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Research Paper

Immune responses and protective effects against Japanese encephalitis induced by a DNA vaccine encoding the prM/E proteins of the attenuated SA14-14-2 strain

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ABSTRACT

Japanese encephalitis virus (JEV) is the causal pathogen of Japanese encephalitis (JE), which has become a severe public health problem and is one of the most rapidly spreading mosquito-borne diseases worldwide. Currently, there is no specific treatment for JEV. A vaccine would be an effective measure for reducing morbidity and mortality. Although the live attenuated vaccine SA14-14-2 has been approved in some countries, it is still necessary to develop safer, more effective, and less costly vaccines. In this study, a DNA vaccine candidate, pV-SA14ME, expressing the prM/E proteins of SA14-14-2 was inoculated into BALB/c mice via intramuscular electroporation, and the immunogenicity and degree of protection were evaluated. We found that administration of 50 μg pV-SA14ME via electroporation via three immunizations could induce persistent humoral and cellular immune responses and effectively protect mice against lethal JEV challenge. This study provides a basis for the subsequent promotion and use of the vaccine and lays the foundation for its further use in swine and humans.

1. Introduction

Japanese encephalitis (JE) is an acute zoonotic disease caused by Japanese encephalitis virus (JEV) infection. There are approximately 67,900 new cases worldwide each year, approximately half of which occur in China (Feng et al., 2013). JEV belongs to the genus *Flavivirus* of the Flaviviridae family and is mainly transmitted by Culex mosquitoes. Swine and herons are the main amplification hosts (Zheng et al., 2013). Swine can develop reproductive system diseases after being infected with JEV, which will affect animal husbandry (Hu et al., 2017). After the host is infected with JEV, the virus can cross the blood–brain barrier of humans. Due to its neurotropism, half of patients will have severe neurological sequelae, such as the rapid onset of high fever, headache, neck stiffness, disorientation, coma, seizures, spastic paralysis, and death (Kulkarni et al., 2018). Currently, vaccination is the best preventive measure for both swine and humans. The affected countries are currently located in Asia. These countries usually carry out vaccination campaigns, but the types of vaccines are different and include a live attenuated vaccine (SA14-14-2 strain), inactivated vaccines (P3 strain and Nakayama strain), and recombinant chimeric vaccines. Among the vaccines used, the live attenuated vaccine based on the SA14-14-2 strain is one of the most widely used and tested vaccines.

Since the advent of the vaccination of the Chinese population in 1989, the total number of vaccination doses has exceeded 1 billion. At the same time, pigs were also vaccinated with the same SA14-14-2 vaccine. Due to its potent immunogenicity and complete protection, this vaccine not only has been integrated into China's National Expanded Program on Immunization but also exported to many affected Asian countries, such as Thailand, Myanmar, and Nepal (Monath, 2002). In 2013, SA14-14-2 passed the precertification process of the World Health Organization, thereby obtaining the procurement qualification of the United Nations and entering the international market. It

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is worth mentioning that although many countries do not directly use the SA14-14-2 vaccine, many variants and vaccines derived from SA14-14-2 have been introduced. For example, Sanofi's recombinant chimeric vaccine JE-CV (IMOJEV®) was licensed for use in Australia for people 12 months of age and older because JEV has also appeared in northern Australia (Kosalaraksa et al., 2017). JE-CV is a new recombinant chimeric virus vaccine that was developed using the yellow fever virus (YFV) vaccine backbone YFV-17D. This vaccine utilizes cDNA encoding the precursor membrane (prM) and envelope (E) proteins of YFV instead of those of SA14-14-2. JE-CV is highly immunogenic and can induce lasting immunity. A single dose is sufficient to induce protective immunity (Appaiahgari and Vrati, 2010). Similarly, IC-51 (IXIARO®), which is sold in the United States, is an inactivated vaccine based on SA14-14-2 (Duggan and Plosker, 2009). In addition, based on the experience with Denvaxia®, a dengue vaccine, the SA14-14-2 backbone has also been used in research of other flavivirus vaccines. The latest example is the Zika vaccine (Li et al., 2018). Therefore, it has been fully proven that SA14-14-2 itself and its skeleton and target genes are safe and effective. To date, there have been few reports of using the prM/E proteins of SA14-14-2 as part of a DNA vaccine. In fact, DNA vaccines are a promising vaccine type, especially in the veterinary field (Francis, 2018), and we have accumulated much experience in the development of flavivirus DNA vaccines against viruses such as dengue virus and Zika virus (Chen et al., 2016; Wang et al., 2018).

In this study, we used a previous platform to construct a JEV DNA vaccine based on the cloning of the sequences of the SA14-14-2 prM/E proteins into the pVAX1 (pV) vector, which was named pV-SA14ME, and evaluated the immunogenicity and protective effect of the vaccine in mice. Compared with live attenuated vaccines, DNA vaccines are less expensive and easier to produce, as they target structural proteins with the strongest antigenicity and can be quickly applied to veterinary use. This study will lay the foundation for future applications to swine and humans.

2. Materials and methods

2.1. Viruses, cells, plasmids, and animals

JEV (strain P3) was stored at −80 °C. It was used as the coating antigen and the stimulus for *in vivo* experiments and for challenge experiments.

HEK 293T cells were used for plasmid transfection. Vero cells were used for plaque assays to detect viral titers, and the plaque reduction neutralization test (PRNT) was used to detect neutralizing antibodies (nAbs).

The pV-SA14ME plasmid was constructed by introducing a *BamH*I enzyme digestion site, Kozak sequence and the signal sequence from vesicular stomatitis virus glycoprotein upstream of the *prM/E* sequence of the SA14-14-2 strain and introducing a *Xho*I digestion site downstream of the *prM/E* sequence into the vector pV (Fig. 1).

Female BALB/c mice (6–8 weeks old) were purchased from Beijing Vital River Experimental Animal Technology Co., Ltd., and used for immunization, obtaining sera, splenocyte collection and challenge tests. All mice were maintained in specific pathogen-free conditions. The results are from a single experiment or are from three independent experiments.

2.2. Ethics statement

We carried out this study in strict accordance with the national guidelines for the care and use of laboratory animals. All animal studies were approved by the Ethics Committee of Beijing Friendship Hospital, Capital Medical University.

2.3. In vitro transfection

One day prior to transfection, HEK 293T cells were plated at 5×10^5 cells/well in a six-well plate. Once they reached approximately 70% confluence in the plate, the cells were transfected with 1 μg/pV-SA14ME or pV per well using Lipofectamine 3000 (Invitrogen, USA) according to the manufacturer's protocols. After incubation for 6 h, the cells were washed with medium, and medium containing 10% fetal bovine serum (FBS) was added to each well. At 48 h post transfection, the cells were fixed with paraformaldehyde for 20 min at room temperature. After washing five times with PBS, the cells were incubated with anti-JEV polyclonal antibody at a dilution of 1:500 with PBS for 1 h at 37 °C. FITC-conjugated goat anti-mouse antibody at a dilution of 1:1000 (TransGen, China) was used as the secondary antibody, and the transfected cells were analyzed by fluorescence microscopy.

2.4. Immunization, sample collection, and challenge

pV-SA14ME or pV was administered intramuscularly via electroporation on days 0, 21, and 42. Each animal received 50 μg of plasmid per dose, which was diluted in sterile saline. The doses were equally divided into two injections of 25 μg, and one was administered into each quadriceps. The schedule of three vaccinations at three-week intervals is based on the research on flavivirus DNA vaccines previously (Chen et al., 2016; Wang et al., 2018). Three weeks after the last dose, the quadriceps femoris muscle at the injection site, sera and splenocytes were collected or challenge tests were performed. The mice in the vaccine and the control group were challenged intraperitoneally with 1×10^5 PFU of JEV. The body weight changes and the survival rate were measured daily after the challenge, and observation continued for 12 consecutive days.

2.5. Immunohistochemical staining

Paraffin sections were incubated with 3% peroxide-methanol and 1% bovine serum albumin (BSA) to block endogenous peroxidase activity. Then, the sections were heated at 100 °C for 15 min in sodium citrate-hydrochloric acid buffer solution for antigen retrieval, followed by incubation with anti-JEV polyclonal antibody at 4 °C overnight. The next day, the sections were incubated with an HRP-conjugated rabbit anti-mouse antibody (ZSGB-BIO, China) for 1 h at room temperature. Finally, diaminobenzidine substrate was used for coloration. The sections were counterstained with hematoxylin, and images were obtained by microscopy.

2.6. Enzyme-linked immunosorbent assay (ELISA)

Sera were prepared from mice on day 21 after the last immunization. Briefly, a 96-well plate was coated with 10^5 PFU of concentrated JEV per well and incubated at 4 °C overnight. The wells were then blocked with PBS containing 5% BSA. The sera were serially diluted in PBS containing 0.05% BSA to concentrations from 1:100 to 1:102,400 and added to the wells, which were incubated with a 1:500 dilution of an HRP-conjugated goat anti-mouse IgG antibody (TransGen, China) for 1 h at 37 °C. The plate was washed, the color developed with TMB, and the reaction was stopped with 1 M H_2SO_4. The plates were read with a microplate reader (Thermo, USA) at 450 nm.

2.7. PRNT

Pooled serum samples were heat-inactivated for 30 min at 56 °C. Mixtures of JEV with final concentrations corresponding to 100 plaque-forming units (PFU) per mixture and two-fold serially diluted serum samples (final serum dilution of 1:10 to 1:1280) were incubated for 1 h at 37 °C in 5% CO_2 and 100 μL virus medium, which was subsequently added to Vero cells in 24-well plates and incubated for 1 h at 37 °C in

5% CO_2. After the mixture was removed, the plates were washed and overlaid with 1.3% methylcellulose containing 5% FBS. After 5 days of incubation, the plates were stained with crystal violet, and the plaques were counted. The nAb titers were calculated as the reciprocal of the maximum dilution of serum that yielded a 50% plaque reduction in comparison with the number of plaques in the controls with JEV infection alone.

2.8. Splenocyte isolation for enzyme-linked immunospot (ELISPOT) assays

Three weeks after the last immunization, the mice were euthanized, and the spleens were aseptically removed. After obtaining single splenocyte suspensions, the frequency of IL-4- and IFN-γ-secreting cells was determined by incubating splenocytes (2×10^5 cells/well) from immunized mice with concentrated JEV for 60 h at 37 °C in 5% CO_2. The ELISPOT assay was performed using a mouse IL-4 or IFN-γ kit according to the manufacturer's instructions (BD, USA). The number of JEV-specific cells was expressed as the spot-forming units (SFUs). The positive and negative controls were generated in this experiment by stimulation with concanavalin A (Sigma-Aldrich, USA) and medium only, respectively. Spots were counted using an ELISPOT reader (CTL, USA).

2.9. Data analysis and statistics

The statistical significance (*P* values) was calculated by using SPSS 17.0 software (USA), and the body weight change was analyzed by repeated multivariate analysis of variance. The survival rates were compared using the log-rank test, and the differences between the groups were compared using one-way ANOVA. Quantitative data are expressed as the mean ± standard deviation. $P < 0.05$ was considered statistically significant. $P < 0.05$ (*), $P < 0.01$ (**), and $P < 0.001$ (***) were regarded as indicating a significant difference and a highly significant difference.

3. Results

3.1. Plasmid DNA can be successfully expressed in vitro and in vivo

We first constructed the plasmid pV-SA14ME according to the flow chart . Before the immunization of mice, we determined whether the target protein of the plasmid could be successfully expressed *in vitro*. We transfected the plasmid pV-SA14ME or pV into HEK 293T cells. The cells transfected with the former displayed specific fluorescence after the binding of JEV antiserum, but the control did not (Fig. 2A and 2B), indicating that pV-SA14ME can successfully express the target protein. Similarly, we obtained the quadriceps muscle from the inoculation site and generated paraffin sections to test whether the target proteins prM/E could be successfully expressed *in vivo*, which would indicate whether it is immunogenic and can induce an immune response. We found that the tissue of the vaccine group showed specific staining indicating the recognition of JEV antiserum; in contrast, the control did not (Fig. 2C and 2D), suggesting the immunogenicity of the plasmid pV-SA14ME. Therefore, the plasmid we constructed can be used in subsequent research.

3.2. pV-SA14ME immunization triggered a JEV-specific antibody response

The immunized mice were not observed to have adverse symptoms such as somasthenia, shagginess, or even paralysis. As one of the important indicators of vaccine-induced immunity, we evaluated the antibody levels produced by mice after three immunizations with pV-SA14ME, including the levels of IgG antibodies and nAbs. In the sera obtained three weeks after the last vaccination, IgG antibody production was successfully induced in the vaccine group and was much greater than that in the control group (1:2628 vs. 1:214, $P < 0.01$, Fig. 3A). NAbs play an important role in antiviral infection and reflect the effectiveness of the vaccine to a certain extent. In the vaccinated mouse sera, the nAb titer reached 1:60, while the control group titer was only 1:11 ($P < 0.01$, Fig. 3B). The above results suggest that pV-SA14ME successfully induced a JEV-specific humoral immune response in mice.

3.3. Splenocytes of immunized mice can generate cytokines upon JEV stimulation

To test the ability of splenocytes from immunized mice to secrete cytokines, we pulsed mouse splenocytes three weeks after the last immunization with concentrated JEV. The vaccine group mice generated higher levels of IL-4 and IFN-γ than the control group ($P < 0.01$, Fig. 4A; $P < 0.001$, Fig. 4B, respectively). IL-4 and IFN-γ are considered representative cytokines of the Th2 and Th1 types, respectively, suggesting that the splenocytes of pV-SA14ME-immunized mice can produce a Th1/Th2 type cytokine response upon stimulation with the JEV antigen.

3.4. pV-SA14ME immunization confers complete protection against JEV

Finally, the challenge test can determine the protective effect of the vaccine. The results showed that the weight of mice in the vaccine group was generally stable after the challenge during the 14-day observation period, whereas the weight of the mice in the control group dropped by more than 30%, which represented a decrease that was far greater than that observed in the vaccine group ($P < 0.01$, Fig. 5A). Similarly, the survival rate also reflects the protective effect. The mice in the vaccine group all survived (7/7), while the mice in the control group all died (0/7, $P < 0.001$, Fig. 5B), indicating that pV-SA14ME can confer strong specific protection against JEV in mice.

4. Discussion

In this study, the *prM/E* gene of the JEV SA14-14-2 strain was subcloned into the eukaryotic expression vector pV and presented in the form of DNA vaccine variants. In general, the results were expected and showed that the vaccine provides another alternative for the attenuated vaccine SA14-14-2, which is used in regions where JEV is endemic.

In fact, live attenuated vaccines do have many unique advantages (Gary and Weiner, 2020), such as similarity to natural infection, long-lasting immunity, and strong humoral and cellular immune responses [30172933]. However, the DNA vaccine also has unique advantages. The greatest advantages of DNA vaccines are their simple

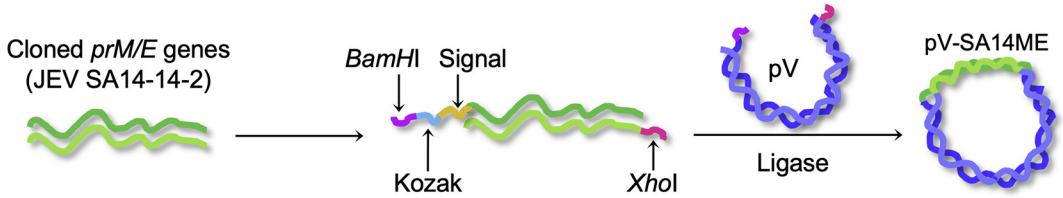

Fig. 1. Flow chart showing the construction of pV-SA14ME.

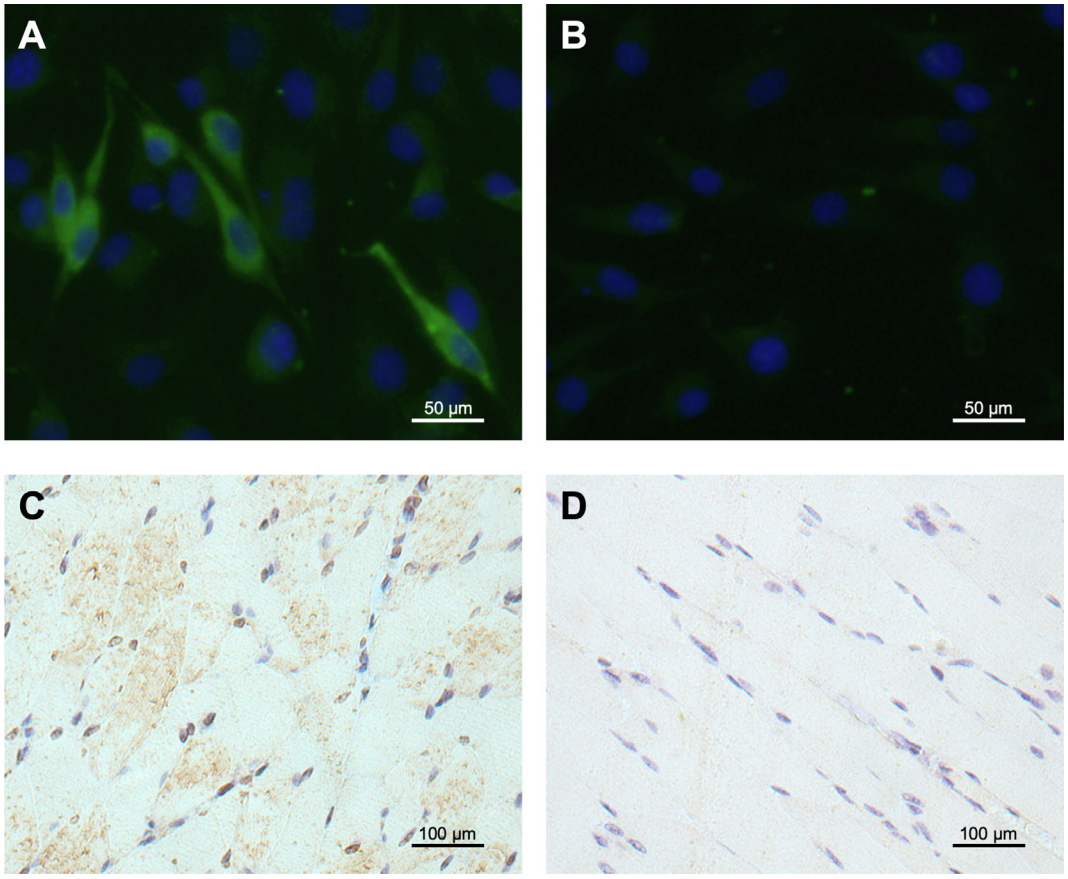

Fig. 2. Representative images of immunofluorescence in Vero cells after transfection with plasmid DNA and immunohistochemical staining of the quadriceps at the injection site. After HEK 293 T cells were transfected with pV-SA14ME or pV, JEV antiserum was used as the primary antibody, and goat anti-mouse FITC-IgG was used as the secondary antibody for staining. Image (A) shows specific green fluorescence but (B) does not. Similarly, the quadriceps was inoculated with (C) pV-SA14ME or (D) pV. Blue fluorescence indicates nuclear staining. The scale is marked with a bar.

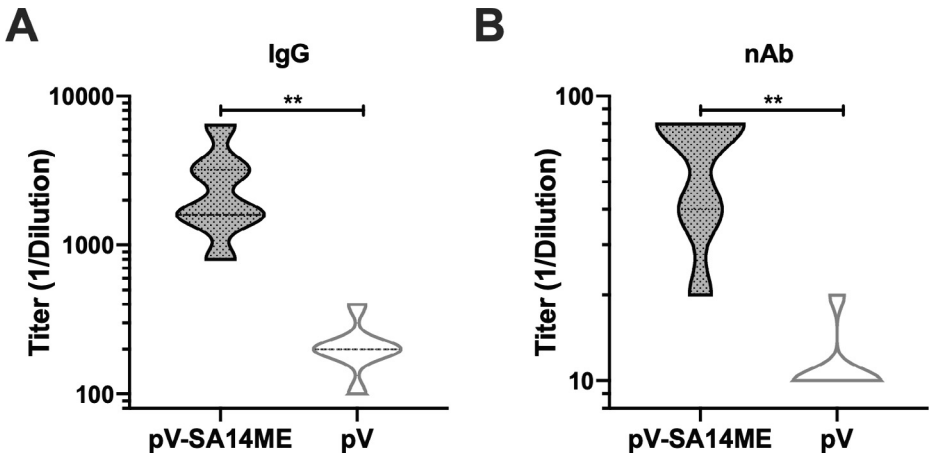

Fig. 3. Specific humoral immune response produced in mice after immunization. In the third week after the mice were immunized three times with pV-SA14ME or pV, (A) IgG antibodies and (B) nAbs were produced at higher titers in the vaccine group than in the control group. Each experiment was independently repeated three times. $^{**}P < 0.01$, $^{***}P < 0.001$, $n = 7$ per group.

manufacturing process and low production cost. For example, for both the Zika virus and SARS-CoV-2 (Gaudinski et al., 2018; Pang et al., 2019), the first vaccine developed was a DNA vaccine, and this confirmed the advantages of this vaccine type, which was also the original intention of our research. Although DNA vaccines still have a long way to go before they can be used for human applications, many veterinary applications have been developed (Jazayeri and Poh, 2019), such as the equine West Nile virus DNA vaccine (Jimenez de Oya et al., 2019) and the avian influenza DNA vaccine for poultry (Swayne, 2020), suggesting that DNA vaccines are becoming increasingly favored by researchers; their applications in the future will serve as an important foundation for the broader use of DNA vaccines.

China has been affected by JEV for a long time. Due to the continuous vaccination campaign, the number of JE cases has dropped dramatically. On the other hand, the pig industry is an important part of China's agriculture and animal husbandry industries. Pigs not only play a role as amplification hosts for JEV, but infected pigs also have adverse symptoms that cause significant losses to farmers (Ladreyt et al., 2019; Young et al., 2020). Thus, pigs are also routinely vaccinated with the SA14-14-2 vaccine. Based on the experience of the use of the West Nile vaccine for horses (Minke et al., 2004), we believe that it is necessary to develop a JEV DNA vaccine with the *prM/E* proteins of SA14-14-2 as

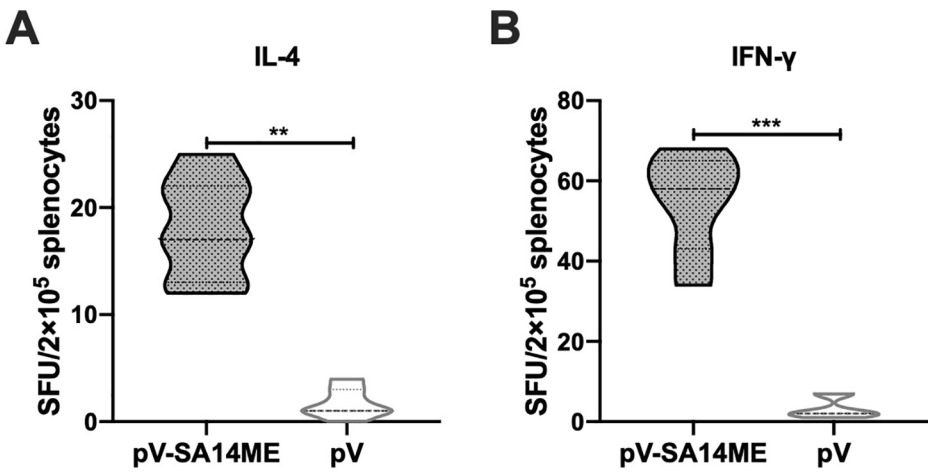

Fig. 4. Specific cytokine secretion by mice after immunization. In the third week after the mice were immunized three times with pV-SA14ME or pV, mouse splenocytes produced high levels of c) IL-4 and d) IFN-γ upon JEV antigen stimulation. Each experiment was independently repeated three times. $^{**}P < 0.01$, $^{***}P < 0.001$, $n = 7$ per group.

soon as possible. In this study, mice were first vaccinated with the pV-SA14ME vaccine to initially evaluate the immunogenicity and protective efficacy of the vaccine. A strong humoral immune response and effective triggering of Th1/Th2 cytokine production all contribute to protection. The encouraging data are also in line with our expectations because we have successfully constructed many flavivirus DNA vaccines using similar platforms and concepts.

The main limitation of this study is the failure to perform a comparative experiment between pV-SA14ME and the live attenuated SA14-14-2 vaccine. It might be interesting to compare our experimental vaccines with the licensed SA14-14-2 vaccine in a different study. The purpose of our study is to offer a new concept for the development an efficacious JEV vaccine. And, our proof-of-concept study provides an alternative vaccine strategy that could be further utilized in nonhuman primates and even in human clinical trials, but it is not meant to represent a replacement for the current SA14-14-2 vaccine. Nonetheless, our results show that mice are conferred with full protection after being vaccinated three times, suggesting that the protective efficacy is similar to that of attenuated strains. The SA14-14-2 strain is currently used as part of a two-shot regimen administered at 8 months and 2 years of age, respectively, while this DNA vaccine used a three-shot regimen, which is also one of the improvements to the vaccine immunization schedule. Alternatively, the prime-boost strategy of heterologous immunity may be utilized.

In conclusion, we used the *prM/E* gene of the live attenuated JEV vaccine SA14-14-2 to construct a DNA vaccine variant. After the mice were vaccinated with this vaccine, the potent immune response and protective efficacy that were observed showed the promising prospects of this vaccine, which laid an important foundation for subsequent swine and human use.

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Credit author statement

Xiaoyan Zheng designed and performed experiments, analyzed data, and wrote the manuscript. Xiaozheng Yu and Yan Wang helped to perform experiments. Min Cui provided valuable suggestions for manuscripts and experiments. Ran Wang designed the experiment, interpreted data and reviewed the manuscript. Chenghong Yin reviewed and guided the overall experiment. All authors have critically read and edited the manuscript.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to

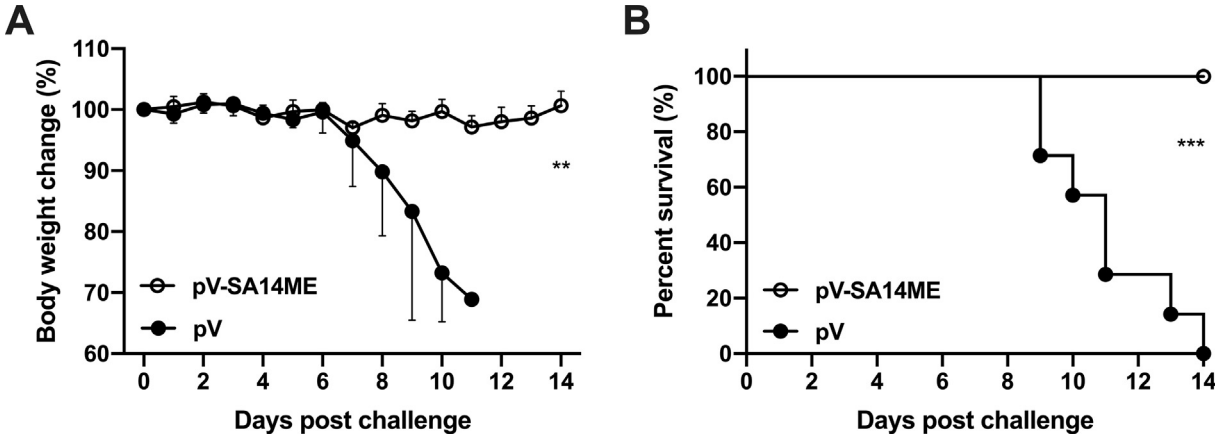

Fig. 5. Protective effect induced in mice after immunization. In the third week after the mice were immunized with three pV-SA14ME or pV doses, the challenge test was performed. The mice in the vaccine group had no significant body weight changes after 14 days, while the control group showed a continual decline in body weight. Similarly, the mice in the vaccine group were completely protected, and the survival rate was as high as 100%, while the mice in the control group all died. Each experiment was independently repeated three times. $^{**}P < 0.01$, $^{***}P < 0.001$, $n = 7$ per group.

influence the work reported in this paper

References

Appaiahgari, M.B., Vrati, S., 2010. Imojev((r)): A yellow fever virus-based novel japanese encephalitis vaccine. Expert. Rev. Vaccines 9, 1371–1384.

Chen, H., Zheng, X., Wang, R., Gao, N., Sheng, Z., Fan, D., Feng, K., Liao, X., An, J., 2016. Immunization with electroporation enhances the protective effect of a dna vaccine candidate expressing prme antigen against dengue virus serotype 2 infection. Clin. Immunol. 171, 41–49.

Duggan, S.T., Plosker, G.L., 2009. Japanese encephalitis vaccine (inactivated, adsorbed) [ixiaro]. Drugs 69, 115–122.

Feng, Y., Fu, S., Zhang, H., Petersen, L.R., Zhang, B., Gao, X., Yang, W., Zhang, Y., Dao, B., Li, K., Li, N., Yin, Z., Liu, Y., Nasci, R., Wang, H., Liang, G., 2013. High incidence of japanese encephalitis, southern china. Emerg. Infect. Dis. 19, 672–673.

Francis, M.J., 2018. Recent advances in vaccine technologies. Vet. Clin. North Am. Small Anim. Pract. 48, 231–241.

Gary, E.N., Weiner, D.B., 2020. Dna vaccines: prime time is now. Curr. Opin. Immunol. 65, 21–27.

Gaudinski, M.R., Houser, K.V., Morabito, K.M., Hu, Z., Yamshchikov, G., Rothwell, R.S., Berkowitz, N., Mendoza, F., Saunders, J.G., Novik, L., Hendel, C.S., Holman, L.A., Gordon, I.J., Cox, J.H., Edupuganti, S., McArthur, M.A., Rouphael, N.G., Lyke, K.E., Cummings, G.E., Sitar, S., Bailer, R.T., Foreman, B.M., Burgomaster, K., Pelc, R.S., Gordon, D.N., DeMaso, C.R., Dowd, K.A., Laurencot, C., Schwartz, R.M., Mascola, J.R., Graham, B.S., Pierson, T.C., Ledgerwood, J.E., Chen, G.L., Vrc, teams VRCs, 2018. Safety, tolerability, and immunogenicity of two zika virus dna vaccine candidates in healthy adults: randomised, open-label, phase 1 clinical trials. Lancet 391, 552–562.

Hu, P., Chen, X., Huang, L., Liu, S., Zang, F., Xing, J., Zhang, Y., Liang, J., Zhang, G., Liao, M., Qi, W., 2017. A highly pathogenic porcine reproductive and respiratory syndrome virus candidate vaccine based on japanese encephalitis virus replicon system. PeerJ 5, e3514.

Jazayeri, S.D., Poh, C.L., 2019. Recent advances in delivery of veterinary dna vaccines against avian pathogens. Vet. Res. 50, 78.

Jimenez de Oya, N., Escribano-Romero, E., Blazquez, A.B., Martin-Acebes, M.A., Saiz, J.C., 2019. Current progress of avian vaccines against west nile virus. Vaccines Basel, 7.

Kosalaraksa, P., Watanaveeradej, V., Pancharoen, C., Capeding, M.R., Feroldi, E., Bouckenooghe, A., 2017. Long-term immunogenicity of a single dose of japanese encephalitis chimeric virus vaccine in toddlers and booster response 5 years after primary immunization. Pediatr. Infect. Dis. J. 36, e108–e113.

Kulkarni, R., Sapkal, G.N., Kaushal, H., Mourya, D.T., 2018. Japanese encephalitis: a brief review on Indian perspectives. Open Virol J 12, 121–130.

Ladreyt, H., Durand, B., Dussart, P., Chevalier, V., 2019. How central is the domestic pig in the epidemiological cycle of japanese encephalitis virus? A review of scientific evidence and implications for disease control. Viruses 11.

Li, X.F., Dong, H.L., Wang, H.J., Huang, X.Y., Qiu, Y.F., Ji, X., Ye, Q., Li, C., Liu, Y., Deng, Y.Q., Jiang, T., Cheng, G., Zhang, F.C., Davidson, A.D., Song, Y.J., Shi, P.Y., Qin, C.F., 2018. Development of a chimeric zika vaccine using a licensed live-attenuated flavivirus vaccine as backbone. Nat. Commun. 9, 673.

Minke, J.M., Audonnet, J.C., Fischer, L., 2004. Equine viral vaccines: the past, present and future. Vet. Res. 35, 425–443.

Monath, T.P., 2002. Japanese encephalitis vaccines: current vaccines and future prospects. Curr. Top. Microbiol. Immunol. 267, 105–138.

Pang, J., Wang, M.X., Ang, I.Y.H., Tan, S.H.X., Lewis, R.F., Chen, J.I., Gutierrez, R.A., Gwee, S.X.W., Chua, P.E.Y., Yang, Q., Ng, X.Y., Yap, R.K., Tan, H.Y., Teo, Y.Y., Tan, C.C., Cook, A.R., Yap, J.C., Hsu, L.Y., 2020. Potential rapid diagnostics, vaccine and therapeutics for 2019 novel coronavirus (2019-ncov): a systematic review. J. Clin. Med. 9.

Swayne, D.E., 2020. Laboratory methods for assessing and licensing influenza vaccines for poultry. Methods Mol. Biol. 2123, 211–225.

Wang, R., Liao, X., Fan, D., Wang, L., Song, J., Feng, K., Li, M., Wang, P., Chen, H., An, J., 2018. Maternal immunization with a dna vaccine candidate elicits specific passive protection against post-natal zika virus infection in immunocompetent balb/c mice. Vaccine 36, 3522–3532.

Young, C.L., Lyons, A.C., Hsu, W.W., Vanlandingham, D.L., Park, S.L., Bilyeu, A.N., Ayers, V.B., Hettenbach, S.M., Zelenka, A.M., Cool, K.R., Peterson, G.J., Higgs, S., Huang, Y.S., 2020. Protection of swine by potent neutralizing anti-japanese encephalitis virus monoclonal antibodies derived from vaccination. Antiviral Res. 174, 104675.

Zheng, H., Shan, T., Deng, Y., Sun, C., Yuan, S., Yin, Y., Tong, G., 2013. Molecular characterization of japanese encephalitis virus strains prevalent in chinese swine herds. J. Vet. Sci. 14, 27–36.