A Bivalent Inactivated Vaccine Prevents Enterovirus 71 and Coxsackievirus A16 Infections in the Mongolian Gerbil

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Abstract

Hand-foot-and-mouth disease (HFMD) is a viral infectious disease that occurs in children under 5 years of age. Its main causes are coxsackievirus (CV) and enterovirus (EV). Since there are no efficient therapeutics for HFMD, vaccines are effective in preventing the disease. To develop broad coverage against CV and EV, the development of a bivalent vaccine form is needed. The Mongolian gerbil is an efficient and suitable animal model of EV71 C4a and CVA16 infection used to investigate vaccine efficacy following direct immunization. In this study, Mongolian gerbils were immunized with a bivalent inactivated EV71 C4a and inactivated CVA16 vaccine to test their effectiveness against viral infection. Bivalent vaccine immunization resulted in increased Ag-specific IgG antibody production; specifically, EV71 C4a-specific IgG was increased with medium and high doses and CVA16-specific IgG was increased with all doses of immunization. When gene expression of T cell-biased cytokines was analysed, Th1, Th2, and Th17 responses were found to be highly activated in the high-dose immunization group. Moreover, bivalent vaccine immunization mitigated paralytic signs and increased the survival rate following lethal viral challenges. When the viral RNA content was determined from various organs, all three doses of bivalent vaccine immunization were found to significantly decrease viral amplification. Upon histologic examination, EV71 C4a and CVA16 induced tissue damage to the heart and muscle. However, bivalent vaccine immunization alleviated this in a dose-dependent manner. These results suggest that the bivalent inactivated EV71 C4a/CVA16 vaccine could be a safe and effective candidate HFMD vaccine.

Key Words: Bivalent vaccine, Hand-foot-and-mouth disease, Enterovirus 71, Coxsackievirus A16, Gerbil

INTRODUCTION

Hand-foot-and-mouth disease (HFMD) is a viral infectious disease that mainly affects young children. It is most common in children under 5 years of age and has a high mortality rate in those under 2 years of age (Yi et al., 2017). HFMD is characterized by highly contagious rashes or blisters on the hands, feet, and groin around the mouth (Goksugur and Goksugur, 2010). Most affected individuals recover easily, but rarely, neuronal or cardiorespiratory complications such as encephalitis, brainstem encephalitis, aseptic meningitis, and polio-like syndrome can develop, and in severe cases, death can occur (Ooi et al., 2010). Although symptoms in adults are mild, close contact with young children can be a latent source of HFMD infection (Yu et al., 2019). Infection is transmitted through direct contact with mucus, saliva, faeces, and vesicles from an infected person (Sarkar et al., 2016; Yi et al., 2017). HFMD mainly occurs in warm temperatures in spring, summer, and autumn, because its incidence increases owing to increased infection by the causative virus in hot and humid environments (Yi et al., 2017).

Human enteroviruses belonging to the Picornaviridae family are the causative agents of HFMD (Coates et al., 2019). Among the 12 species in the enterovirus genus, enterovirus A–D are human enteroviruses (Ogi et al., 2017). There are more than 100 types of human enterovirus, including enterovirus, poliovirus, coxsackie A and B viruses, and echovirus (Coates et al., 2019). Among human enteroviruses, coxsacki-
evirus A16 (CVA16) and enterovirus 71 (EV71) are the main causes of HFMD, but the incidence of CVA16 infection is higher than that of EV71 (Yi et al., 2017). EV71 and CVA16 are very similar in structure (Ren et al., 2015). Both viruses are positive single-stranded RNA viruses, comprised of a non-enveloped, icosahedral virion (Ren et al., 2015; Yi et al., 2017). The coding region is divided into three sub-regions, specifically P1, P2, and P3. The P1 region encodes four structural proteins (VP1, VP2, VP3, and VP4), whereas non-structural proteins are encoded by the P2 (2A, 2B, and 2C) and P3 (3A, 3B, 3C, and 3D polymerase) regions (Ogi et al., 2017). VP1, VP2, and VP3 are present on the capsid surface, but VP4 is present on the inside (Ren et al., 2015; Yi et al., 2017). EV71 and CVA16 mainly exhibit genetic differences in VP1 and VP4 (Li et al., 2011), and these differences in structure and immunogenicity necessitate the production of a bivalent safe and effective HFMD vaccine with broad coverage (Ren et al., 2015).

The main causative virus of HFMD is CVA16. It usually results in a rash in the form of large vesicles, but rarely, meningitis, myelitis, encephalitis, and respiratory failure can occur (Legay et al., 2007; Aswathyraj et al., 2016). CVA16, although rare, can cause myocarditis in children and pneumonia in adults (Wang et al., 2004; Legay et al., 2007). Further, EV71 is the second leading cause of HFMD. In HFMD caused by EV71 infection, petechiae and characteristic rashes appear mainly on the trunk and limbs (Aswathyraj et al., 2016). In general, these comprise typical symptoms of HFMD, but in severe cases, neurological complications such as meningitis, myelitis, encephalitis, and acute flaccid paralysis could occur (Aswathyraj et al., 2016; Yi et al., 2017). In addition, co-infection with CVA16 and EV71 increases disease severity (Aswathyraj et al., 2016).

Animal models of EV71 and CVA16 virus infections include AG126 transgenic mice, cynomolgus and rhesus monkeys, human scavenger receptor 2 (hSCARB2) transgenic mouse models, and the Mongolian gerbil (Liu et al., 2011). Unlike that in humans, viral replication occurs in muscle and adipose tissue in AG126 transgenic mice (Yi et al., 2017). The disease signs and neurological complications observed with cynomolgus and rhesus monkey infection are similar to those in humans, but there are ethical and economic problems associated with their use (Arita et al., 2005; Liu et al., 2011; Yi et al., 2017). One of the viral receptors, hSCARB2, is a receptor associated with EV71 and CVA16 infections (Kobayashi and Koike, 2020).

Neonatal hSCARB2 transgenic mice show signs such as paralysis and death after infection (Fujii et al., 2013). However, since neonatal mice are used, stable experiments are not easy, and these animals are not suitable for evaluating the efficacy of direct immunization with vaccine candidates. A small rodent, the Mongolian gerbil, is also used as a sensitive animal model for neurotrophic viruses (Nakamura et al., 1999; Porres et al., 2017). EV71 and CVA16 infections also result in muscle paralysis and central nervous system damage in 3-week-old gerbils (Yao et al., 2012; Sun et al., 2016). Moreover, the Mongolian gerbil can be infected even at the young adult stage and therefore is advantageous to test vaccine efficacy via viral infection after immunization.

There are no efficient therapeutics for HFMD, and in severe cases, it can lead to death; thus, vaccines are effective in preventing this disease. Current commercially available vaccines are licensed and effectively used publicly only in China, and this includes a monovalent vaccine against EV71 C4a. (Yi et al., 2017; Liu et al., 2020). Three monovalent inactivated EV71 vaccines are licensed for children 6-71 months of age in China. Their efficacy for protection against HFMD is greater than 90%, showing clinical effectiveness and safety (Zhu et al., 2013; Guan et al., 2020). Moreover, a monovalent EV71 C4a vaccine also exhibits cross-neutralization reactivity with other sub-genotypes (A, B3, B4, B5, C1, C2, C3, and C5) (Liu et al., 2015).

For efficient protection against HFMD, broad coverage, including that against coxsackievirus and enterovirus, is needed. We developed a bivalent inactivated HFMD vaccine form and used it to challenge a Mongolian gerbil model. In this study, we immunized Mongolian gerbils with a mixture of inactivated EV71 (iEV) and inactivated CVA16 (iCV) and then tested their effectiveness against EV and CV infection.

MATERIALS AND METHODS

Animals and virus challenge

In this study, all animal experiments were performed with the approval of the Institutional Animal Care and Use Committee of Ajou University (IACUC No. 2019-0042). The Mongolian gerbils, purchased from JANVIER Labs (Mayenne, France), were bred in the Laboratory Animal Research Center of Ajou University Medical Center (Suwon, Korea). Sterile water and food were provided ad libitum. One-week-old Mongolian gerbils was immunized via intramuscular injection with 100 μL of the inactivated EV71 (iEV) and inactivated CVA16 (iCV) mixed with aluminium hydroxide (Croda, Snaith, UK). Boosting injections were performed 1 week later. The administered dose of the vaccine antigen was based on the VP1 content. The immunization doses were tested as iEV 0.5 ng+iCV 2.5 ng for low dose, iEV 1.0 ng+iCV 5.0 ng for mid dose, and iEV 2.0 ng+iCV 10 ng for high dose. One week after the last immunization, 100 μL of the virus was used for intraperitoneal infection. EV71 C4a and CVA16 were distributed from Korea Disease Control and Prevention Agency (KCDC) (Cheonju, Korea) for research and vaccine development. The Mongolian gerbils were infected with EV71 C4a or CVA16 at 3×10^5 TCID50/gerbil or 2×10^5 TCID50/gerbil, respectively. The animals were monitored daily for mortality, body weight, and signs of infection. HFMD-like signs were assessed based on a pathological scoring system (0, healthy; 1, ruffled hair; 2, weakness in hind limbs; 3, paralysis in a single hind limb; 4, paralysis in both hind limbs; 5, death).

Manufacturing of inactivated virus vaccines

EV71 C4a or CVA16 strain were inoculated onto Vero cells and incubated at 36.5°C for 3 days. The cultured supernatant was filtered with a depth filter (Sartorius, Göttingen, Germany), concentrated and dialyzed using a 100 kDa ultrafiltration membrane (Sartorius). The virus pool was inactivated using β-propiolactone (Tokyo Chemical Industry Co., Tokyo, Japan) and further purified using Affinity resin/multimodal resin (Cytiva, MA, USA) for EV71 C4a or ionexchange resin/multimodal resin (Cytiva) for CVA16. For completion of viral inactivation, column eluates were additionally reacted with β-propiolactone and then concentrated, dialyzed using a 100 kDa ultrafiltration membrane.

The antigen titre of the iEV vaccine was determined using
Fig. 1. Immunization with a bivalent inactivated vaccine increases Ag-specific antibodies and helper T cell cytokines. (A) Mongolian gerbils were immunized twice with a bivalent EV71 C4a/CVA16 vaccine via the intramuscular route (n=6-8). (B) At 1 week following the final immunization, Ag-specific IgG in the sera were determined. A Student’s t-test was performed, comparing the vaccine group with the vehicle group. (C) The gene expression of T cell cytokines was analysed in the spleen. Graphs show the mean ± SEM. Analysis was based on a one-way ANOVA with multiple comparisons; ns, not significant; *p<0.05, ***p<0.001, compared with vehicle group.

Antibody analysis
One week after the last immunization, antibody analysis was performed via ELISA. Immuno 96-well plates (Thermo Fisher Scientific, MA, USA) were coated with 50 pg/well of inactivated EV71 or CVA16 standard foam in 0.05 M bicarbonate buffer (pH 9.6) and incubated overnight at 4°C. After washing three times with phosphate-buffered saline (PBS), blocking was performed for 1 h at 37°C with 1% bovine serum albumin (BSA) in PBS. Samples were then washed with PBS, diluted with 0.1% BSA/PBS, and incubated overnight at 4°C. After washing with 0.05% Tween20/PBS, Rabbit Anti-Mongolian Gerbil IgG Antibody H+L (Bioss, Woburn) was added at 4°C for 18 h. After the last wash, 3,3′,5,5′-tetramethylbenzidine (TMB, Invitrogen, Waltham, MA, USA) were coated with 50 pg/well of inactivated EV71 or CVA16 standard foam in 0.05 M bicarbonate buffer (pH 9.6) and incubated overnight at 4°C. After washing three times with phosphate-buffered saline (PBS), blocking was performed for 1 h at 37°C with 1% bovine serum albumin (BSA) in PBS. Samples were then washed with PBS, diluted with 0.1% BSA/PBS, and incubated overnight at 4°C. After washing with 0.05% Tween20/PBS, Rabbit Anti-Mongolian Gerbil IgG Antibody H+L (Bioss, Woburn) was added at 4°C for 18 h.

Cytokine analysis
One week after the last immunization, total RNA was extracted from spleen tissue using TRIzol reagent (Invitrogen, Waltham, MA, USA). Total RNA was synthesized as cDNA using SuperScript II Reverse Transcriptase (Invitrogen). Gene expression of gerbil cytokines was analyzed via real-time PCR using SYBR Green PCR Master Mix (Applied Biosystems, Waltham, MA, USA). Primer sequences of gerbil genes were as follows: GAPDH forward, 5′-CATGCGGCTCCGAGTTCCT-3′ and reverse, 5′-TCTGCAGTCGGCATGT-3′; TNF-α forward, 5′-TTCTGCAGTCGGCATGT-3′ and reverse, 5′-TTCTGCAGTCGGCATGT-3′; IFN-γ forward, 5′-CATGGCCTTCC-3′ and reverse, 5′-TTCTGCAGTCGGCATGT-3′; IL-4 forward, 5′-GACCATGAGGACAGAAGACC-3′ and reverse, 5′-GGTGCTGGACACCGTGTAC-3′; IL-10 forward, 5′-GATGGCGCCGGCTCACT-3′ and reverse, 5′-GATGGCGCCGGCTCACT-3′; IL-17 forward, 5′-GGGGTGGCTGCGAGCTCTG-3′ and reverse, 5′-GGGGTGGCTGCGAGCTCTG-3′.
Viral RNA determination

Five days after EV71 C4a or CVA16 infection, gerbil brain, muscle, spleen, and heart tissues were obtained. Viral RNA was extracted using the Qiagen viral RNA kit (Qiagen, Hilden, Germany). Viral RNA was synthesized into cDNA using SuperScript II RT (Invitrogen). Viral RNA contents of each tissue were analysed via real-time PCR using SYBR Green PCR Master Mix. The sequences of Enterovirus primers were forward, 5′-GGCCCCTGAATGCGGCTAATCC-3′ and reverse, 5′-GCGATTGTCACCATWAGCAGYCA-3′; IFN-γ forward, 5′-TTGGGCCCTCTGACTTCGT-3′ and reverse, 5′-CTTG- γCA-3′; IFN-γ forward, 5′-TTGGGCCCTCTGACTTCGT-3′ and reverse, 5′-GACCCCGGAGTTGTTCTTCA-3′; IL-10 for-ward, 5′-CAAGGCAGCCTTGCAGAAG-3′ and reverse, 5′-TTGGGCCCTCTGACTTCGT-3′; TNF-α forward, 5′-CAAGGCAGCCTTGCAGAAG-3′ and reverse, 5′-TC-ward, 5′-CAAGGCAGCCTTGCAGAAG-3′ and reverse, 5′-GACCCCGGAGTTGTTCTTCA-3′; IL-4 forward, 5′-CAGGGTGCTCCGCAAATTT-3′ and reverse, 5′-GCTCCCCCAGAAGTCGGCG-3′; IL-17 forward, 5′-AGCTCCAGAGTTGTTCTTCA-3′ and reverse, 5′-TC-ward, 5′-CAAGGCAGCCTTGCAGAAG-3′ and reverse, 5′-GACCCCGGAGTTGTTCTTCA-3′; IL-10 forward, 5′-CAAGGCAGCCTTGCAGAAG-3′ and reverse, 5′-GCTCCCCCAGAAGTCGGCG-3′; IL-4 forward, 5′-CAGGGTGCTCCGCAAATTT-3′ and reverse, 5′-GCTCCCCCAGAAGTCGGCG-3′; IL-17 forward, 5′-AGCTCCAGAGTTGTTCTTCA-3′ and reverse, 5′-TC-

Histology

Five days after virus infection, the brainstem, hind limb muscle, spleen, and heart of gerbils were obtained and fixed with formalin for 24 h. The fixed tissues were prepared with paraffin blocks and stained with haematoxylin-eosin at T&P Bio (Gwangju, Korea). In the muscle and heart, inflammatory cell infiltration and muscle fibre degeneration were scored (0, normal; 1, mild; 2, moderate; 3, severe; total score, 6) (Nugra-heni and Saputri, 2017; Sun et al., 2022).

Statistics

The Student’s t-test was used to compare the differences between two groups. To compare multiple groups, we performed one-way analysis of variance (ANOVA) followed by Tukey’s post hoc test. Survival rates were compared using the log-rank test. Statistical significance was set at p<0.05.

RESULTS

Immunization with the bivalent inactivated vaccine induces Ag-specific IgG antibody production and T cell immune responses

To induce an effective immune response against viral Ag, immunization with inactivated viruses needs to be performed at least twice. Preliminary experiments were conducted to determine the interval and frequency of immunizations. First, considering the timing and interval of immunization, the possibility of viral infection in various aged adult gerbils was investigated. Since infection with both EV71 C4a and CVA16 is required, we first tested this using CVA16, which results in
relatively severe signs of infection. However, CVA16 did not infect 60-day-old gerbils (Supplementary Fig. 1). As such, we challenged 3-week-old young gerbil with the virus and confirmed infectivity. Therefore, as an optimized regimen, the first immunization was performed at 1 week of age to ensure two immunizations prior to infection. The first immunization was performed via the intra-muscular route to 1-week-old gerbils, and additional immunization was performed 1 week later (Fig. 1A).

To determine whether the bivalent inactivated vaccine could effectively induce an immune response in gerbils, antibody and cytokine levels were analysed 1 week after the last immunization. When Ag-specific IgG in the sera was analysed, EV71-specific IgG was not significantly increased with the low dose but was significantly increased with mid and high doses (Fig. 1B). CVA16-specific IgG was significantly increased with all doses. It was confirmed that the bivalent inactivated vaccine could effectively result in the production of Ag-specific IgG. Cytokine gene expression was then analysed in the spleen to determine whether it also affects the T cell immune response (Fig. 1C). Th1 (IFN-γ, TNF-α), Th2 (IL-4, IL-10), and Th17 (IL-17) immune responses were highly increased upon high-dose vaccine immunization. These results suggested that immunization with the bivalent inactivated vaccine can increase Ag-specific IgG production and induce T cell cytokine responses.

**Immunization with the bivalent inactivated vaccine effectively protects against EV71 C4a and CVA16 infection**

To determine whether the bivalent inactivated vaccine could effectively protect against viral infection, EV71 C4a or CVA16 virus was infected intraperitoneally 1 week after the last immunization. We then analysed the body weight, morbidity, and survival rate among infected gerbils. To optimize viral infection, various doses of each virus were tested, and then, the infection concentration was selected as 3×10^6 TCID50/gerbil for EV71 C4a and 2×10^5 TCID50/gerbil for CVA16 (Supplementary Fig. 2). After infection with EV71 C4a and CVA16, signs of infection were apparent within 4 days, and the survival rate was changed within 7 days. In addition to limb paralysis, bleeding from the nose and eyes was also observed (Supplementary Fig. 3), which started 4 days after infection, and the animals did not show signs of recovery. To investigate the protective efficacy of bivalent inactivated vaccine against viral infection, gerbils immunized twice were challenged with each virus. The morbidity of immunized gerbils was decreased, and animals rapidly recovered from EV71 C4a infection (Fig. 2A). In particular, the severity of disease symptom in the immunized group significantly reduced in vaccine dose-dependent manner, and the survival rate was 100% with the mid and high doses (p<0.0005, log-rank test, EV71 C4a infection vs. mid or high dose). In addition, even with a low dose, the survival rate was greater than 75% (p=0.0160, log-rank test, EV71 C4a infection vs. low dose). Signs of disease in gerbils following CVA16 virus infection were severer than those with EV71 C4a, but the recovery was dose-dependent (Fig. 2B). The group immunized with a high-dose vaccine had a 100% survival rate (p<0.0001, log-rank test, CVA16 infection vs. high dose). The group immunized with mid or low doses had survival rates of 75% (p=0.0005, log-rank test, CVA16 infection vs. mid dose) and 85% (p=0.0001, log-rank test, CVA16 infection vs. low dose), respectively. These results suggest that the bivalent inactivated vaccine effectively protects against viral infec-

Fig. 3. Immunization with the bivalent vaccine effectively prevents viral amplification in infected tissues. Mongolian gerbils were immunized twice with the bivalent EV71 C4a/CVA16 vaccine via the intramuscular route (n=3). (A, B) At 1 week following the final immunization, (A) EV71 C4a and (B) CVA16 were used for infection via the intraperitoneal route. On day 5 after infection, viral RNA content was analysed in the brainstem, muscle, spleen, and heart. Graphs show the mean ± SEM. One-way ANOVA was performed; ***p<0.001, vehicle vs virus-infected group; **p<0.01, virus-infected group vs immunized group.
tions by reducing morbidity and mortality after EV71 C4a and CVA16 infections in a dose-dependent manner.

**Bivalent inactivated vaccines prevent EV71 C4A and CVA16 viral amplification and tissue damage**

The bivalent inactivated vaccine could effectively protect against signs of infection caused by viral infection. To investigate whether it could also inhibit viral amplification and tissue damage after infection, relative viral RNA content was analysed in the brainstem, muscle, spleen, and heart of gerbils at day 5 following infection with each virus. Based on these results, all immunization doses significantly inhibited EV71 C4a (Fig. 3A) and CVA16 replication (Fig. 3B). These results suggested that the bivalent inactivated vaccine can efficiently prevent viral replication.

Next, we investigated whether bivalent inactivated vaccine immunization could prevent against tissue damage caused by viral infection. Brainstem, muscle, spleen, and heart tissues were obtained 5 days after infection and stained with haematoxylin-eosin to assess this. EV71 C4a infection resulted in muscle and heart tissue damage (Fig. 4A). In particular, the damage to muscle tissue was very severe. The degree of tissue damage was scored based on inflammatory cell infiltration and muscle fibre degeneration. As a result, the degree of host tissue damage was significant at high doses in both the muscle and heart (Fig. 4B, 4C). However, in the brainstem and spleen, tissue damage was not significant, even with EV71 C4a infection (Supplementary Fig. 4A). These results suggested that bivalent inactivated vaccine immunization can prevent against host muscle and heart damage following EV71 C4a infection in dose-dependent manner.

Similar to that with EV71 C4a infection, CVA16 infection did not affect the brainstem and spleen (Supplementary Fig. 4B), whereas the muscle and heart were severely damaged (Fig. 5A). When the histological grade of tissue damage was scored, based on the same method used for EV71 C4a, bivalent inactivated vaccine immunization was found to prevent muscle and heart damage in a dose-dependent manner (Fig. 5B, 5C). These data show that the bivalent inactivated vaccine can prevent against viral amplification and tissue damage in EV71 C4A- and CVA16-infected gerbils.

**DISCUSSION**

HFMD has been steadily occurring world-wide and recently has become frequent in Asia-Pacific regions, such as China, Japan, and Korea (Yi et al., 2017; Baek et al., 2020).
there are no specific therapeutics for HFMD, safe vaccination is an effective way to prevent its development in young children. EV71 vaccines from three manufacturers have been clinically used in China since 2015 (Liu et al., 2020). The inactivated monovalent EV71 C4a vaccine demonstrated safety and clinical effectiveness in a phase IV study with 89.7% protection and a 4.58% incidence of side effects (Guan et al., 2020). However, since HFMD can be caused by infection predominantly with CVA16, as well as EV71 C4a, bivalent or multivalent vaccines that are more effective than monovalent vaccines and offer broader coverage are needed (Liu et al., 2020). Bivalent or multivalent EV71/CVA16 vaccines are not available yet and are under development (Ku et al., 2014; Liu et al., 2020). In rhesus monkeys, the bivalent inactivated vaccine and live bivalent attenuated vaccine were effective in protecting against EV71 or CVA16 infection (Fan et al., 2020; Yang et al., 2020). In mice deficient in interferon (IFN) α/β (A129) and α/β and γ (AG129) receptors, a trivalent inactivated vaccine containing EV71, CVA16, and A6 also protected against lethal viral challenges (Caine et al., 2015). In addition, tetravalent virus-like particle (VLP) vaccines (EV71-VLP, CVA16-VLP, CVA6-VLP, and CVA10-VLP) were found to effectively protect against viral infection (Zhang et al., 2018).

Animal models of enterovirus infection have been established using AG129 mice, cynomolagus and rhesus monkeys, and hSCARB2 transgenic mice (Arita et al., 2005; Zhang et al., 2011; Fujii, et al., 2013; Meng and Kwang, 2014). In the immune-deficient AG129 transgenic mice, devoid of α/β and γ interferon receptors, virus replication occurs in muscle and adipose tissue, unlike that in humans (Khong et al., 2012; Meng and Kwang, 2014). The use of cynomolagus and rhesus monkeys, which show disease signs similar to those of humans, is unethical and expensive (Arita et al., 2005; Zhang et al., 2011). Meanwhile, hSCARB2 transgenic mice, which exhibit pathological features similar to those of human EV71 encephalitis, are associated with the disadvantage of only being infected during the neonatal period (Yi et al., 2017). Therefore, an evaluation of passive immunity via antiserum delivery can be performed, but this model is limited in its use to evaluate vaccine efficacy. In contrast, the Mongolian gerbil is a suitable model for evaluating the efficacy of vaccines because viral infection can occur after inducing active immunity via vaccination (Xu et al., 2015; Sun et al., 2016).

The Mongolian gerbil is a rodent native to the Mongolian steppe, belonging to the subfamily Gerbillinae (Zorio et al., 2019). Further, it is an animal used to model infections with various viruses, including Borna disease virus, La Crosse encephalitis virus, encephalomyocarditis virus, Puumala and Puumala-related viruses, and human hepatitis E virus (Matsuzaki et al., 1989; Osorio et al., 1996; Nakamura et al., 2019).
In conclusion, bivalent inactivated vaccines can protect Mongolian gerbils against EV71 C4a and CVA16 infections. The bivalent inactivated vaccine induced vaccine Ag-specific antibody responses and T cell cytokine production, especially upon immunization with high doses. When the immunized gerbils were infected with EV71 C4a and CVA16, the bivalent inactivated vaccine could efficiently inhibit viral amplification, protect host organs, and promote survival after lethal viral challenge in a dose-dependent manner. Taken together, we propose that the bivalent inactivated EV71 C4a/CVA16 vaccine could be a safe and effective HFMD vaccine candidate. In addition, the Mongolian gerbil is an efficient and suitable infection animal model for EV71 C4a and CVA16 to investigate vaccine efficacy following direct vaccine immunization.

CONFLICT OF INTEREST

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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REFERENCES


