JOURNAL OF GENERAL VIROLOGY

RESEARCH ARTICLE

Nerome *et al.*, *Journal of General Virology* 2018;99:897–907

DOI 10.1099/jgv.0.001081

MICROBIOLOGY SOCIETY

Development of a Japanese encephalitis virus genotype V virus-like particle vaccine in silkworms

Kuniaki Nerome,[1,*] Ryoji Yamaguchi,[2] Naoyuki Fuke,[2] Uda Zahli Izzati,[2] Kenichi Maegawa,[1] Shigeo Sugita,[3] Kazunori Kawasaki,[4] Kazumichi Kuroda[5] and Reiko Nerome[1]

Abstract

To counter the spread of multiple Japanese encephalitis virus (JEV) variants harboured in alternative host species and highly neurotoxic variants with new antigenicity, such as genotype V (Muar), methods for developing more effective and low-cost vaccines against a variety of epidemic JEV strains are required. Here, we successfully synthesized large amounts of a Muar virus-like particle (MVLP) vaccine for JEV in silkworm pupae by using a *Bombyx mori* nuclear polyhedrosis virus recombinant consisting of JEV codon-optimized envelope (E) DNA. In particular, histopathological examination suggested that MVLP was efficiently synthesized in body fat tissues as well as epithelial cells. Quantitative analysis indicated that one silkworm pupa produced 724.8 µg of E protein in the MVLP vaccine. Electron microscopic examination of purified MVLP vaccine defined a typical MVLP morphological structure. Detailed MVLP antigen assessment by immune-electron microscopy revealed that the majority of MVLPs were covered with approximately 10 nm projections. Boosted immunization with MVLP antigens in mice and rabbits tended to show improved plaque inhibition potency against homologous Muar and heterologous Nakayama, but less potency to Beijing-1 strains. Notably, mixed immune rabbit antisera against Nakayama and Muar VLP antigens led to an increase in the low antibody reaction to Beijing-1. Additionally, a stopgap divalent JEV vaccine consisting of MVLP and Nakayama VLP and its immune mouse serum significantly increased plaque inhibition titre against Muar, Nakayama and Beijing-1 strains. These findings suggested that low-cost MVLP vaccines prepared in silkworm pupae are suitable for providing simultaneous protection of individuals in developing countries against various JEV strains.

INTRODUCTION

Many severe neurological diseases are caused by transmission of Japanese encephalitis viruses (JEVs) from mosquito vectors, although most have shown a tendency of decreasing prevalence in humans upon immunization with suitable vaccines and improvement of ecological circumstance. However, some different JEV variants are still circulating in swine populations, mosquitos and birds, thus maintaining the mosquito–swine or mosquito–bird ecosystem. For example, the active circulation of numerous JEV variants in the swine population was defined following a large-scale virological surveillance in Japan, conducted with the cooperation of local governments [1]. In particular, several antigenic variants exhibiting slightly different evolutionary locations within genotype I evolutional branch clusters were found to co-circulate in the swine population. Furthermore, two new isolates with distinct neurovirulence and neuroinvasiveness were isolated from swine at the same farm on the same day of 2004 [1], meaning there is the potential for JEV to re-emerge in humans despite vaccination.

JEVs have been actively circulating in humans and swine in other South Asian countries as well [2]. In particular, among the numerous isolates analysed, most were reported to belong to genotype III until the 1990s, including isolates from China, Taiwan, India, Japan, Australia, Korea and Indonesia [3]. However, the major genotype of JEV isolated in Japan changed from genotype III to genotype I in the early 1990s and recently a similar genotype shift has also been confirmed in South Korea, northern Vietnam, China, Taiwan and Thailand [4]. Moreover, the isolation of genotype IV virus in Indonesia has attracted further attention owing to the potential for future epidemics [3]. Consistent with these findings, Japanese encephalitis disease has also

Received 5 March 2018; Accepted 5 May 2018

Author affiliations: [1]The Institute of Biological Resources, Okinawa, Japan; [2]Laboratory of Veterinary Pathology, Department of Veterinary, Faculty of Agriculture, University of Miyazaki, Miyazaki, Japan; [3]Equine Research Institute, Japan Racing Association, Tochigi, Japan; [4]National Institute of Advanced Science and Technology (AIST), Osaka, Japan; [5]Division of Microbiology, Nihon University School of Medicine, Tokyo, Japan.

***Correspondence:** Kuniaki Nerome, rnerome_ibr@train.ocn.ne.jp

Keywords: Japanese encephalitis genotype V virus; vaccine; silkworm; virus-like particle.

Abbreviations: CPE, cytopathic effect; E, envelope; HE, haematoxylin-eosin; JEV, Japanese encephalitis virus; MDNA, Muar DNA; MNPV, multinucleocapsid nuclear polyhedrosis virus; MVLP, Muar virus-like particle; NVLP, Nakayama virus-like particles.

been reported in several Asian countries, with 50 000–175 000 confirmed new cases per year consisting of different age groups [5–7] and incidence rates ranging from 0.003 to 3.7 % across various populations in Indonesia, Nepal, India, Bangladesh, Korea and China [8].

Historically, JEV vaccines have played an important role in elimination of JEV epidemics since the first use of a mouse brain-derived genotype III JEV vaccine in Japan in 1945 [9]. Subsequently, vaccine quality has markedly improved, with mouse brain-derived vaccines having been replaced by cell culture-derived vaccines [10–16]. In particular, the safety and efficacy of a Vero cell-derived genotype III JEV vaccine has been confirmed [14]. Conversely, the increased incidence of infection may be attributable in part to decreased protective immunity in humans and constant circulation of the virus in swine and bird vectors. Many antigenic and virulent JEV variants are co-circulating in mosquitos, swine and humans in Southeast Asian countries [7, 8], highlighting the need for wide distribution of more effective and low-cost vaccines against a variety of epidemic JEV strains throughout many parts of Asian countries. In particular, the spread of a genotype V virus possessing new antigenicity and high neurovirulence characteristics necessitates suitable and effective countermeasures against this variant. For example, the high pathogenicity of the genotype V Muar virus, which was first isolated from a patient with encephalitis in Malaysia in 1952 [17], has been investigated in detail in a mouse model [18, 19]. In addition, this virus has been circulating constantly in the mosquito population in different parts of Asia, producing new threats mainly in Asian countries [20, 21]. However, the present JEV vaccine may be ineffective against JEV genotype V Muar strain owing to antigenic differences [18, 22].

Accordingly, in this study, we aimed to develop a genotype V vaccine of JEV using our baculovirus (multinucleocapsid nuclear polyhedrosis virus; MNPV) genetic engineering system [23, 24]. As a result, we achieved successful production of a JEV-Muar virus-like particle (MVLP) vaccine in silkworm *Bombyx mori* pupae. In addition, the biological, morphological and immunological characteristics of the JEV-MVLP vaccine were subsequently characterized.

RESULTS

Design and characterization of chimeric JEV DNA (genotypes III and V)

For production of MVLP vaccines against JEV genotype V (Muar strain), codon-optimized DNA consisting of Muar envelope (E) and frequently used silkworm-specific codons was designed based on computer gene analyses as described previously [23, 24].

Expression of codon-optimized MNPV recombinant DNA gene

Expression of recombinant JEV-MNPV was assessed in *B. mori* ovarian (Bm-N) cells (Fig. 1a, b). The appearance of cytopathic effects (Fig. 1a RV-CPE), which differed markedly from that of normal control cells (Fig. 1a NC), indicated infection of recombinant virus in Bm-N cells. Apparent synthesis of E protein in the recombinant-infected cells was also confirmed in Bm-N cells, showing clear fluorescence in the recombinant virus-infected cells (Fig. 1b Ab-MVLP, Ab-JEV), which was apparently comparable to that of normal cells (Fig. 1b NC).

Histopathological examination in infected pupae

Pathological specimens of recombinant JEV-MNPV-infected pupae were separated into two main tissues. As shown in Fig. 1(c) with haematoxylin-eosin (HE) staining, the epithelium under the skin showed expanded nuclei from 4 days after infection, with epithelial cell nuclei (indicated by an arrow) showing marginalized chromatin with single prominent nucleoli (Fig. 1c E). A similar nuclear change (indicated by an arrow) was detected in body fat cells (Fig. 1c F). In the course of the 4 day infection, the number of recombinant JEV-MNPV-immunoreactive epithelial cells increased (Fig. 2a E, indicated by arrows). Similar positive cells were also confirmed in body fat tissues (Fig. 2a F, indicated by arrows). As shown in Fig. 2(a) F, the number of positive epithelial cells reactivated with anti-Tag monoclonal antiserum (indicated by arrows) increased over time in the body fat tissue. The above positive cells were quite different from those of negative (noninfected) control cells.

Extraction of MVLPs and their morphology

For extraction and preparation of MVLPs, JEV-MNPV was infected into 30 silkworm pupae, and MVLPs were extracted via three cycles of sonication [23, 24]. As shown in Table 1 60 ml of crude pupae homogenates exhibited a haemagglutination (HA) titre of 512 in an ordinary PBS sample containing Mg^{++} and Ca^{++} ions, with a total protein concentration of 906 mg ml^{-1} (Table 1). Conversely, authentic Nakayama and Muar strains of JEV did not show HA activity in PBS containing Mg^{++} and Ca^{++} ions, whereas Muar-infected homogenates exhibited marked HA activity. The migration profile in slab gel electrophoresis is shown in Fig. 2(b); the 53 kDa E protein was estimated to be present at a concentration of 21 744 μg ml^{-1}, with an estimated production level of 724 μg $pupa^{-1}$, indicating that abundant amounts of E protein were produced (Table 1). Unfortunately, we could not directly identify the above 53 kDa protein in the present study, however, as shown in Fig. 2(c), the position of MVLP E protein was indirectly confirmed with antiserum to the Beijing-1 control virus in Western blot analysis (Fig. 2c). This partially purified sample was further purified by 10–50 % sucrose gradient centrifugation [23, 24] and MVLPs were determined as a marked white band in Fig. 2(d) (indicated by an arrow). As a result, the homogenate produced two protein peaks in the sucrose gradient (Fig. 2e). The two peaks of protein concentration and HA activity coexisted in the same 12 fractions, suggesting that the Muar E protein apparently exhibited HA activity.

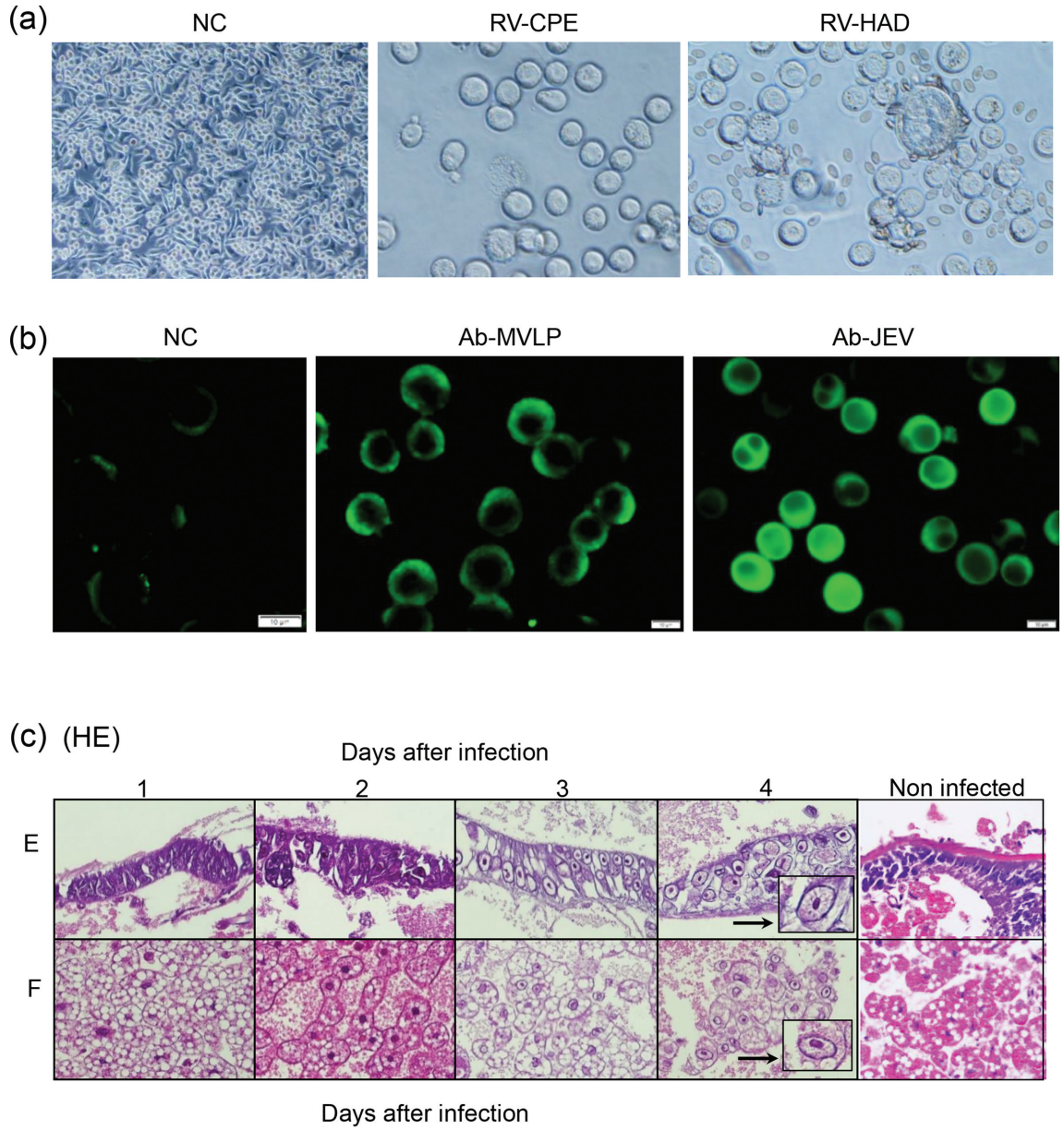

Fig. 1. Examination of expression of MVLP antigens in Bm-N cells and their synthesis in recombinant-infected pupae. (a) Expression of recombinant MVLP in Bm-N cells based on cytopathic effect (CPE) and haemadsorption (HAD) tests in normal control cells (NC), recombinant-infected CPE (RV-CPE) and recombinant-infected HAD (RV-HAD) cells. (b) Expression test based on fluorescent (FA) antibody test in normal (NC), recombinant-infected cells treated with antibody to MVLP (Ab-MVLP) and recombinant-infected cells treated with an antibody to the JEV vaccine (Ab-JEV). (c) MVLP production analysis in MVLP-recombinant-infected pupae based on histopathological examination during a 4 day period. E, epithelium. F, body fat. (c) E, marginalized chromatin and single dense nucleoli observed in epithelial cells from 4 days after infection and noninfected (normal) control cell. (c) F, lack of eosinophilic cytoplasmic granules, marginalized chromatin and single dense nucleoli observed from 4 days after infection in body fat tissue and also noninfected (normal) control cell. (c) E, arrows indicate epithelial nucleus and (c) F, body fat nucleus.

Morphological examination of JEV-MVLP

As marked, HA titres were present in the crude silkworm homogenate (Fig. 2c) and fractions 9–13 (Fig. 2c) were pooled and examined by electron microscopy. As shown in Fig. 3(a), MVLPs exhibited a variety of shapes under high-magnification electron microscopy. Notably, we observed many clear, long projections (spikes) on the surfaces of MVLPs that measured approximately 80–100 nm in diameter. For estimation of the lengths of spikes on the surface of VLPs, 10 spikes were selected and their length was

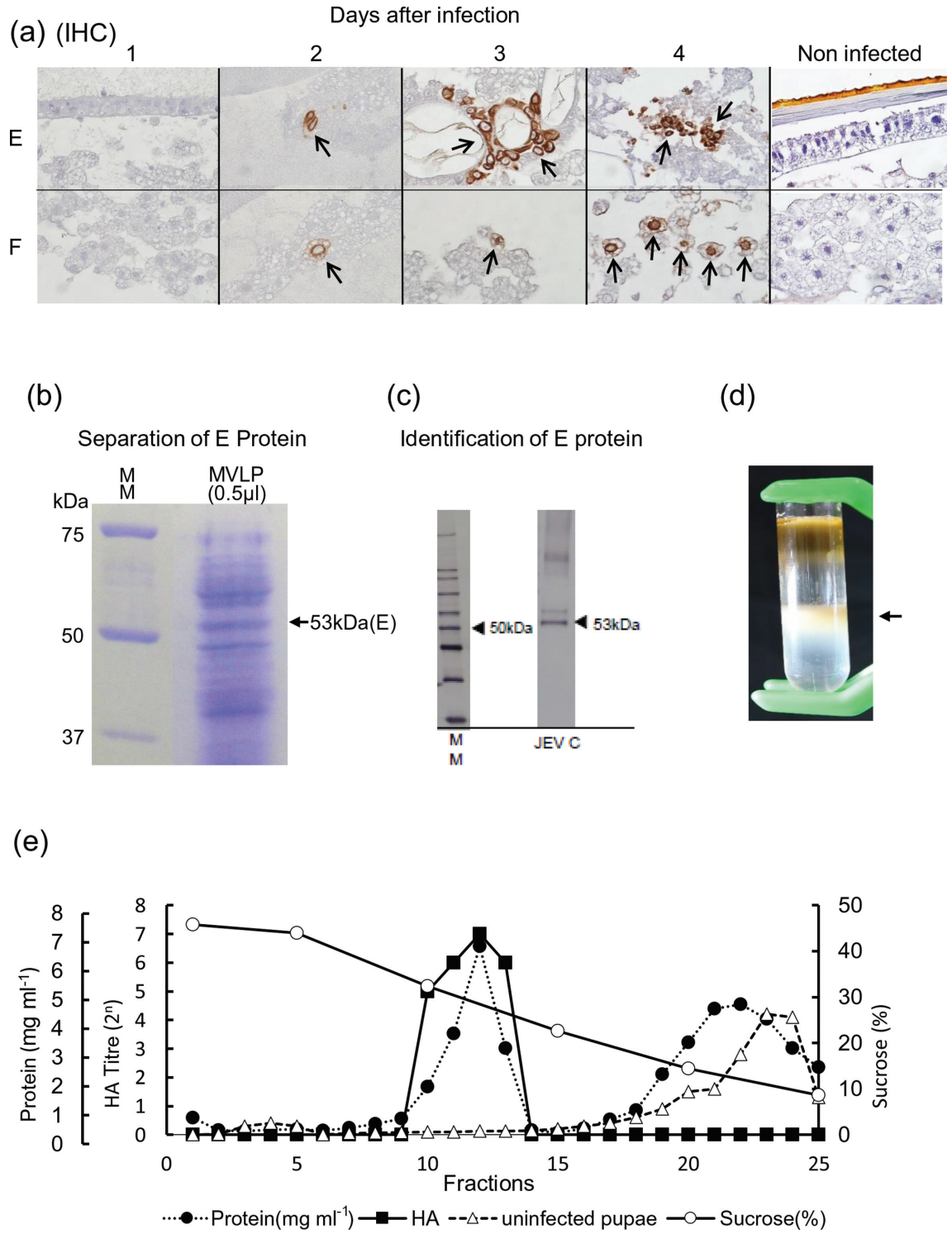

Fig. 2. Immunopathology of epithelial cells and body fat tissues in silkworm pupae infected with recombinant MVLP. (a) IHC. E, intense nuclear membrane and cytoplasmic immunoreactivity with an anti-DYKDDDK Tag monoclonal antibody in infected epithelial cells and noninfected (normal) control cell. F, nuclear membrane and cytoplasmic immunoreactivity with an anti-DYKDDDK Tag monoclonal antibody in infected body fat tissue and also noninfected (normal) control cell. Arrows in E and F, epithelial cells stained with Tag monoclonal antibody. (b) Separation of MVLP antigen and identification of E protein. (c) Identification of E protein of JEV control (JEV C) by Western blot analysis with antiserum to Beijing-1 strain. (d) Separation of MVLP antigen by sucrose density gradient centrifugation. (e) Fractionation of MVLP antigen and examination of biological activities.

Table 1. Recovery of MVLP-E protein from recombinant virus-infected pupae

No. of pupae	Vol (ml)	HA titre	Protein		
			Total (mg ml^{-1})	Total E (μg ml^{-1})	E (μg $head^{-1}$)
30	60	512	906	21 744	724

measured (Fig. 3a, b). The lengths were distributed between 7.89 and 16.62 nm, with a mean length estimated as approximately 11 nm (Fig. 3b).

Next, we used an immuno-gold electron microscope to confirm the presence of E protein on these spikes. Immuno-gold-treated MVLPs showed clear distributions across a large number of gold spots around MVLPs (Fig. 3c, indicated by white arrows). Electron micrographic imaging showed that the immune-gold bound to a large number of MVLPs. The clear immune-gold binding to the surface of MVLP suggested the presence of specific E proteins on the MVLP particles. For further detailed observation, two MVLP particles were selected and their spikes measured (Fig. 3d, e). For short distances of approximately 9–14 nm, these spikes were considered to have bound directly to MVLP structures.

Plaque neutralization using immune antisera prepared in mice and rabbits immunized with MVLP and Nakayama (N)VLP antigens

Plaques produced by Muar, Nakayama and Beijing-1 strains of JEV in the presence of rabbit antiserum clearly distinguished at a 40–10, 240-fold antiserum dilution as exhibiting plaque inhibition titres against homologous Muar and heterologous Nakayama and Beijing-1 strains (Fig. 4a). For example, plaques of Muar and Nakayama strains at ×40 serum dilution had completely disappeared, indicating 100 % plaque inhibition. In contrast, the plaques of the Beijing-1 strain at ×40 serum dilution were similar to those of the virus control. This result indicated potential antigenic differences between Muar or Nakayama strains and Beijing-1 strains (Fig. 4a). Similarly, the plaque inhibition curve of the Muar strain markedly differed from those of the Nakayama and Beijing-1 strains (Fig. 4b–d). The plaque inhibition titres are summarized in Fig. 4(e). The plaque inhibition titres against the Muar, Nakayama and Beijing-1 strains were 10, 240, 256 and 40, respectively.

The immune responses of anti-Muar VLP antisera obtained from mice immunized with three doses of MVLP vaccines are shown in Fig. 5(a). Preimmune control mouse serum did not show apparent inhibition (Fig. 5a). Plaque inhibition titres against the three strains increased gradually according to dose increase, although the antibody titre against Beijing-1 was low. This tendency was also observed in rabbit antisera against MVLP antigens (Fig. 4e). We also prepared rabbit antiserum against the MVLP vaccine using Freund's complete adjuvant-MVLP vaccine, which demonstrated a considerably higher 50 % plaque inhibition titre against the homologous Muar strain, showing more than 10 000-fold plaque reduction (Fig. 4e); the same antiserum demonstrated a nearly 2560-fold plaque inhibition titre against the Nakayama strain (Fig. 4e). However, this mouse antiserum still showed a low titre against the Beijing-1 virus.

To address whether the low immune response against the Beijing-1 strain might be improved, mouse antiserum obtained from three-dose mice and rabbit antiserum were mixed and their antisera were again tested. As shown in Fig. 5(b), the antibody titre against the Beijing-1 strain increased nearly twofold. Additionally, preimmune rabbit serum did not show apparent inhibition.

Experimental preparation of the divalent JEV vaccine

To confirm this finding, Nakayama VLP and Muar VLP antigens were mixed and their antigen contents were adjusted to contain 20 μg E protein/ml, and the mixture was used to immunize mice three times at 1 week intervals. As shown in Fig. 5(c), the immune serum obtained after 4 weeks showed almost similar antibody responses to the three strains at levels similar to those of the mixed antiserum (Fig. 5c).

DISCUSSION

Recently available JEV vaccines have played an important role in decreasing JEV epidemics caused by JEV variants belonging to genotypes I–IV. Nevertheless, the development of additional, low-cost vaccines is required to provide protection for populations in developing countries. In this context, silkworm-derived vaccines may constitute suitable prophylactic agents. However, genotype V viruses were first isolated from a human in Malaysia (1952) and from mosquitoes in China (2009), with the genotype V JEV genome identified in mosquitoes in China (2009) and in South Korea as well, whereas immune efficacy against this virus is very low. Specifically, Tajima *et al.* [18] found that mouse antisera to the inactivated Nakayama genotype III JEV vaccine showed efficient plaque neutralization titres to the homologous Nakayama strain, whereas the antibody titres to genotype V Beijing-1 and Muar strains were much lower [18]. Consistent with the above report, in JEV-vaccinated mice, the protection levels against genotype III and V JEV were high and low, respectively [24]. Furthermore, seroconversion levels in different age groups of healthy individuals immunized with the currently available JEV vaccine were extremely low against genotype V JEV [22].

To address this issue, in the present study we demonstrated that potent antibodies against both the Muar genotype V virus and the Nakayama strain could be produced through

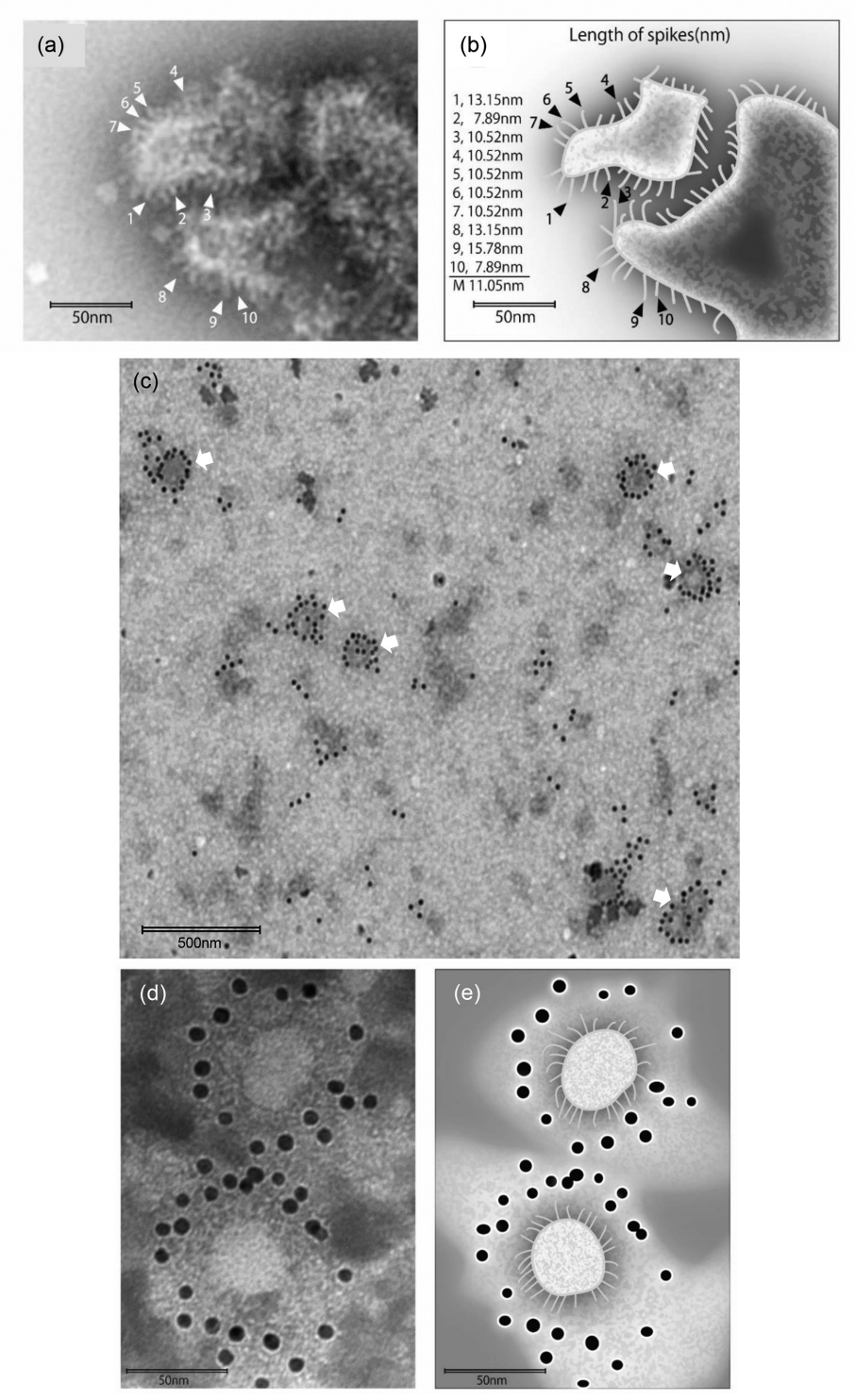

Fig. 3. Electron microscopic examination of purified MVLP antigens produced in silkworm pupae. (a–c) Electron micrograph of a sample treated with immune-gold. White arrows indicate typical MVLP antigens covered with black immune-gold (indicated by white arrows). (d), (e) Estimation of the length of E projection through transmission of MVLP vaccine detected with electron microscopy. Identification of E protein spikes with a gold-conjugated JEV antibody; black points indicate the edges of the E protein projection reacted with the JEV-specific antibody.

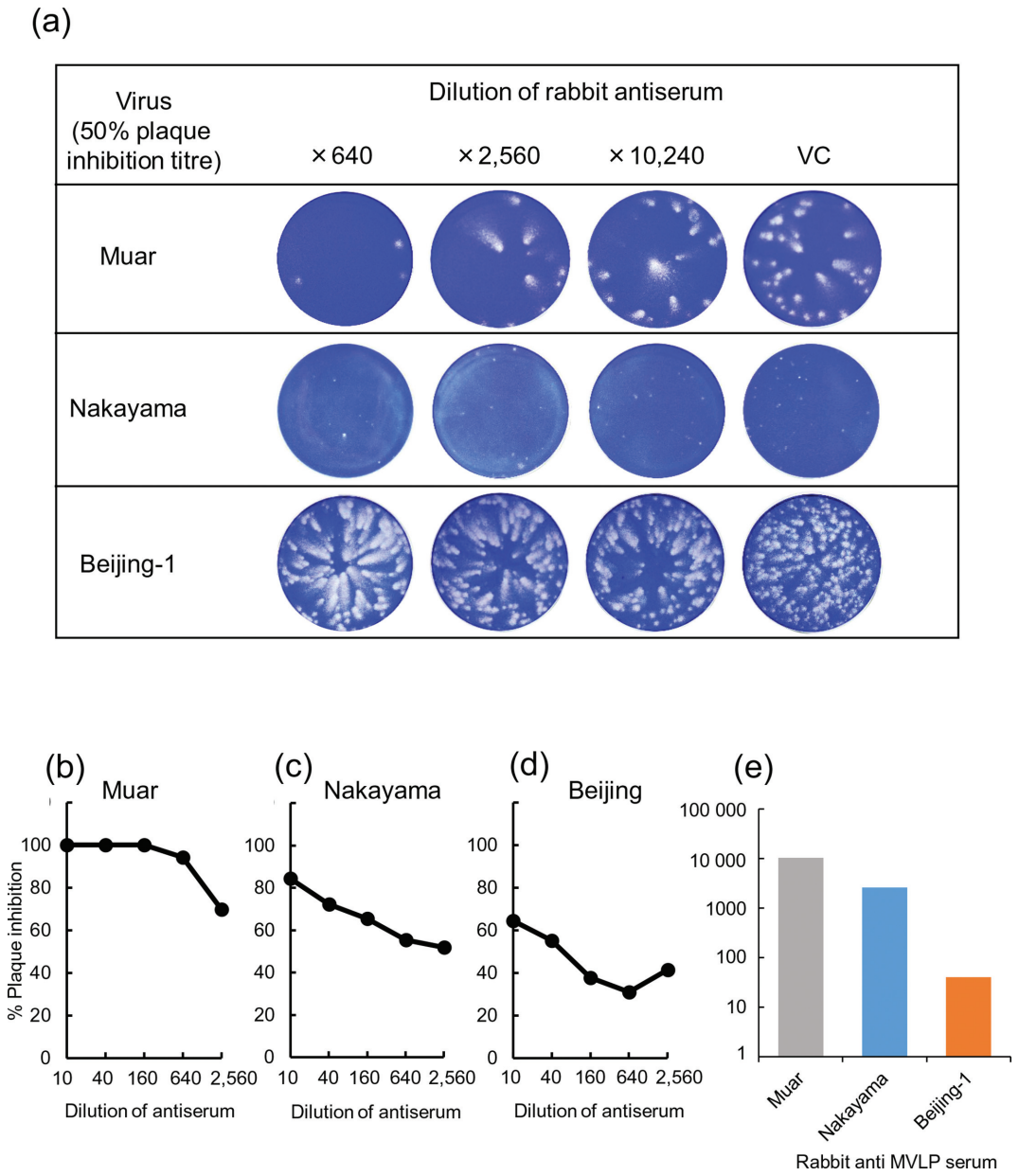

Fig. 4. Plaque inhibition profiles of antisera prepared in rabbits immunized with the partially purified MVLP vaccine against Muar, Nakayama and Beijing-1 strains of JEV. (a) Challenge viruses and plaque inhibition patterns. VC, virus control. (b–d) Plaque inhibition curves of Muar, Nakayama and Beijing-1 strains. (e) Summary plaque inhibition titres of Muar, Nakayama and Beijing-1 strains.

immunization with MVLP vaccines prepared in silkworm pupae. First, a series of experiments demonstrated that the codon-optimized and synthetic Muar DNA (MDNA) gene of the JEV Muar strain allowed increased production of an E protein-associated MVLP vaccine. We also found that a single pupa could produce 724 µg of E protein. It was further evident that nuclear changes could be detected in the epithelium and body fat tissues, suggesting increased production of MVLP. These changes were consistent with the immunoreactivity observed in both tissues, indicating active production of MVLP antigens in the epithelium and body fat.

Additionally, in this study we evaluated the tentative length of the E protein on the surface of MVLPs. Consistent with previous evidence, most E protein spikes were found to stretch upward from the surface of MVLPs and could be characteristically identified using immune-electron microscopy. Although the VLP structures of JEV and other flaviviruses have been reported in other vaccine studies, the detailed structures of VLP E proteins have not

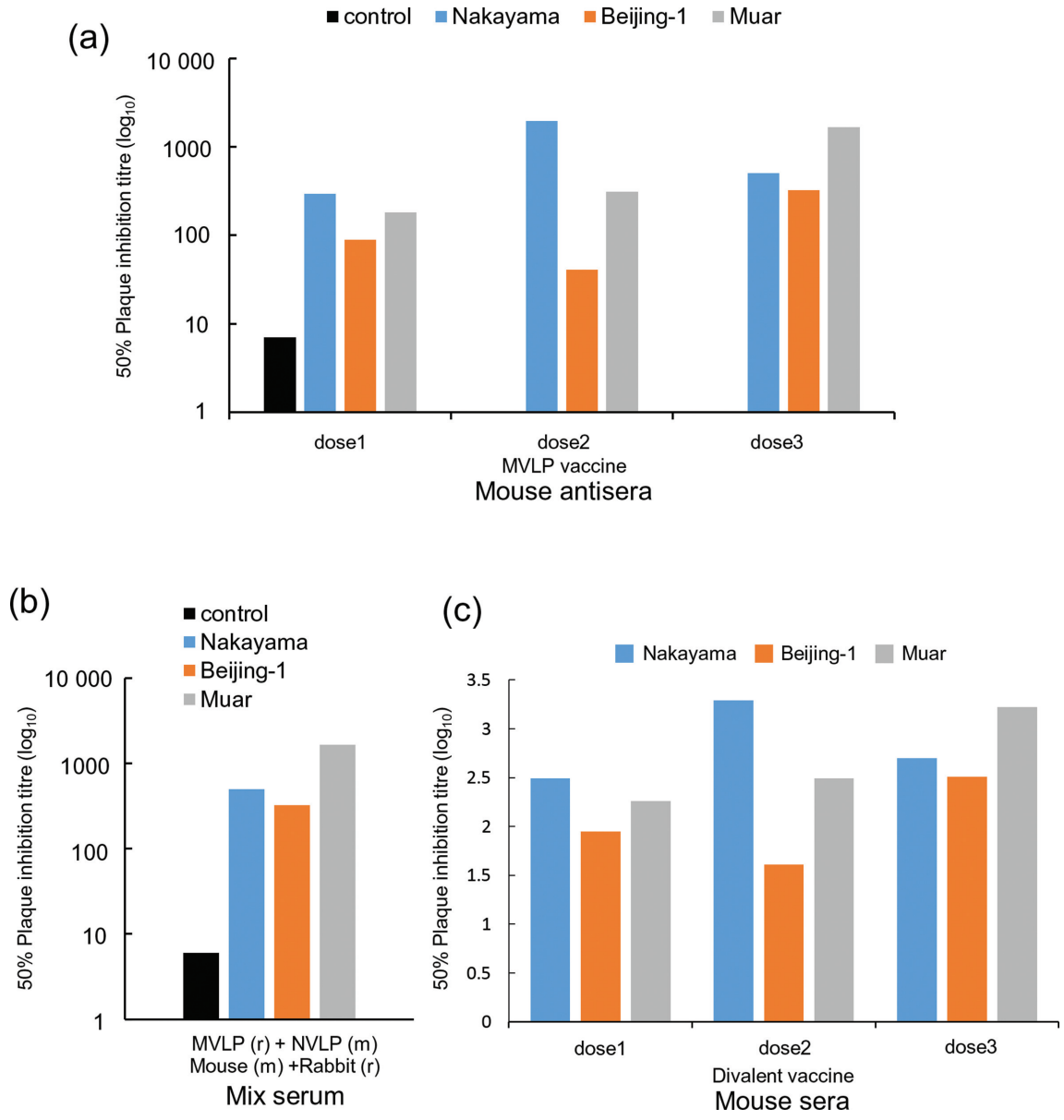

Fig. 5. Immune responses in mice and rabbits immunized with MVLP and NVLP vaccines produced in silkworm pupae. (a) 50 % plaque inhibition antibodies produced in mice immunized with three doses of vaccine. (b) 50 % plaque inhibition antibody titres of mixed antisera against MVLP rabbit and NVLP mouse antisera. (c) Serial antibody production in mice immunized with three doses of divalent MVLP and NVLP vaccine.

been extensively studied [25–27]. In the present report, we identified a variety of E protein spikes ranging from 7.8 to 16.6 nm in length (mean length: 11 nm). However, virion surface structures of flaviviruses have been reported to change during growth from an immature to mature stage [28, 29], with the arrangement of the spikes also influencing virus particle maturation [30]. For example, the surface structure of immature flavivirus shows prominent and irregular spikes, which markedly differ from those of mature particles [28]. The spikes observed in the MVLPs in the present study were somewhat similar to those of the surface of immature flaviviruses, showing clear and long E spikes.

Anti-mouse serum to MVLPs tended to exhibit a low immune response against both homologous Muar and heterologous Nakayama strains after one dose of immunization, with considerably less reaction to the Beijing-1 strain; however, this lower activity tended to increase after three immunization doses, reaching high reactivity with the former strains. Notably, such cross-potency, in contrast to the

standard JEV vaccines, may afford the potential for developing potent, divalent vaccines consisting of Muar and Beijing-1 strains or Muar and Nakayama strains. Indeed, test divalent JEV vaccines prepared in the present study could substantively mutually raise antibody titres against all of the above three strains of JEV. Together, our findings thus suggest low-cost MVLP vaccines prepared in silkworm pupae as suitable for providing simultaneous, potent protection of individuals in developing countries against various JEV strains.

Because the antibody to the Muar strain highly reacted with homologous Muar and heterologous Nakayama strains, but reacted to a lesser extent with the Beijing-1 strain, a divalent vaccine consisting of Muar and Beijing-1 strains or Muar and Nakayama strains would be ideal. In fact, the test divalent JEV vaccine prepared in the present study apparently could produce a mutual antibody titre against the above three strains of JEV.

METHODS

Construction of chimeric JEV-MDNA and generation of JEV-recombinant MNPV

Silkworm codon-optimized chimeric MDNA was designed using the E gene of genotype V JEV-Muar strain by introduction of frequently used silkworm codons. In total, 433 silkworm codons and prM plus 1500 Muar virus E codons were used for preparation of target chimeric MDNA for vaccine production. The resulting codon-optimized synthetic MDNA containing 2001 codons was used for preparation of recombinant baculovirus (JEV-MNPV), and subsequent procedures were performed as described previously [23, 24, 31].

Cells and viruses

The cells and viruses used in the present study were basically similar to those described in our previous reports [23, 24, 31]. Vero cells (cat. no. JCRB 9013) were purchased from the Japanese Collection of Research Bioresources (JCRB) cell bank and were used for propagation of the Nakayama, Beijing-1 and Muar JEV strains, which were obtained from the National Institute of Infectious Diseases (Tokyo, Japan).

Description of fluorescent-labelled antibodies and Western blot

Bm-N cells infected with the recombinant virus (JEV-MNPV) were maintained in MEM containing 10 % calf serum at 25 °C in a 5 % CO_2 incubator for 1–3 days. Briefly, experimental procedures were as described previously [24, 31]. The wased Bm-N cells on the cover slip glass were treated with mouse or rabbit antisera against JEV, followed by treatment with fluorescent isothiocyanate (FITC) conjugated anti-mouse or rabbit antibodies (West Grove, PA, USA). For Western blot analysis, goat polyclonal anti-JEV rabbit serum was obtained from NIID (Tokyo, Japan) and FITC-conjugated rabbit anti-goat IgG-AP antibodies (Santa Cruz Biotechnology) were used. The final signal was detected using ECL plus reagents (GE, Healthcare, Port Washington, NY, USA).

Histopathological examination

Infected silkworm pupae from 1 to 4 days after infection were collected and fixed in 10 % neutral buffered formalin or Methyl Carnoy's fixative solution, processed by conventional methods, and embedded in paraffin. For histopathological examination, 4 µm sections were cut and stained with haematoxylin-eosin (HE). For detection of recombinant JEV-MVLP, continuous sections of each sample were subjected to immunohistochemistry analysis. Antigen retrieval was omitted in Methyl Carnoy's fixed samples. Following blocking steps, sections were incubated overnight at 4 °C with an anti-DYKDDDDK Tag monoclonal antibody as the primary antibody, and for 30 min at 37 °C with the EnVision+Dual Link System-HRP (Dako) as a secondary antibody, with PBS rinses prior to each step. Colour was developed by reaction with 3,3′-diaminobenzidine substrates and counterstaining with haematoxylin.

Extraction and purification of MVLP antigen from recombinant-infected silkworm pupae homogenates

The recombinant baculovirus (JEV-MNPV) was inoculated into silkworm pupae. After 3–4 days, 0.01 % formalin and phenylthiourea were added and the inactivated pupae were homogenized with three cycles of sonication, as described previously [24]. The resulting homogenates were treated as described previously [23, 24, 31].

Haemagglutination test

In a recent study, we found that silkworm-derived MVLP and NVLP could apparently agglutinate chicken RBC in PBS free of Mg^{++} and Ca^{++} ions [PBS (−)] and we performed the haemagglutination (HA) test as described previously [23, 24].

Morphological characterization of MVLPs using electron microscopy

Purified MVLP fractions showing high HA activity were collected, and the sucrose component was removed through column filtration (PD-10 column; GE Healthcare). The resulting HA-MVLP samples were negatively stained with 2 % phosphotungstic acid. Samples were observed under an H-7600 electron microscope for identification of spikes on the surface of MVLPs, and another sample was treated with 5 % skim milk in PBS blocking solution. 10 µl of primary polyclonal JEV rabbit sera antibody (100×dilution) obtained from NIID (Tokyo, Japan) was applied on the grid for 10 min at 37 °C, and the grids were then rinsed twice with blocking solution. Secondary antibodies [10 nm gold-conjugate goat polyclonal anti-rabbit IgG (HTL; BBI Solutions); 20×dilution] in blocking solution were applied on the grid for 30 min at room temperature. After rinsing with distilled water, the grids were stained with 2 % phosphotungstic acid solution for 90 s. The grids were then observed under a transmission electron microscope (Hitachi-HT

7700) at 80 kV. Spike length was observed and measured based on the electron micrographs through reciprocation from electron micrographs, and 10 spikes were selected for calculation.

Immunization with JEV-MVLP antigens in rabbits and mice

Preparation of antisera against JEV-MVLP and JEV-Nakayama VLP antigens was performed as described previously [31]. Briefly, partially purified MVLP antigen was adjusted to contain 40 μg ml^{-1} $dose^{-1}$ and this MVLP antigen was immunized into mice. The immunizations were repeated three times at weekly intervals and all blood samples were obtained 1 week after the last immunization. In the case of divalent JEV vaccines, 40 μg each of Nakayama and MVLP antigens were mixed together and immunized into mice as described for the Muar VLP vaccine.

50 % plaque reduction neutralization tests ($PRNT_{50}$)

Growth inhibition of immune sera against MVLP and NVLP prepared in mice and rabbits was undertaken by $PRNT_{50}$ in Vero cells as described previously [31]. Briefly, each challenge virus (Nakayama, Beijing-1, Muar) was mixed with an equal volume of fourfold diluted antisera (1 : 40–1 : 10 240) and then incubated at 35 °C for 90 min. Subsequently, overlay medium containing 1 % methylcellulose was added and the cells were incubated at 35 °C for 4–6 days. The cells were fixed using a 3.7 % formalin/PBS solution and stained using methylene blue. The $PRNT_{50}$ titre was estimated as described previously [31].

Funding information
This work was supported by a grant from the Okinawa Communicable Disease Research Hub Formation Promotion Project by Okinawa Prefectural Government Commissioned Project for fiscal year 2016 and 2017.

Acknowledgements
Authors are grateful to Ms Sayaka Matsuda (Institute of Biological Resources) for her skilful technical support.

Conflicts of interest
We are applying for a patent based on the results described in the present report.

Ethical statement
Mouse and rabbit experiments were approved and performed in accordance with the Fundamental Rules for Animal Experiments and the Guidelines for Animal Experiments Performed at The Institute of Biological Resources published by the Animal Welfare and Animal Care Committee including the Animal Ethics Committee of the Institute of Biological Resources, Okinawa, Japan.

References

1. Nerome R, Tajima S, Takasaki T, Yoshida T, Kotaki A *et al.* Molecular epidemiological analyses of Japanese encephalitis virus isolates from swine in Japan from 2002 to 2004. *J Gen Virol* 2007;88:2762–2768.
2. Yun SI, Lee YM. Japanese encephalitis. *Hum Vaccin Immunother* 2013;10:2263–2279.
3. Solomon T, Ni H, Beasley DW, Ekkelenkamp M, Cardosa MJ *et al.* Origin and evolution of Japanese encephalitis virus in southeast Asia. *J Virol* 2003;77:3091–3098.
4. Tsai TF. New initiatives for the control of Japanese encephalitis by vaccination: minutes of a WHO/CVI meeting, Bangkok, Thailand, 13–15 October 1998. *Vaccine* 2000;18:1–25.
5. Burke DS, Leake CJ. Japanese encephalitis. In: Monath TP (editor). *The Arboviruses: Epidemiology and Ecology*. Boca Raton, FL: CRC Press; 1988. pp. 63–92.
6. Campbell GL, Hills SL, Fischer M, Jacobson JA, Hoke CH *et al.* Estimated global incidence of Japanese encephalitis: a systematic review. *Bull World Health Organ* 2011;89:766–774.
7. Nga PT, del Carmen Parquet M, Cuong VD, Ma SP, Hasebe F *et al.* Shift in Japanese encephalitis virus (JEV) genotype circulating in northern Vietnam: implications for frequent introductions of JEV from Southeast Asia to East Asia. *J Gen Virol* 2004;85:1625–1631.
8. Tiwari S, Singh RK, Tiwari R, Dhole TN. Japanese encephalitis: a review of the Indian perspective. *Braz J Infect Dis* 2012;16:564–573.
9. Nerome R, Kurane I. Japanese encephalitis virus vaccine. *Rinsho Kensa* 2004;48:399–415.
10. Chen HL, Chang JK, Tang RB. Current recommendations for the Japanese encephalitis vaccine. *J Chin Med Assoc* 2015;78:271–275.
11. Kuzuhara S, Nakamura H, Hayashida K, Obata J, Abe M *et al.* Non-clinical and phase I clinical trials of a vero cell-derived inactivated Japanese encephalitis vaccine. *Vaccine* 2003;21:4519–4526.
12. Ishikawa T, Yoshii H, Onishi T, Ishibashi M, Imagawa T. Development of an inactivated Japanese encephalitis vaccine by utilizing a continuous cell line. *Clin Virol* 1998;26:340–350.
13. Schuller E, Klingler A, Dubischar-Kastner K, Dewasthaly S, Müller Z. Safety profile of the Vero cell-derived Japanese encephalitis virus (JEV) vaccine IXIARO®. *Vaccine* 2011;29:8669–8676.
14. Miyazaki C, Okada K, Ozaki T, Hirose M, Iribe K *et al.* Phase III clinical trials comparing the immunogenicity and safety of the vero cell-derived Japanese encephalitis vaccine Encevac with those of mouse brain-derived vaccine by using the Beijing-1 strain. *Clin Vaccine Immunol* 2014;21:188–195.
15. Lobigs M, Pavy M, Hall RA, Lobigs P, Cooper P *et al.* An inactivated Vero cell-grown Japanese encephalitis vaccine formulated with Advax, a novel inulin-based adjuvant, induces protective neutralizing antibody against homologous and heterologous flaviviruses. *J Gen Virol* 2010;91:1407–1417.
16. Zhang F, Ma W, Zhang L, Aasa-Chapman M, Zhang H. Expression of particulate-form of Japanese encephalitis virus envelope protein in a stably transfected Drosophila cell line. *Virol J* 2007;4:17.
17. Hale JH, Lim KA, Chee PH. Japanese type B encephalitis in Malaya. *Ann Trop Med Parasitol* 1952;46:220–226.
18. Tajima S, Yagasaki K, Kotaki A, Tomikawa T, Nakayama E *et al.* *In vitro* growth, pathogenicity and serological characteristics of the Japanese encephalitis virus genotype V Muar strain. *J Gen Virol* 2015;96:2661–2669.
19. de Wispelaere M, Frenkiel MP, Desprès P. A Japanese encephalitis virus genotype 5 molecular clone is highly neuropathogenic in a mouse model: impact of the structural protein region on virulence. *J Virol* 2015;89:5862–5875.
20. Takhampunya R, Kim HC, Tippayachai B, Kengluecha A, Klein TA *et al.* Emergence of Japanese encephalitis virus genotype V in the Republic of Korea. *Virol J* 2011;8:449.
21. Li MH, Fu SH, Chen WX, Wang HY, Guo YH *et al.* Genotype v Japanese encephalitis virus is emerging. *PLoS Negl Trop Dis* 2011;5:e1231.
22. Cao L, Fu S, Gao X, Li M, Cui S *et al.* Low protective efficacy of the current Japanese encephalitis vaccine against the emerging genotype 5 Japanese encephalitis virus. *PLoS Negl Trop Dis* 2016;10:e0004686.
23. Nerome K, Sugita S, Kuroda K, Hirose T, Matsuda S *et al.* The large-scale production of an artificial influenza virus-like particle vaccine in silkworm pupae. *Vaccine* 2015;33:117–125.

24. Nerome K, Matsuda S, Maegawa K, Sugita S, Kuroda K *et al.* Quantitative analysis of the yield of avian H7 influenza virus haemagglutinin protein produced in silkworm pupae with the use of the codon-optimized DNA: a possible oral vaccine. *Vaccine* 2017; 35:738–746.
25. Schalich J, Allison SL, Stiasny K, Mandl CW, Kunz C *et al.* Recombinant subviral particles from tick-borne encephalitis virus are fusogenic and provide a model system for studying. *J Virol* 1996; 70:4549–4557.
26. Kojima A, Yasuda A, Asanuma H, Ishikawa T, Takamizawa A *et al.* Stable high-producer cell clone expressing virus-like particles of the Japanese encephalitis virus e protein for a second-generation subunit vaccine. *J Virol* 2003;77:8745–8755.
27. Hua RH, Li YN, Chen ZS, Liu LK, Huo H *et al.* Generation and characterization of a new mammalian cell line continuously expressing virus-like particles of Japanese encephalitis virus for a subunit vaccine candidate. *BMC Biotechnol* 2014;14:62.
28. Mukhopadhyay S, Kuhn RJ, Rossmann MG. A structural perspective of the flavivirus life cycle. *Nat Rev Microbiol* 2005;3: 13–22.
29. Zhang X, Ge P, Yu X, Brannan JM, Bi G *et al.* Cryo-EM structure of the mature dengue virus at 3.5-Å resolution. *Nat Struct Mol Biol* 2013;20:105–110.
30. Zhang Y, Corver J, Chipman PR, Zhang W, Pletnev SV *et al.* Structures of immature flavivirus particles. *Embo J* 2003;22:2604–2613.
31. Matsuda S, Nerome R, Maegawa K, Kotaki A, Sugita S *et al.* Development of a Japanese encephalitis virus-like particle vaccine in silkworms using codon-optimised prM and envelope genes. *Heliyon* 2017;3:e00286.