 days using the same criteria as described above. The procedure was shown in a timeline in Fig. 2. The survival rate for each experimental group of gerbils was calculated and the results analyzed using the GraphPad Prism software package, version 5.02 (SPSS Inc.; http://www.graphpad.com).

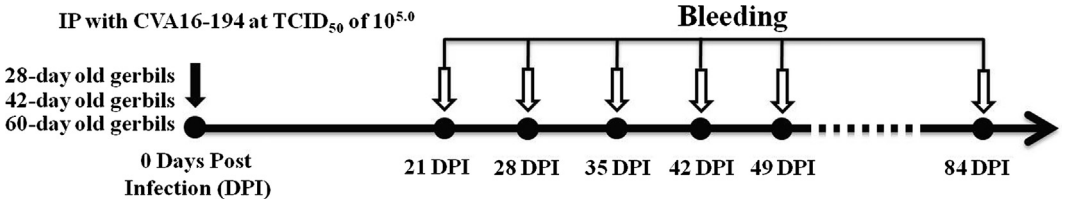

Fig. 1. Timeline of neutralizing antibody detection. 28-, 42-, and 60-day-old gerbils (n = 6 for each age group) were inoculated intraperitoneally with CVA16-194 at a $TCID_{50}$ of $10^{5.0}$. Blood samples were collected at given time points and serum neutralizing antibody titers were detected by micro-neutralization assay.

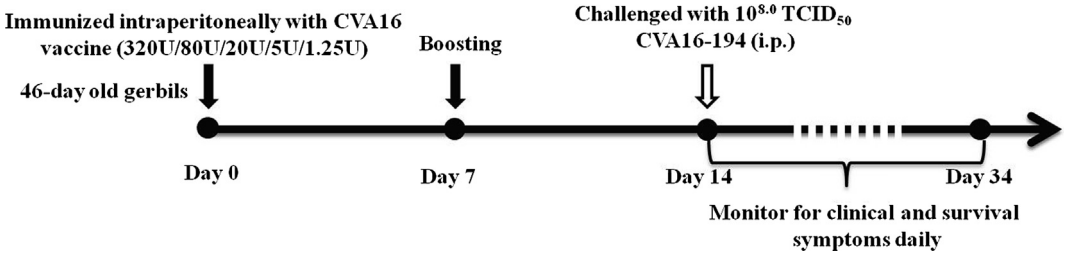

Fig. 2. Timeline of the immunization and challenge assay. 46-day-old gerbils (n = 50) were divided equally into 5 groups, and immunized intraperitoneally at 5 different doses (approximately 320 U/gerbil, 80 U/gerbil, 20 U/gerbil, 5 U/gerbil and 1.25 U/gerbil) of CVA16 vaccine mixed with aluminum hydroxide adjuvant, boosted 7 days later in all 5 groups. 14 days after first immunization, all 60-day-old gerbils were challenged with CVA16-194 using $10^{8.0}$ $TCID_{50}$ injected intraperitoneally. Following the challenge, all of the gerbils were monitored daily for survival and clinical symptoms for 20 days.

3. Results

3.1. Infection studies in adult gerbils

To investigate the susceptibility of 60-day-old gerbils to CVA16 infection, gerbils were infected with CVA16-194 at $TCID_{50}$ ranging from $10^{6.0}$ to $10^{8.0}$. Ten 60-day-old gerbils inoculated intraperitoneally with $10^{8.0}$ $TCID_{50}$/mL, which resulted in significant clinical symptoms, including hind limb weakness, paralysis of one or both hind limbs or partial forelimb paralysis, loss of balance, tremors, walking by dragging hind limbs, rigidity in the hind limbs, and loss of normal motor function (Fig. 3B). Six out of 10 gerbils developed rapidly progressing weakness and lethargy and died within a 12–24 h period at 5–6 days post-infection. In addition, 20% (2/10) exhibited hind limb paralysis and died 3–4 days after the onset of symptoms, 10% (1/10) experienced sequelae of paralysis, which they could not recover from, and 10% (1/10) demonstrated no clinical symptoms during the 20-day monitoring period. Significant weight loss was also observed in all gerbils. Further, 90% (9/10) of the gerbils exhibited clinical signs of disease and 80% (8/10) died 5–10 days after infection. At a lower dose of $10^{6.0}$ CVA16-194, the onset of symptoms occurred 6 days after infection, when only 20% (2/10) of the gerbils exhibited clinical signs of disease, 80% had no symptoms, and no gerbils had died (Fig. 4A and B). In addition, 70% (7/10) of the gerbils had detectable CVA16 antibody seroconversion. Compared to 21-day-old gerbils, the time of onset was delayed 1–2 days for all adult gerbils. The mortality rate in adult gerbils was 80%, compared to the 100% mortality rate in the 21-day-old gerbils.

3.2. CVA16 replication in tissues

Five days after 60-day-old gerbils were infected with CVA16-194, various tissue samples, including brain, brainstem, spinal cord, skeletal muscle, heart, liver, lung, kidney, spleen, stomach, and intestine were collected. Viral loads were measured using RT-PCR. The highest load values of approximately $10^{6.5\sim6.75}$ $TCID_{50}$/g were detected in the spinal cord and brainstem (Fig. 5). Organ tissues with moderately high viral loads ($10^{4.5\sim5.5}$ $TCID_{50}$/g) were from the heart, spleen, and brain. Relatively low viral loads ($10^{2.5\sim3.75}$ $TCID_{50}$/g) were found in liver, lung, kidney, stomach, and intestine tissues. The lowest viral load ($10^{1.25}$ $TCID_{50}$/g) was detected in the skeletal muscle. The viral loads in various tissues of infected gerbils differed in 14-, 21- and 60-day-old gerbils. The highest viral loads were detected in 3 types of tissue (spinal cord, brainstem, and skeletal muscle) in 14- and 21-day-old gerbils. However, the highest viral loads were only detected in the spinal cord and brainstem of 60-day-old gerbils. Relatively high viral loads in the liver, lung, kidney, stomach, skeletal muscle, and intestine were found in 14-day-old gerbils compared to corresponding values in adult gerbils. The viral loads in brain, heart, and spleen tissue were comparable among the 14-, 21-, and 60-day-old

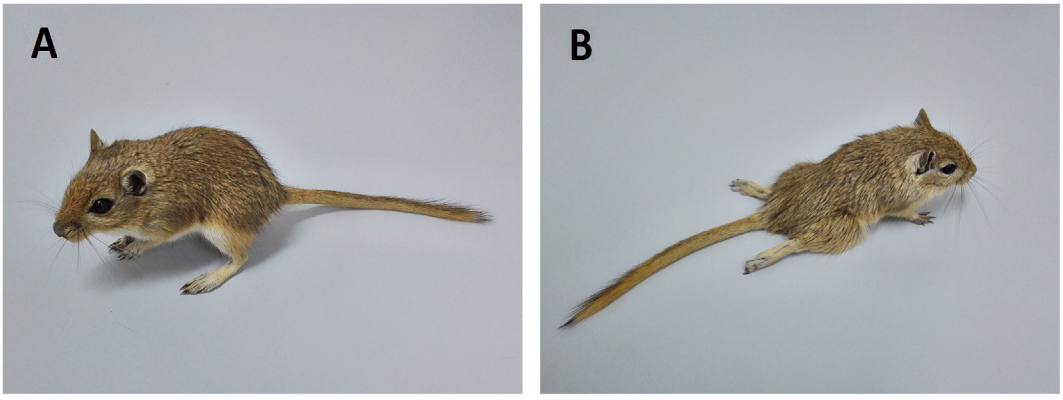

Fig. 3. 60-day-old gerbils were infected with CVA16 intraperitoneally at a dose of $10^{8.0}$ $TCID_{50}$. (A) Normal healthy 60-day-old gerbil; (B) 60-day-old gerbil exhibiting paralysis of both hind limbs 5 d post-infection.

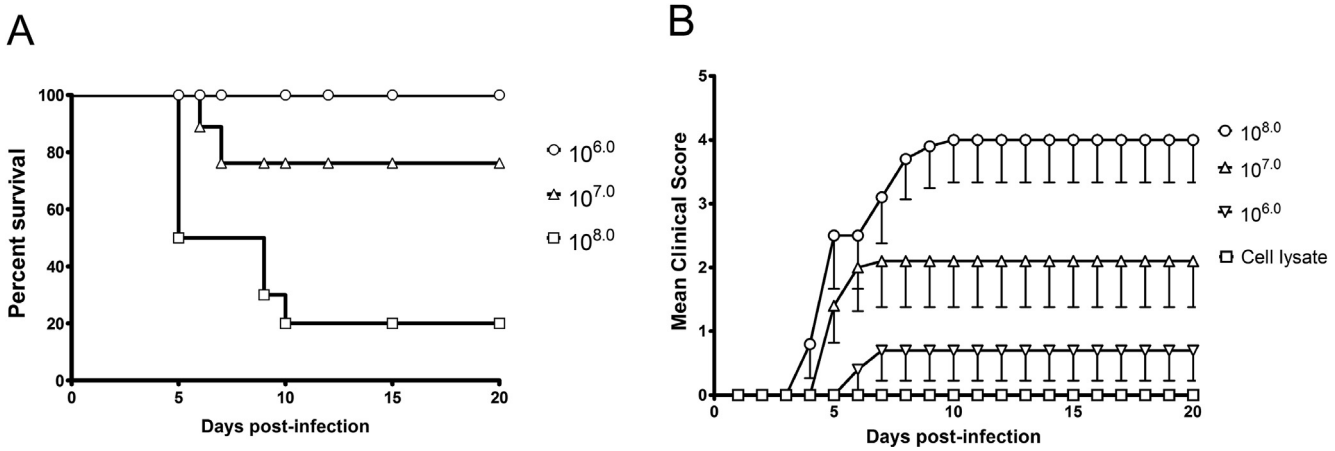

Fig. 4. Survival rates and clinical scores of CVA16-infected 60-day-old gerbils.(A) Survival curves for 60-day-old gerbils (n = 10) infected with CVA16-194 from a $TCID_{50}$ of $10^{6.0}$ to $10^{8.0}$; (B) Mean clinical scores for 60-day-old gerbils (n = 10) infected with CVA16-194 from a $TCID_{50}$ of $10^{6.0}$ to $10^{8.0}$. Clinical scores were defined as: 0 = no clinical symptoms; 1 = hunchbacked or reduced mobility; 2 = limb weakness; 3 = paralysis in 1 hind limb; 4 = paralysis in both hind limbs; and 5 = moribund or dead.

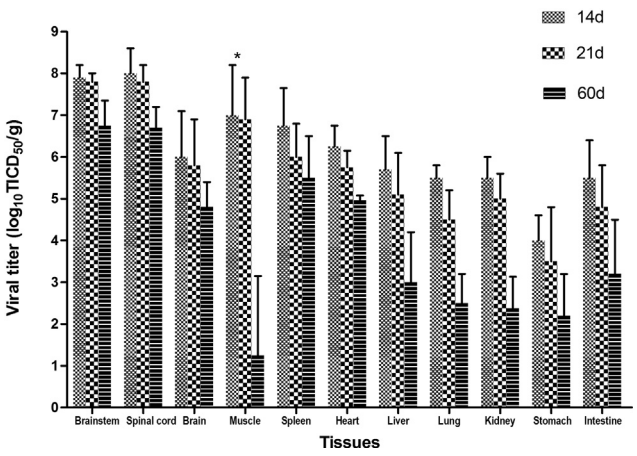

Fig. 5. Virus replication in tissues of CVA16-infected gerbils in different age groups. The 60-day-old gerbils were inoculated with CVA16-194 at $10^{8.0}$ $TCID_{50}$. 14- and 21-day-old gerbils were inoculated with CVA16-194 at $10^{5.5}$ $TCID_{50}$. The bar graph illustrates the viral loads in the brainstem, spinal cord, brain, muscle, spleen, heart, liver, lung, kidney, stomach, and intestine from CVA16-infected 14-, 21-, and 60-day-old gerbils. Viral loads were determined based on the standard curve of real-time RT-PCR obtained with 10-fold serial dilutions of CVA16-194. The virus titer was expressed as log_{10} $TCID_{50}$ per gram of tissue, which represents the mean ± SEM for 2 independent experiments each generated with 3 technical replicates. *Significantly different from the 60-day-old group ($p < 0.0001$).

gerbils. The largest difference in viral load was in the skeletal muscle of 14- and 21-day-old gerbils versus 60-day-old gerbils.

3.3. Pathology characteristics of CVA16-infected gerbils

To identify the histological factors associated with various clinical symptoms, 60-day-old gerbils received $10^{8.0}$ $TCID_{50}$ of CVA16-194. 5 or 6 days post-infection, the brainstem, spinal cord, heart, and other tissues samples were harvested for histopathological assessment. Histologic examination of the tissues revealed evidence of CVA16 infection-induced lesions in some gerbil tissues. Severe degenerative changes in neural cells, widespread swelling of neuronal cell bodies, neuronal degeneration and loss, as well as neuronophagia by microglial cells were observed in the spinal cord and brainstem. Few inflammatory cell infiltrations were observed in infected heart muscle tissue. The myocardial fibers exhibited edema and necrotizing myositis (Fig. 6A). Viral antigens were diffusely observed within the spinal cord, brainstem, and heart muscle fibers (Fig. 6B). Histopathologic examination showed that the heart muscle exhibited histopathologic changes. These findings were consistent with the histopathological analysis of humans and were identical to human symptoms.

3.4. Dynamic changes of neutralizing antibodies in response to CVA16 infection

The 60-day-old gerbils inoculated with $10^{8.0}$ $TCID_{50}$ CVA16 experienced an 80% mortality rate. Previous experiments had demonstrated that relatively low viral load did not cause gerbil death (data not shown). Three groups of 28-, 42-, and 60-day-old gerbils (6 in each group) were inoculated intraperitoneally with $10^{5.0}$ $TCID_{50}$ CVA16-194. CVA16 stimulated high levels of neutralizing antibodies among the 3 groups and specific CVA16 neutralizing antibody levels could last for 84 days. Antibodies were present on the eighth day post-infection and increased to a peak level of 1:2962.97. The antibody titer then declined slowly in the 3 groups of gerbils (Fig. 7). Average neutralizing antibodies had geometric mean titers of 1:2109.22, 1:1303.39, and 1:499.65 for the groups of 28-, 42-, and 60-day-old gerbils, respectively. The highest CVA16-specific neutralizing antibody titers elicited by CVA16 infection were detected in 28-day-old gerbils. Relatively low neutralizing antibody titers induced by CVA16 infection were found in 60-day-old gerbils.

3.5. Neutralizing antibody responses to the CVA16 vaccine

To investigate whether gerbils were able to elicit strong neutralizing antibody responses to a CVA16 vaccine derived from CVA16-393, 30-day-old gerbils were immunized with CVA16 vaccine-adsorbent aluminum hydroxide adjuvant. Experimental results revealed that a single dose of MRC-5 cell-prepared CVA16 vaccine at a dose of 3200 U/gerbil could elicit an effective neutralizing antibody response. Seroconversion in the 10 gerbils was 90–100% 2 weeks after immunization. The average neutralizing antibody titer was 74.60 (Table 1).

3.6. Protection of vaccinated gerbils against lethal challenge

To evaluate the potential of the CVA16 vaccine candidate and the utility of the adult gerbil model for vaccine efficacy studies, 60-day-old gerbils immunized with the CVA16 vaccine twice at doses of 320 U/gerbil, 80 U/gerbil, 20 U/gerbil, 5 U/gerbil, and 1.25 U/gerbil, along with a negative control group, were all administrated CVA16-194 at $10^{8.0}$ $TCID_{50}$. 5 to 6 days post-challenge, gerbils in the negative control group began to exhibit clinical symptoms of CVA16 infection, including slow movement, progressive hind limb paralysis, and eventual death. In addition, 80% of the gerbils in the control group had died 10 days after the challenge (a survival rate of 20%). In contrast, few gerbils died in the CVA16 vaccine immunized groups. Specifically, when gerbils were immunized with a dose of 320 U/gerbil, 80 U/gerbil, and 20 U/gerbil, all gerbils survived (a survival rate 100%). No gerbils experienced weight loss, paralysis, or death. For the group immunized at a dose of 5 U/gerbil, 1 gerbil exhibited paralysis and eventually died. Thus, the morbidity rate was 10%. In the 1.25 U/gerbil group, the morbidity rate increased to 50% (5/10). The survival rates for the groups receiving vaccine doses of 320 U/gerbil, 80 U/gerbil, and 20 U/gerbil were significantly greater than those of the control and 1.25 U/gerbil groups ($p < 0.0001$, Mantel-Cox log-rank test) (Fig. 8A). These results indicate that CVA16 vaccine doses greater than 20 U/gerbil produce a good protective effect against the CVA16-194 challenge.

Meanwhile, the serum neutralization antibody response to the CVA16 virus was measured in individual serum samples collected 2 weeks after the first vaccination (before the CVA16 challenge). Receiving 2 doses of vaccine induced a neutralizing antibody response of 100% seroconversion for a dose of 320 U/gerbil, 80 U/gerbil, or 20 U/gerbil, with geometric mean titers (GMTs) of 1:272.27, 1:160.08, and 1:72.12, respectively. Further, 80% (8/10) seroconversion with a neutralizing antibody GMT of 1:38.59 was detected in the 5 U/gerbil group. In this group, the gerbils with serum neutralization antibody titers > 1:16 were completely protected from death after challenge with the CVA16-194 virus. These results indicated that serum neutralization antibody titers > 1:16 may be sufficient to protect gerbils from a lethal CVA16 challenge.

4. Discussion

To date, several animal models have been developed for CVA16 research, most utilizing mice [24–27]. Although newborn mice are known to be susceptible to CVA16 infection, mice older than 7 days are no longer susceptible [22]. Such a short susceptibility period to CVA16 infection is an obvious disadvantage of the mouse model. Our previous research demonstrated that CVA16 is highly

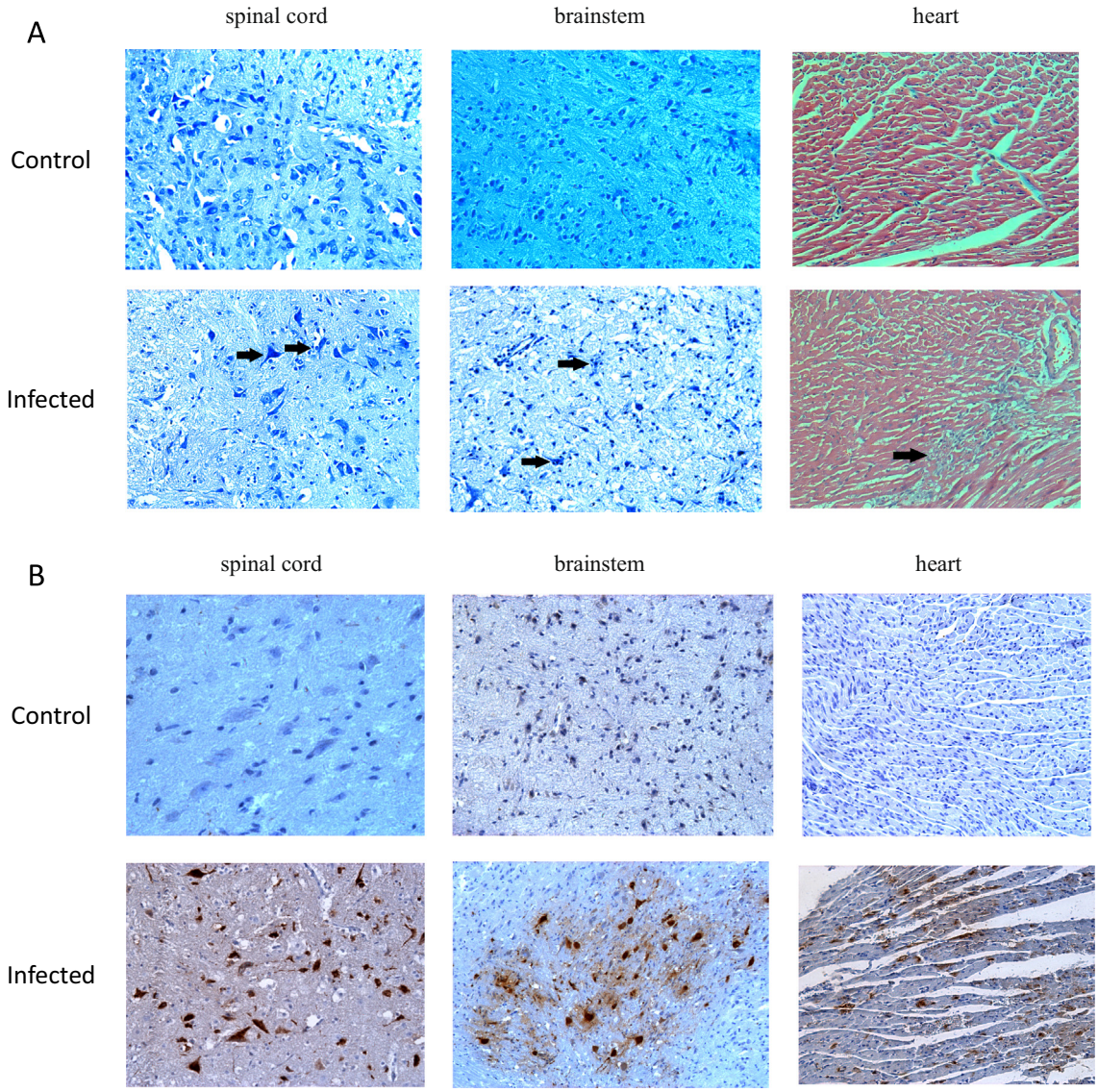

Fig. 6. Pathological findings for CVA16-infected gerbils. The 60-day-old gerbils were infected with CVA16-194 at $10^{8.0}$ $TCID_{50}$. (A) Representative images of hematoxylin and eosin (H&E) or Nissl stained brainstem, spinal cord, and heart muscle tissue harvested from CVA16-infected gerbils 5 days post-infection and control gerbils. The arrows indicate focal shrunken neurons or neuronophagia in the infected brainstem and spinal cord, and inflammatory cell infiltration, swelling and necrotizing myositis in the infected heart muscle. (B) Representative IHC images showing brainstem, spinal cord, and heart muscle tissue harvested from CVA16-infected gerbils 5 days post-infection and control gerbils. The viral antigens (arrows) in paraffin-embedded tissues were detected with HRP-conjugated antibodies and visualized with DAB followed by counter-staining with hematoxylin. Magnification: 100×.

pathogenic to 21-day-old gerbils, causing neurologic complications including hind limb paralysis associated with a 100% mortality rate, thus providing a new avenue for CVA16 studies [23]. In this investigation, we further showed that adult (60-day-old) gerbils are also susceptible to infection by higher virus titers of CVA16. We tried many routes of inoculation such as the oral route and the subcutaneous route to infect gerbils, and found the intraperitoneal inoculation route was the most effective way to infect gerbils (Supplementary Table S3). Intraperitoneal inoculation of adult gerbils with a clinical CVA16 isolate strain induced 80% lethality, with clinical symptoms including hind limb weakness, hind limb paralysis, tremors, and eventual death due to myocarditis. The mortality of male gerbils was higher than that of female gerbils (Supplementary Table S1). Histopathological changes, such as neuronal loss and neuronophagia, were observed in the spinal cord, brainstem, and heart muscle, along with necrotizing myositis. Among the 11 types of tissue analyzed from infected gerbils, the highest viral loads were found in the spinal cord and brainstem. Analysis further indicated that central nervous system (CNS) tissue was a major target of CVA16 virus replication. Both infant and adult gerbils were discovered to be susceptible to CVA16 infection. The greatest advantage of this model is that adult gerbils are susceptible to CVA16 infection from the clinical isolate strain and not the adaptable strain, which contains some mutations of the original strain. Thus, all clinical symptoms manifested in adult gerbils after infection completely reflect the original viral virulence.

Mice have many advantages over other model animals and are often used to reproduce human diseases [28–32]. Like mice, gerbils are also small, facilitating large-scale throughput studies [33–38]. In previous research, we demonstrated that adult gerbils exhibit no obvious clinical symptoms when inoculated intraperitoneally with $10^{5.5}$ $TCID_{50}$ CVA16 [23]. In this study, when the virus titration increased to $10^{6.0}$ $TCID_{50}$, 2 of 10 adult (60-day-old) gerbils showed clinical symptoms in the form of paralysis of one or both

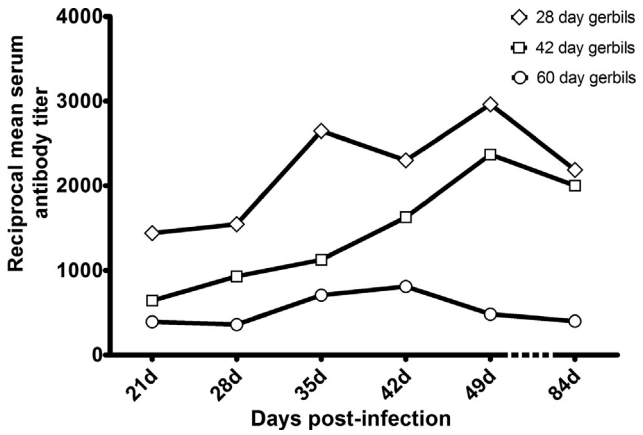

Fig. 7. Neutralizing antibodies generated in CVA16-infected gerbils. 28-, 42-, and 60-day-old gerbils were inoculated intraperitoneally with CVA16-194 at $10^{5.0}$ $TCID_{50}$. Blood samples were collected on days 21, 28, 35, 42, 49, and 84 post-infection. CVA16 neutralizing antibodies were measured using the conventional CPE-based assay in Vero cells. The lines represent the mean antibody titers from 6 gerbils per group for each day.

Table 1
Neutralizing antibody titers in gerbils immunized with CVA16 vaccine.

Age	Neutralizing antibody (1/GMT) after 2 weeks
30-day-old	74.60 ± 6.09

Note: The 30-day-old gerbils were immunized with CVA16-393 vaccine (3200 U/gerbil) and their blood sera were drawn 2 weeks after immunization.

hind limbs over the 20-day monitoring period, and all survived. After inoculation with $10^{7.0}$ $TCID_{50}$ CVA16, 5/10 (50%) of the gerbils experienced hind limb paralysis and 2/10 (20%) died, one 7 days post-infection and the other 13 days post-infection. The mortality rate increased to 80% (8/10) following inoculation with $10^{8.0}$ $TCID_{50}$, with gerbils dying 5–10 days post-infection. All of the surviving gerbils had detectable CVA16 seroconversion. Thus, the incidence and severity of clinical symptoms in gerbils were positively correlated with infection dose. Although the highest viral loads were detected in the CNS (approximately $10^{7.0\sim8.0}$ $TCID_{50}$ in the brainstem and spinal cord), the non-CNS tissues (muscle, spleen, heart, live, lung, kidney, and intestine) of 14-day-old gerbils also had relatively high viral loads, reaching as much as $10^{6.0}$–$10^{7.0}$ $TCID_{50}$. High viral loads were mainly found in the CNS in 60-day-old gerbils and non-CNS tissues showed comparatively low viral loads. This indicates that CVA16 more easily infects the non-CNS tissues of 14-day-old gerbils than 60-day-old gerbils, thus demonstrating that infection sensitivity differs slightly between 14-day-old and 60-day-old gerbils. Similarly, infant gerbils are more sensitive to CVA16 than adult gerbils.

Enterovirus A71 (EVA71) and CVA16 are consistently the 2 major causative agents of HFMD [39–43]. Severe neurological diseases, including meningitis, encephalitis, acute flaccid paralysis, and acute cardiopulmonary dysfunction, are often caused by EVA71 [44–47]. CVA16 infection is generally considered to be mild and self-limiting, with symptoms such as fever, vesicle blisters, vesicular maculopapular rash, ulcers, and pharyngitis. However, several reports have shown that CVA16 is also associated with more severe clinical symptoms such as heart, pericardium, brain, and lung disease, resulting in fatal myocarditis, encephalitis, and pneumonia [18–21]. One case was documented in which a severe inflammatory process occurred in the myocardium, from which CVA16 was isolated [18,19]. Infection with CVA16 in adult gerbils induces neurological lesion-related symptoms such as limb paralysis, slowness, ataxia, and lethargy. Infected gerbils also exhibit pathological myocardial changes characterized by scattered inflammatory cell infiltration. The associated myocardial damage is consistent with human infection with CVA16, in which CVA16 has also been isolated from the myocardium. In contrast, in the neonatal mouse model, no obviously positive CVA16 antigen or pathological change was found in the CNS, which showed that CVA16 had no significant neurotropism in this model [22,25]. Therefore, the neonatal mouse model does not completely reproduce the associated human clinical pathology characteristics. In infected gerbil model, however, both CNS damage (neuronophagia in the spinal cord and brainstem) and myocardium damage (necrotizing myositis and inflammatory cell infiltrations in the heart muscle) are observed, which correspond in extent to the clinical symptoms observed in human patients. Therefore, the adult gerbil

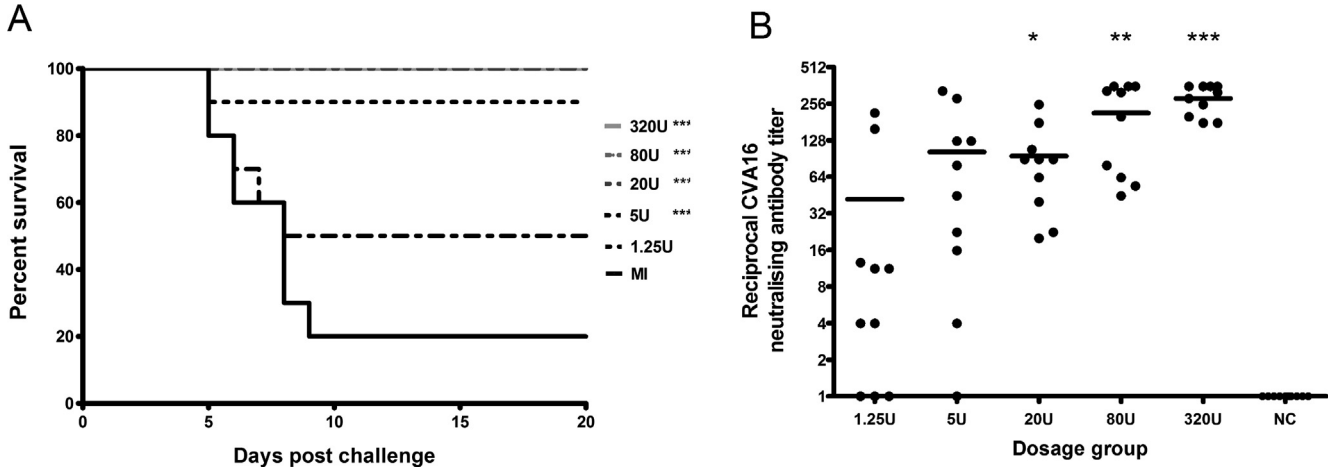

Fig. 8. Analysis of protected 60-day-old gerbils. (A) Gerbils protected from a challenge with a lethal dose of CVA16-194. The 46-day-old gerbils were immunized with 100 μL CVA16-393 vaccine twice in 7 days interval. Seven days after the second dose, all gerbils were challenged intraperitoneally with $10^{8.0}$ $TCID_{50}$ of CVA16-194. Gerbils receiving aluminum hydroxide adjuvant alone served as the negative control group. Gerbils were then observed for 20 days, during which time all illnesses and deaths were recorded. The Mantel-Cox log-rank test was used to compare the survival of immunized gerbils to that of the control gerbils. *** denotes a significance value of $p < 0.0001$. (B) Neutralizing antibody titers against CVA16 in the serum collected 2 weeks after the first vaccination. The Mann-Whitney test was used to compare the titers of each dosage group to that of the 1.25 U group. * denotes a significance value of $p < 0.05$, ** denotes a significance value of $p < 0.01$, and *** denotes a significance value of $p < 0.0001$. NC = negative control.

is a suitable animal model for investigating the pathogenesis of nervous system damage during CVA16 infection.

The characteristics of CVA16 infection in adult gerbils, including the effects of age on susceptibility to infection, viral loads, and pathological changes to various tissues, have not been adequately identified. In the present study, CVA16 was highly virulent to 60-day-old gerbils at $10^{8.0}$ $TCID_{50}$. Although adult gerbils exhibited pronounced hind limb paralysis, relatively low viral loads in the hind limbs were detected, even lower than in other tissues. Histopathological examination revealed that muscle fibers remained intact, and no inflammatory cell infiltration was found. This indicates that the muscle cells were not damaged in the infected hind limbs, damage that would have directly resulted in motor dysfunction and paralysis. Instead, we hypothesize that CVA16 infects the central nervous system, causing nerve degeneration, destruction, or death. As a result, nerve signals are not transmitted to the legs, resulting in the clinical manifestation of hind limb paralysis. Previous studies have shown 2 possible routes for the poliovirus (PV) to reach the central nervous system (CNS): from the bloodstream across the blood-brain barrier (BBB) or through peripheral nerves via retrograde axonal transport [48,49]. Yang et al. demonstrated that the PV replicates in muscle cells that maintain constant viremia and spreads to the CNS from peripheral nerves. Che-Szu Chen et al. demonstrated that hematogenous transport might not represent a major transmission route. Since skeletal muscle has persistent viral titers and is a vigorous viral source for entry into the bloodstream or CNS in EVA71, this indicates that retrograde axonal transport is a major transmission route of EVA71 in mice [30]. Both EVA71 and CVA16 belong to the enterovirus genus and cause similar clinical symptoms. The detailed mechanism of CVA16 infection has not been clarified yet. Based on our studies of adult gerbils infected with CVA16, we hypothesize that the bloodstream may be the major route of viral spread in the gerbil model. Meanwhile, the spread of CVA16 from muscle to CNS may represent a secondary transmission pathway. In our studies, gerbils exhibit more susceptibility to infection by both EVA71 and CVA16 than mice [50,51]. This result implies that the CVA16 transmission route in gerbils differs from that in mice. Further research, however, should be undertaken to confirm this inference.

In order to examine the dynamic profiles of specific antibody response to CVA16 infection, 28-, 42-, and 60-day-old gerbils were inoculated intraperitoneally with $10^{5.0}$ $TCID_{50}$/gerbil of CVA16-393 in a 100 μL volume. The data revealed that neutralizing antibodies could be induced in all 3 groups of gerbils and that the antibody response to CVA16 infection varied among 28-, 42-, and 60-day-old gerbils. Protective neutralizing antibody levels increased over time following challenged with CVA16, with neutralizing antibodies peaking 49 days post-infection, then subsequently waning. The virus-specific antibody responses were sustained at relatively high levels for 84 days. Specifically, 28-day-old gerbils exhibited the highest antibody titer of the 3 groups, presumably indicating that the strongest immune response against CVA16 occurs in younger animals. These results also indicate more generally that a gerbil's age may affect its susceptibility to CVA16 infection. Neutralizing antibody response to CVA16 infection in 28-day-old gerbils rose quickly from 1:1546 to 1:2648 during the fifth week post-infection. In contrast, neutralizing antibody titers rose slowly in 60-day-old gerbils. Therefore, age might account for the variety of dynamic profiles seen in the antibody response. The 28-day-old gerbils were found to be more susceptible to viral infection than 60-day-old gerbils and typically suffered more severe clinical symptoms. These results demonstrate that specific antibody response to CVA16 is age-dependent, with adult gerbils being less affected by viral infection. Therefore, 28-day-old gerbils are best suited for observing antibody responses to CVA16 infection.

For vaccines, the specific antibody response induced by the same dose of CVA16 was found to be lower in infant (7-day-old) gerbils than in 30-day-old gerbils (Supplementary Table S4). This indicates that a mature immune system is critical for a vaccine-induced immune response. A single-dose of CVA16 vaccine was discovered to induce 90–100% seroconversion in 10 30-day-old gerbils 2 weeks after immunization. In contrast, a single-dose of CVA16 vaccine immunization only elicited few or no neutralizing antibodies in ICR or Balb/c mice (data not shown). Therefore, CVA16 vaccines are more efficient triggers for neutralizing antibodies in a gerbil model than in a mouse model. Meanwhile, gerbils immunized with the prime-boost vaccine elicited higher neutralizing antibodies, and all survived after a viral challenge with no significant clinical symptoms. Viral loads were found to be significantly reduced in the brainstem, spinal cord, and heart muscle tissue of vaccinated gerbils compared with the control group. Few or no neutralizing antibodies were observed in ICR or Balb/c mice undergoing the same immune procedure (data not shown). Therefore, gerbils are significantly better than ICR or Balb/c mice for evaluating the immune effects of CVA16 vaccines. Thus, the gerbil model provides us with a new CVA16 vaccine evaluation tool. In addition, gerbils are fully susceptible to both EVA71 and CVA16, which belong to the same enterovirus genus and cause similar clinical symptoms. Therefore, the gerbil model is a better tool for evaluating potential EVA71 and CVA16 bivalent vaccines.

In conclusion, we successfully established an adult gerbil model of CVA16 infection using a clinical isolate strain. These infected gerbils developed neurological lesion-related symptoms and severe necrosis in the CNS, eventually resulting in death. The data presented in this study indicate that a formaldehyde-inactivated CVA16-393 vaccine could provide highly effective protection from CVA16 infection in a gerbil model and could be a potential promising vaccine candidate. Therefore, an adult gerbil model provides us with a valid tool for studying the pathogenesis and evaluating antiviral reagents of CVA16 infection.

Declaration of Competing Interest

The authors declared that there is no conflict of interest.

Acknowledgements

This work was supported by the Major Science and Technology Project of the Science and Technology Department of Zhejiang Province (Grant Number 2018C03070), the Commonwealth Project of the Science and Technology Department of Zhejiang Province (LGF18H100002), the Zhejiang Provincial Foundation for Scientific Research in Medicine and Health (WKJ-ZJ-1816), and the Zhejiang Provincial Natural Science Foundation of China (LY18H190003).

Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.vaccine.2019.07.046.

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