[17]. Existing JE vaccines are produced from infectious agents (JEV), thus posing safety concerns in their production. As containment facilities are required, this increases the price of the final product. Inactivated vaccines require purification and/or concentration processes, further increasing their costs. Live attenuated vaccines can be produced less expensively but have a potential risk of reversion to virulence. To address these concerns, genetically engineered vaccines have been developed [18,19].

DNA vaccines are gene-based and have advantages of durability, heat-stability, safety and inexpensiveness [20]; thus they constitute one of the most appropriate strategies for developing countries. However, only two DNA vaccines have so far been licensed for infectious diseases, and these are only for veterinary use; against West Nile virus or infectious hematopoietic necrosis virus [21]. The major drawback hampering the development of DNA vaccines is the lower immunogenicities seen in large animals and/or humans than those expected from results obtained in rodent models [22]. Relatively high DNA vaccine doses of 0.5–2.5 mg are generally used in large animals and humans when immunized by the intramuscular route [23,24]. Decreasing vaccine doses with the efficacy maintained may lead to reductions in cost. Therefore, several approaches to enhance immunogenicity of DNA vaccines have been investigated: optimization of the transcriptional elements on the plasmid backbone [25], immune plasmid adjuvant [26,27], use of new-generation delivery methods [28,29], and prime-boost strategies [20].

In our laboratory, strategies to increase immunogenicity of DNA vaccines have been investigated in flavivirus models using mice. We first found that simultaneous immunization with protein-based vaccines synergistically increased the ability of DNA vaccines to induce neutralizing antibodies in models of JEV and dengue type 2 virus [30]. Second, a needle-free jet-injection system was shown to enhance immunogenicity of DNA vaccines in a model of JEV [31]. Third, we demonstrated that needle-free injection of mixture of DNA and protein vaccines significantly increased their own immunogenicities in models of JEV [32], dengue type 1–4 viruses [33] and West Nile virus [34].

Neutralizing antibodies are the most important protective immunity against JEV [35]. Our DNA vaccines involve a strategy that coexpresses the premembrane (prM) and envelope (E) genes of JEV. Coexpression of flavivirus prM/E genes is known to induce the production of extracellular subviral particles in transfected mammalian cells that are an excellent immunogen for inducing strong neutralizing antibody responses in animals [36]. Our previous studies have demonstrated that a pcDNA3-based plasmid encoding the JEV prM/E genes (designated pcJEME) induced neutralizing antibodies and protection from lethal challenge in mice [37]. In addition, another JE DNA vaccine (designated pNJEME) based on pNGVL4a, a vector addressing field applications, had the ability to induce neutralizing antibodies also in swine [38] and monkeys [39].

The present study aimed to examine if the effect of our immunization strategy (needle-free injection of the DNA-protein vaccine mixture), already shown in mice, would also be effective in miniature pigs. Our results indicate a similar enhancing effect in these animals. Pregnant sows immunized twice with a mixture of 10 or 1 μg of the JE-DNA vaccine and a 1/100 dose of JEVAX developed neutralizing antibodies and were protected against fetal death and mummification.

## 2. Materials and methods

### 2.1. Cells

Mammalian Vero and mosquito C6/36 cells have been described [33]. Briefly, Vero cells were cultivated in a growth medium composed of Eagle's minimal essential medium supplemented with 10% heat-inactivated fetal bovine serum at 37 °C in a humidified atmosphere of 5% $CO_2$–95% air. C6/36 cells were grown under the same conditions as in Vero culture except for the addition of nonessential amino acids to the medium and that the cultivation temperature was 28 °C.

### 2.2. Viruses

The Nakayama strain of JEV [37] was used. Culture fluids harvested from C6/36 cells infected with the Nakayama strain were used for neutralization tests. The Sw/Mie/40/2004 strain [40] of JEV isolated from swine sera, which had been passaged four times in C6/36 cells, was used for protection experiments.

### 2.3. DNA vaccine

The pNGVL4a-based vaccine plasmid containing the *prM* and *E* genes of the JEV Nakayama strain (pNJEME) has been described [38]. The pNGVL4a vector was supplied by the National Gene Vector Laboratory (currently the Vector Core Laboratory), the University of Michigan: pNGVL4a was renamed to pUMVC4a. All DNA were purified using a Qiagen endotoxin-free DNA purification kit (EndoFree Plasmid Mega Kit; Qiagen, Hilden, Germany).

### 2.4. Protein vaccine

The formalin-inactivated, mouse brain-derived JE vaccine for human use (JEVAX) was purchased from Takeda Pharmaceutical (Osaka, Japan).

### 2.5. Animals

Miniature pigs of the Clawn strain were purchased from Japan Farm Clawn Institute (Kagoshima, Japan) and kept in the Institute for Experimental Animals, Kobe University Graduate School of Medicine (Kobe, Japan) and/or the facility for experimental animals, National Institute of Animal Health (Tsukuba, Japan). Prior to their use for experiments, the absence of hemagglutination-inhibiting (HAI) antibodies against JEV was confirmed in all animals. Four-week-old piglets (3–7 kg/pig) and 8- to 10-month-old pregnant sows (6–7 weeks after mating, 25–33 kg/pig) were used for immunization and/or protection experiments. Prior to all handlings, animals were sedated with a 1 mg/kg of azaperon (Stresnil; Daiichi-Sankyo, Tokyo, Japan) and 0.05 mg/kg of atropine sulfate (Atropine sulfate injection 0.5 mg; Mitsubishi Tanabe Pharma Corporation, Osaka, Japan), and were anesthetized with a mixture of 5 mg/kg of ketamine (Ketalar for intramuscular injection 500 mg; Daiichi-Sankyo) and a 0.05 mg/kg of xylazine (Celactal 2% injection; Bayer Medical, Tokyo, Japan). Pigs were kept in group cages at 28 ± 2 °C under a light (12 h) and dark (12 h) protocol, and fed powdered diet once a day. At the end of the experiment, pigs were pre-treated with azaperon and euthanized with an overdose intravenous (i.v.) injection of thiopental sodium (Ravonal 0.5 g for injection; Mitsubishi Tanabe Pharma Corporation) or pentobarbital sodium (Nembutal 5% injection; Dainippon Sumitomo Pharma, Osaka, Japan). All animal experiments at Kobe University were approved by the Institutional Animal Care and Use Committee (Permission number: P-060304) and conducted according to the Kobe University Animal Experimentation Regulations, while those at National Institute of Animal Health were carried out following the Guide for the Care and Use of Laboratory Animals in the National Institute of Animal Health under approval of the biosafety, animal care and ethical committees of National Institute of Animal Health (Permission number: 2006-781).

### 2.6. Immunization and challenge

Miniature pigs were immunized once or twice with a 3 or 7 week interval with DNA and/or protein vaccines by injecting 0.5 ml of the vaccine solution into the thigh or the base of the right ear lobe using a spring-powered needle-free jet-injector (ShimaJET attached with a 0.26 mm-diameter orifice nozzle; Shimadzu, Kyoto, Japan) or a normal syringe with a needle. The doses of pNJEME were 1–100 μg and those of JEVAX were 1/10 or 1/100 of a human dose: the inoculum size was adjusted with phosphate-buffered saline (PBS). Groups of pigs were immunized with a mixture of pNJEME and JEVAX: as controls, those inoculated with pNJEME alone, a mixture of JEVAX and pNGVL4a, pNGVL4a alone, or PBS were used. Pigs were bled from the ear vein and individual sera were examined for neutralizing and/or HAI antibodies.

Protection experiments were performed by monitoring levels of viremia of JEV after challenge with 6.3 $\log_{10}$ PFU of the JEV Sw/Mie/40/2004 strain inoculated into the base of the ear lobe by a subcutaneous route using a normal syringe with needle. Pigs were bled daily until 7 days after challenge and also on day 14 in some experiments. Plasma was isolated and examined for neutralizing antibodies and viremia levels. Pregnant sows were bled twice at pre-challenge, 2 weeks after the second immunization and post-challenge, 5 weeks after the second immunization. Pregnant sows were immunized twice with a 3-week interval, bled and challenged 2 weeks after the second immunization, and euthanized by an overdose of anesthetic agent. Fetal conditions in the uterus were assessed at 3 weeks following challenge (5 weeks after the second immunization). Presumed gestation dates on which fetuses died were calculated from their crown-to-rump lengths (CRLs) by the following formula; $X = 3 \times (Y + 21)$, where $X$ is the presumed period (day) between pregnancy and the death and $Y$ is the CRL (cm) obtained from the dead fetus.

### 2.7. Serological tests

Neutralizing antibodies in heat-inactivated sera/plasma were titrated using plaque reduction assays performed with JEV (Nakayama) without complement as previously described [37]. The neutralizing antibody titer was expressed as the highest serum dilution yielding a 90% reduction in plaque number. The hemagglutination-inhibition tests were performed as previously described [31]. For treatment with 2-mercaptoethanol (2-ME), a 0.4 ml aliquot of serum was mixed with an equal volume of 0.2 M 2-ME in 0.01 M PBS. After 1 h incubation at 37 °C, the sera were extracted twice with acetone, dried in vacuo for 5 min and then restored in 0.4 ml of PBS [41]. Some sera were subjected to sucrose density gradient centrifugation to obtain IgG and IgM fractions as described previously [31]. Briefly, sera were applied to a 10–40% continuous sucrose gradient. Following centrifugation, fractions were collected from the bottom. Each fraction was tested for IgG and IgM class antibody levels by a conventional enzyme-linked immunosorbent assay using peroxidase-conjugated anti-swine IgG (whole IgG-reactive; MP Biomedicals, Irvine, US). A "synergistic" effect was defined as a "more than additive" effect: this was used when the mean neutralizing antibody titer obtained from the group of interest was significantly higher than the sum of those obtained from another two groups.

### 2.8. Statistical analysis

Significance of differences in geometric mean antibody titers or infective titers were evaluated by Student's $t$-test. For this evaluation, titers less than the detection limit (<1:10) were assigned a value of 1:5. The chi-square test with the Yates' correction factor was used to compare the percentages of abnormal fetuses between vaccinated and unvaccinated sows. Probability levels ($P$) of less than 0.05 were considered significant.

## 3. Results

### 3.1. Preliminary experiments

Since the DNA/protein vaccine mixture had been injected into the thigh in our mouse experiments, this site was selected for the first preliminary experiment using miniature pigs. Two 4-week-old piglets were immunized twice at a 7-week interval: one with a mixture of 100 μg of pNJEME and a 1/10 dose of JEVAX and another with a mixture of 1 μg of pNJEME and a 1/100 dose of JEVAX. As shown in Fig. 1A, the piglet immunized with a higher dose induced a neutralizing antibody titer of 1:20 at 2 weeks after the first immunization, which was increased to 1:640 at 3 weeks after the second immunization. In addition, even 1 μg of pNJEME induced a neutralizing antibody titer of 1:160 at 2 weeks after the second immunization.

In our second preliminary experiment, the base of the ear lobe was used as an injection site for vaccination. Each of three piglets received either a single immunization with a mixture of 100 μg of pNJEME and a 1/10 dose of JEVAX, or a mixture of 10 or 1 μg of pNJEME and a 1/100 dose of JEVAX (Fig. 1B). The former two piglets induced neutralizing antibody titers of 1:10–1:40, while the latter did not induce detectable levels until the end of this experiment. Based on these results, the base of the ear lobe was used as an injection site in subsequent experiments.

To confirm the reproducibility of the above experiments, a third preliminary experiment was performed using another three piglets and exactly the same immunization protocol as used in the above experiment (Fig. 1B). As shown in Fig. 1C, all piglets developed higher neutralizing antibody titers than those shown in Fig. 1B, probably due to individual variations. To analyze these quick and high neutralizing antibody responses, sera collected at 1 and 5 week were examined for IgM and IgG class antibodies separated on a sucrose density gradient. While the neutralizing antibody titers of the IgM fractions were higher than those of the IgG fractions at 1 week after immunization, the IgG fractions showed higher titers than the IgM fractions did at 5 weeks after immunization (Fig. 1D). The presence of IgM antibodies in the 1-week samples was confirmed by 8-fold decrease of HAI antibody titers after treatment of the sera with 2-ME (Fig. 1E). These results suggested that despite the considerable individual variations the needle-free immunization with the mixture of DNA/protein vaccines could induce neutralizing antibodies in miniature pigs.

### 3.2. Effect of needle-free injection and co-immunization with JEVAX on immunogenicity of DNA vaccine

To evaluate the effect of needle-free injection and co-immunization with JEVAX on immunogenicity of pNJEME in miniature pigs, six groups of two 4-week-old piglets were immunized twice with a 3-week interval with DNA and/or protein vaccines using a needle-free injector or a normal syringe with a needle (Fig. 2A). The DNA was 10 μg of pNJEME, while the protein was a 1/100 dose of JEVAX with or without 7 μg of pNGVL4a. pNGVL4a was added to adjust the adjuvant effect of the CpG motif contained in this vector plasmid on the immunogenicity of JEVAX. The molarity of CpG motifs contained in 10 μg of pNJEME corresponded to that contained in 7 μg of pNGVL4a.

Fig. 2B shows the time courses of the neutralizing antibody titers induced in individual piglets until 8 weeks after the first immunization. Co-immunization with the pNJEME-JEVAX mixture using a normal syringe with a needle (group 3) induced neutralizing antibodies after the second immunization in both piglets with geo-

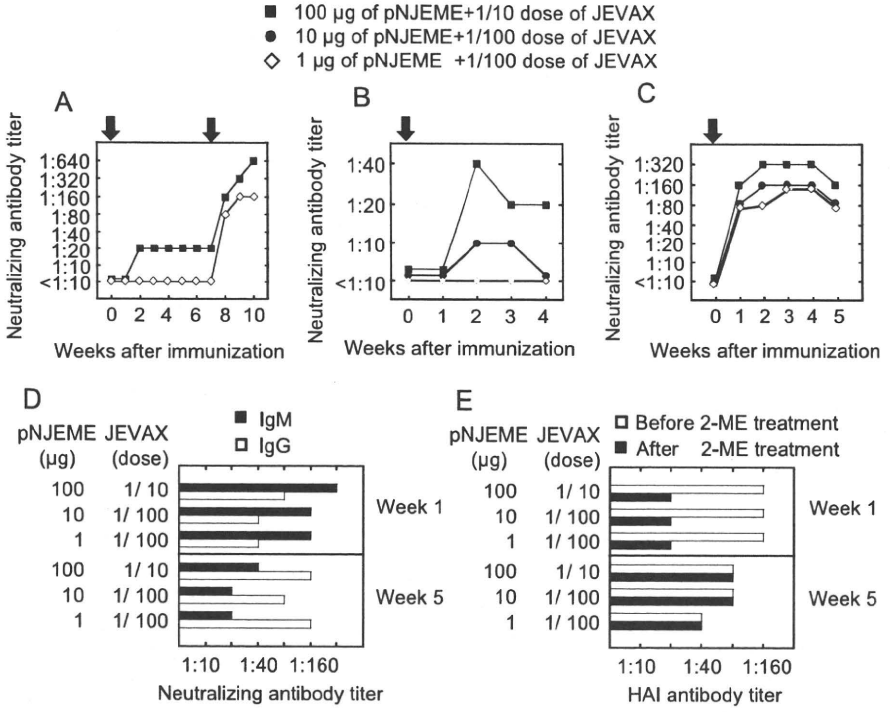


**Fig. 1.** Preliminary evaluation of the needle-free immunization protocol. (A) to (C) Four-week-old miniature pigs were immunized with a mixture of 100 μg of pNJEME and a 1/10 dose of JEVAX (closed squares), 10 μg of pNJEME and a 1/100 dose of JEVAX (closed circles) or 1 μg of pNJEME and a 1/100 dose of JEVAX (open diamonds). Arrows indicate the timing of immunization. The vaccine mixture was inoculated using a needle-free injector at the thigh (A) or the base of the ear lobe (B, C). (D) Sera obtained from piglets shown in Fig. 1C at week 1 and 5 were separated into IgG (open bars) and IgM (closed bars) fractions on a sucrose density gradient, and examined for neutralizing antibodies. (E) Sera used in Fig. 1D were examined for HAI antibodies with (closed bars) or without (open bars) 2-ME treatment.

metric mean neutralizing antibody titers of 1:14–1:20, while those immunized with the same dose but by using a needle-free injector (group 4) showed significantly higher mean titers of 1:95–1:190 ($P<0.01$ by the Student's $t$-test at any of the 1–5 week periods after the second immunization), indicating the effectiveness of the needle-free injection method. In addition, since piglets immunized with DNA or protein vaccine alone (groups 1, 2 and 5) did not develop detectable levels of neutralizing antibodies at most time points, the titers shown by co-immunization with the pNJEME-JEVAX mixture using a needle-free injector (1:95–1:190) indicated a synergistic increase in neutralizing antibody responses, after the second immunization ($P<0.01$ by the Student's $t$-test, comparing the titers obtained in group 4 with the sum of those obtained in groups 2 and 5). These results were consistent with the HAI antibody titers obtained with the same samples (Fig. 2C). Thus, the highest neutralizing antibody responses in group 4 indicated a combined effect of the needle-free injection and co-immunization with JEVAX on immunogenicity of pNJEME in miniature pigs.

During or at the end of the immunization experiment, one piglet immunized with JEVAX alone (group 5) and two piglets inoculated with pNGVL4a (group 6) suffered accidental deaths that were unrelated to the experiments. Although the piglets in group 6 were supposed to serve as a control for the challenge experiment, we did challenge the rest of the piglets at 5 weeks after the second immunization to compare viremia levels among animals immunized with different immunogens by different injection methods. Following challenge, all three piglets immunized with pNJEME by a normal syringe/needle injection (Fig. 2D, group 1) or JEVAX by a needle-free injection (group 5) showed viremia between 2 and 4 days after challenge at the maximum infective titer of 3.4 $\log_{10}$ PFU/ml. None of these piglets showed detectable levels of neutralizing antibodies prior to challenge. On the other hand, all piglets immunized with the pNJEME-JEVAX mixture, and showing individual neutralizing antibody titers of 1:10–1:320, were fully protected from viremia using any injection system (groups 3 and 4). Piglets immunized with pNJEME alone by a needle-free injector (group 2) showed lower viremia levels than those immunized with the same immunogen by a normal syringe/needle injection: one showed only 1.0 $\log_{10}$ PFU/ml at maximum and another did not develop detectable levels of viremia. These results indicated that immunization with the pNJEME-JEVAX mixture at the present dose provided miniature pigs full protection from viremia.

Fig. 2E shows time courses of neutralizing antibody titers following challenge. Piglets immunized with the pNJEME-JEVAX mixture by a needle-free injector (group 4) started increasing their antibody titers on day 3 or 4 postchallenge, reaching ≥1:1280 on day 5 or 6. By contrast, the increases in antibody titers in piglets immunized with either pNJEME or JEVAX (groups 1, 2 and 5) started on day 5 or 6 in most cases and the titers shown on day 7 were lower than those shown in group 4 piglets. Piglets immunized with the pNJEME-JEVAX mixture by a normal syringe/needle injection (group 3) showed an intermediate pattern: the start of increase in antibody titers was seen on day 4 or 5. These results indicated that miniature pigs immunized with the pNJEME-JEVAX mixture by a needle-free injector displayed strong anamnestic neutralizing antibody responses to the challenge.

### 3.3. Viremia protection by 1 μg of pNJEME mixed with JEVAX

To examine if a lower dose of the DNA vaccine would induce viremia protection, 1 μg was used as the dose of pNJEME. In this experiment, only the DNA and protein mixture was used as an immunogen, and was inoculated by using only a needle-free injector. Specifically, three groups of 4-week-old piglets were

A

| Group | pNJEME | JEVAX | pNGVL4a | Needle |
|---|---|---|---|---|
| 1 | 10 μg | - | - | + |
| 2 | 10 μg | - | - | - |
| 3 | 10 μg | 1/100 dose | - | + |
| 4 | 10 μg | 1/100 dose | - | - |
| 5 | - | 1/100 dose | 7 μg | - |
| 6 | - | - | 7 μg | - |

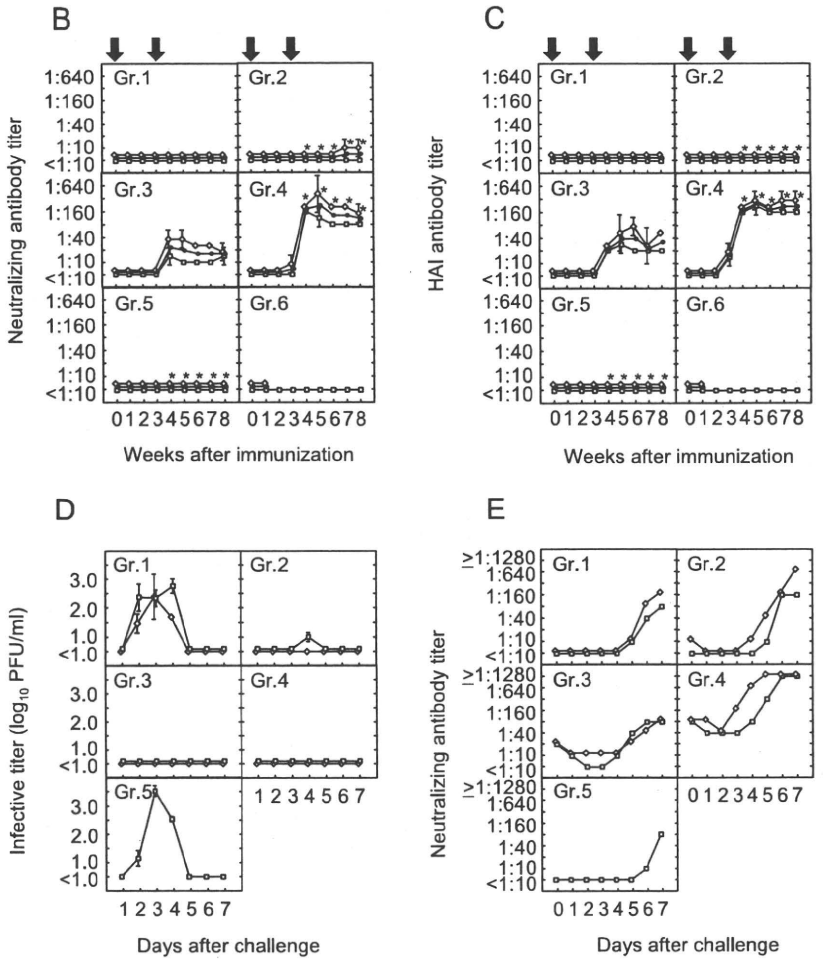


**Fig. 2.** Effect of the needle-free co-immunization strategy on immunogenicity and protective capacity of pNJEME in miniature pigs. Six groups of two 4-week-old piglets were immunized twice with a 3-week interval at the base of the ear lobe. Five weeks after the second immunization, piglets were challenged with 6.3 $\log_{10}$ PFU of JEV. (A) Immunogens and the injection methods used in each group. In the "Needle" column, "+" and "−" indicate the use of a normal syringe/needle and needle-free injector, respectively. (B) Time course of neutralizing antibody titers. (C) Time course of HAI antibody titers. Arrows indicate the timing of immunization. (D) Plasma infective titers following challenge. (E) Postchallenge neutralizing antibody titers. Constant symbols (open diamonds or open squares) were used in panels B–E to represent data obtained from the same individuals in each group. In panels B–D, individual data are indicated by the mean and standard error (indicated by bars) obtained from two repeated tests. Closed circles indicate the mean antibody titers obtained from two piglets in each group. The asterisks in panel B and C indicate the significance of differences detected by the Student's *t*-test ($P < 0.01$) between the mean titers shown by piglets of group 4 and the sum total of those shown by piglets of groups 2 and 5 at each time point.

immunized twice with a 3-week interval with 10 ($n = 3$) or 1 μg ($n = 4$) of pNJEME mixed with a 1/100 dose of JEVAX, as well as PBS as a control ($n = 3$). These piglets were challenged at 2 weeks after the second immunization and examined for viremia (Fig. 3). Prior to the challenge, all three piglets immunized with 10 μg of pNJEME or three of the four piglets immunized with 1 μg of pNJEME had detectable levels of neutralizing antibodies (Fig. 3, upper panels, day 0).

Piglets inoculated with PBS showed viremia titers of 2.1–5.0 $\log_{10}$ PFU/ml during days 1–4 postchallenge (Fig. 3, lower panel). All three piglets immunized with 10 μg of pNJEME were protected from viremia: the full protection induced by 10 μg of pNJEME was consistent with the results in Fig. 2D. The 1 μg of pNJEME protected viremia in three of the four piglets: the three possessed detectable prechallenge neutralizing antibodies. These results indicated that the presence of detectable levels of neutralizing antibodies in prechallenge sera correlated with protection against viremia in miniature pigs following challenge.

Neutralizing antibody titers started increasing on days 2–4 postchallenge in piglets immunized with 10 μg of pNJEME, while

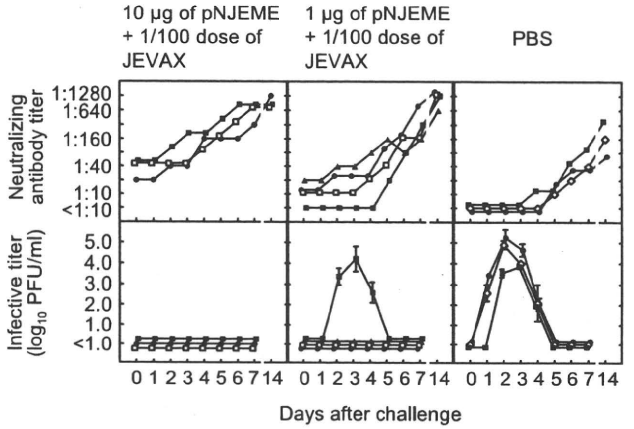


**Fig. 3.** Protective capacity of 1 μg of pNJEME against viremia in miniature pigs applying a needle-free co-immunization strategy. Three groups of 4-week-old piglets were immunized twice with a 3-week interval at the base of the ear lobe. Immunogens were a mixture of 10 μg of pNJEME and a 1/100 dose of JEVAX (left panels: $n=3$), 1 μg of pNJEME and a 1/100 dose of JEVAX (middle panels: $n=4$) or PBS as a control (right panels: $n=3$). Two weeks after the second immunization, pigs were challenged with 6.3 $\log_{10}$ PFU of JEV. Constant symbols were used for representing data obtained from the same individuals in each group. For infective titers, each datum indicates the mean and the standard deviation (indicated by bars) obtained from two repeated tests.

the increase in piglets inoculated with PBS started on days 4–5; immunized piglets displayed anamnestic antibody responses (Fig. 3, upper panels). Three piglets immunized with 1 μg of pNJEME that had prechallenge antibodies and were protected from viremia showed increases in antibody levels starting from days 2–4. By contrast, the start of the increase in antibody titers in the other piglet, which was immunized with 1 μg of pNJEME but did not develop detectable prechallenge antibodies and was not protected from viremia, was on day 5.

Collectively, in most cases no viremia was detected in sera showing neutralizing antibody titers of 1:10 or higher during the course of infection. Viremia disappeared when neutralizing antibody titers started increasing. Induction of anamnestic neutralizing antibody responses correlated with the prechallenge neutralizing antibody titers. These results indicated that the dose of 1 μg of pNJEME is effective for inducing neutralizing antibodies and protection against viremia in three of the four 4-week-old miniature pigs, when the DNA was mixed with a 1/100 dose of JEVAX.

### *3.4. Protection of pregnant sows from fetal death following challenge with JEV*

To evaluate the ability of the pNJEME-JEVAX mixture to prevent miniature pigs from JEV-induced abortion, vaccinated or unvaccinated pregnant sows were challenged. In this experiment, three groups of two 8- to 10-month-old pregnant sows (6–7 weeks after mating) were immunized twice with a 3-week interval, and challenged 2 weeks after the second immunization. Immunogens were 10 or 1 μg of pNJEME mixed with a 1/100 dose of JEVAX, or PBS as a control, and were inoculated using a needle-free injector. Pregnant sows were bled twice with a 3-week interval before and after challenge (2 and 5 weeks after the second immunization), respectively. Immediately after the second bleeding, sows were euthanized and fetus conditions in the uterus were observed (Table 1).

Pregnant sows immunized with a mixture of 10 or 1 μg of pNJEME and a 1/100 dose of JEVAX developed neutralizing antibody titers of ≥1:40 at 2 weeks after the second immunization. On the other hand, inoculation with PBS did not induce detectable levels of antibodies. Following challenge, none of the twelve fetuses in immunized animals were dead or abnormal, whereas six (50%) of twelve fetuses in unimmunized sows were mummified: statistical significance ($P<0.05$) was detected in the population of dead fetuses between immunized (0%: 0 of 12 fetuses) and unimmunized (50%: 6 of 12 fetuses) groups. Although the average fetus number in a sow in the vaccinated group (3 fetuses) was different from that of the unvaccinated group (6 fetuses), both numbers were within the individual variations occurring in the Clawn strain of miniature pigs: the mean and standard error are reported as 4.7 and 2.2, respectively [42]. These results indicated that two immunizations with a mixture of 10 or 1 μg of pNJEME and a 1/100 dose of JEVAX induced neutralizing antibodies in pregnant sows and protected their fetuses from fetal death.

Attempts to demonstrate the virus or viral RNA in the brain of mummified fetuses failed, probably because of the necrosis of this tissue at the time of euthanasia (3 weeks following challenge). Since miniature pigs of the specific pathogen-free grade were used for the experiment in an appropriate animal facility, it is highly probable that JEV infection was the cause of the fetal deaths. To support this, a further attempt was done to assess the date on which fetuses died, by measuring the CRL, which is generally used for estimating the age of pig fetuses during the various developmental stages [43]. Based on the CRL, it was suggested that the mummified fetuses had died between 3 and 7 days after challenge (Table 1). The presumed death dates were within a limited period (within 5 days) and also a few days after the viremic period that was shown in the same strain of this animal (Figs. 2 and 3). These results suggested that these fetuses died of infection following challenge of the pregnant sows.

## 4. Discussion

Swine are the most important amplifiers of JEV in the peridomestic environment: they develop levels of viremia high enough to supply the virus to vector mosquitoes. Humans are infected

**Table 1**
Protective capacity of the pNJEME-JEVAX mixture against fetal death in pregnant sows.

| Group | Immunogen | | Pig Number | Neutralizing antibody titer | | HAI antibody titer | | Fetus status 3 weeks after challenge | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | pNJEME (μg) | JEVAX (dose) | | 2 weeks[a] | 5 weeks[a] | 2 weeks[a] | 5 weeks[a] | Mummy | Normal | % Mummies | Presumed death date[b] |
| 1 | 10 | 1/100 | AN35 | 1:57 | ≥1:1280 | 1:40 | ≥1:1280 | 0 | 3 | 0 | |
| | | | AO28 | 1:320 | >1:1280 | 1:320 | ≥1:1280 | 0 | 3 | 0 | |
| 2 | 1 | 1/100 | AO51 | 1:80 | 1:226 | 1:57 | 1:226 | 0 | 2 | 0 | |
| | | | AO64 | 1:40 | 1:905 | 1:40 | 1:905 | 0 | 4 | 0 | |
| Total | | | | | | | | 0 | 12 | 0[c] | |
| 3 | PBS | | AN42 | <1:10 | 1:80 | <1:10 | 1:160 | 4 | 3 | 57 | 3, 4, 6, 7 |
| | | | AP41 | <1:10 | 1:40 | <1:10 | 1:40 | 2 | 3 | 40 | 3, 6 |
| Total | | | | | | | | 6 | 6 | 50[c] | |

[a] Weeks after the second immunization. "2 weeks" indicates the timing immediately before JEV challenge. "5 weeks" indicates the time of euthanasia.
[b] Presumed death dates were estimated individually from the CRL of the mummified fetus.
[c] $P<0.05$.

by infective mosquito bites and can develop clinical symptoms at subclinical: clinical infection rates of 1:25 to 1:1,000 [44]. Therefore, mass immunization of swine is theoretically a logical strategy to reduce the viral transmission cycle in peridomestic areas, thus potentially contributing to public health and the domestic economy. Although birds or other potential amplifiers such as wild boars [45] or bats [46] may also increase the population of infected mosquitoes, a field trial conducted in an isolated small island in Japan suggested the possible effect of swine immunization on the prevention of human JEV infections [47].

In addition to its important role in virus transmission, JEV viremia has a role in virus dissemination in the host body. In humans and horses, JEV replicated at or near the mosquito bite site is transported via the blood stream to the brain where the virus may penetrate the blood-brain barrier and cause encephalitis. Similarly, intrauterine infection in swine is considered to result from virus dissemination through viremia. Therefore, the ability of neutralizing antibodies to protect against abortion/stillbirth in pigs is attributed to the reduction in viremia to levels that will not encourage virus dissemination to the uterus: similar to their ability to protect against encephalitis in humans and horses. Vaccination that can induce neutralizing antibodies is an effective measure to suppress viremia and protect humans and animals from contracting the disease. In the present study, all pigs showing a neutralizing antibody titer of 1:10 or higher were protected from viremia following challenge, consistent with a titer (1:10) known to be required for disease protection in vaccinated humans [48].

Miniature pigs have been developed as useful laboratory animals for their size. To the best of our knowledge, this is the first study using miniature pigs as an animal model to evaluate DNA vaccines for flaviviruses. In this study, pregnant sows (Table 1; approximately 9 months old) induced higher neutralizing antibody titers than did younger pigs (Fig. 2B; approximately 2 months old): immune systems of swine are known to develop as they age [49]. Thus, swine of reproductive ages may effectively induce neutralizing antibodies even by immunization with 1 μg of DNA (with a small amount of protein); however, the present study showed the ability of this low dose (1 μg) to induce neutralizing antibodies in just two pregnant sows. Optimization of the timing for immunization would be an important issue as a next step.

Needle-free injections have been used to improve the immunogenicity of DNA vaccines [29]. They include particle bombardment by a gene gun [50,51], electroporation [52,53], and high-pressure jet-injection delivery [54,55]. Both gene gun and electroporation devices have advantages of providing DNA vaccines with high immunogenicity and transfection efficiency; thus, only a small amount of DNA is required. With a gene gun, several studies have demonstrated that 1–10 μg of DNA vaccines induced neutralizing antibody titers against hepatitis B virus [56] and influenza virus [57]. However, gene guns require an expensive apparatus with gold particles and a special source of compressed helium gas, and thus are considered difficult to introduce into developing countries. As for electroporation, potential genome integration presents serious safety concerns [58]. On the other hand, spring-powered jet-injection devices are convenient and inexpensive and do not require special apparatus. Also, several studies have proven that the use of a jet-injection delivery system increased immune responses in large animals [59,60] and humans [55].

In conclusion, the present study verified the effectiveness in miniature pigs of needle-free jet injection of a JE DNA/protein vaccine mixture. While our earlier study, which did not use a needle-free injector, used 100–450 μg of pNJEME for inducing neutralizing antibodies in swine [38], the present study showed that only 10 or 1 μg of pNJEME induced neutralizing antibodies when mixed with a small amount of JEVAX and inoculated using a needle-free injector. The protective effect of the induced neutralizing antibodies against fetal death was demonstrated using pregnant sows. Field trials using a larger number of pigs will progress the evaluation of the effectiveness of the present vaccination strategy for preventing abortion and stillbirth in swine, and potentially for reducing human JEV infections.

## Conflict of Interest statement

The needle-free injector, ShimaJET, used in this study is a product of Shimadzu Corporation that the authors H. Udagawa and Y. Mukuta are employees of. However, sale of the apparatus has been halted since 2007. The injector was remodeled, in part for this study, with support from the above noted JST grant. The other authors declare they have no conflicts of interest.

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Review
Future Virology

# Combating Japanese encephalitis: Vero-cell derived inactivated vaccines and the situation in Japan

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Japanese encephalitis (JE) is a major public health threat in Asia, because of its high mortality and high incidence of psychoneurological sequelae in survivors. It is caused by JE virus (JEV) infection, transmitted by vector mosquitoes. The disease is vaccine preventable, and has been well controlled in some countries. Since no specific antivirals have been approved, prevention with vaccine is important in this disease. This article provides a general overview of JE and JEV, but special focus has been put on recently developed Vero cell-derived formalin-inactivated JE vaccines, and the situation in Japan relating to these vaccines. In Japan, where JE has been well controlled, the strong governmental recommendation of the mouse brain-derived vaccine for routine immunization was suspended in 2005, owing to a patient suffering severe postvaccination events. In 2010, the recommendation was reinstated, targeting a limited population utilizing a Vero cell-derived vaccine.

Japanese encephalitis (JE) is an important public health issue; in Asia, it is a major cause of viral encephalitis. The causative agent, JE virus (JEV), a positive-strand RNA virus, is a member of the genus flavivirus in the family *Flaviviridae*. Despite the severe manifestation associated with JEV infection, JE is a vaccine-preventable disease. In fact, countries that have conducted nationwide vaccine programs, such as Japan, Korea and Taiwan, have successfully reduced the number of JE cases since the introduction of JE vaccines (from the 1950s to the 1970s) [1]. However, since internationally available formalin-inactivated vaccines are expensive and require multiple doses, many developing countries in endemic regions face difficulties in implementing nationwide vaccine programs. Therefore, improved vaccines are required, as well as the development of effective antivirals. On the other hand, in areas where JE is well controlled, the necessity of continued vaccination programs has been questioned, since, in these regions the number of mosquito vectors has also reduced. In this article, an overview of the transmission cycle, pathogenesis, geographic distribution and epidemiology of JEV is presented. Then, recently developed antiviral drugs and currently available vaccines, including a Vero cell-derived formalin-inactivated JE vaccine, and the issues surrounding these vaccines, are discussed in detail. In particular, the current status of natural JEV activity amongst vaccinated populations in Japan is considered as a model for reconsidering the necessity for continued vaccination. Specific information about recent advances in JE vaccine development is available elsewhere [2], and is, therefore, not dealt with in this article. A thorough literature search on the subject of antivirals and vaccines was performed using the PubMed and HighWire databases, and findings from those publications are included in this manuscript. In addition, recent domestic publications have been included to address the current status of JE disease control in Japan.

## Transmission cycle

Japanese encephalitis virus was first isolated from the brain of a patient in Japan in 1935; this strain was the prototype Nakayama strain. Then, in 1938, JEV was isolated from *Culex tritaeniorhynchus* mosquitoes, again in Japan [3]. More than 30 species of mosquitoes have been shown to be able to transmit JEV, among which *Cx. tritaeniorhynchus* is considered the most important vector for human infection [4,5]. An infective titer of 1–2 $\log_{10}$ per blood meal is sufficient for *Cx. tritaeniorhynchus* to establish a disseminate infection and transmit the virus [6,7]; this corresponds to a viremia level of approximately $10^4$–$10^5$ per ml, supposing that the blood meal size is 2 µl.

**Keywords**

▪ antivirals ▪ Japanese encephalitis ▪ vaccine

Wild birds are thought to act as reservoirs of JEV in nature (and may also act as amplifiers), since many species of birds have been proven to develop high viremia titers (1–3 × $10^4$ per ml) for as long as 4 days [5,8,9]. Although a number of seroepidemiological studies have shown that antibodies against JEV are detected in many mammalian species [5,10–14], only pigs are considered to be important as amplifiers in the JEV transmission cycle, because of their high viremia titer and the duration of viremia [15]. In fact, JEV has been shown to be transmitted from experimentally infected birds and pigs to mosquito vectors [16]. Although humans and horses develop fatal encephalitis, these two species do not exhibit high enough levels of viremia to infect mosquito vectors. Thus, humans and horses are incidental dead-end hosts [17].

## Pathogenesis

Most JEV infections in humans are asymptomatic. The ratio of symptomatic to asymptomatic infections ranges from 1:25 to 1:1000 [18,19]. Symptomatic infections in humans exhibit a variety of manifestations ranging from febrile illness to meningitis, polio-like acute flaccid paralysis and encephalitis [19]. The incubation period of JEV infection is 5–15 days. Various factors, including the mosquito species, the frequency of exposure to mosquito bites, the circulating strains of JEV [20], and host factors, such as age, gender, genetic background and existing antibodies against JEV and/or other flaviviruses [18,21–24], affect the ratio of symptomatic to asymptomatic infections. Generally, the case fatality rate ranges from 5 to 40% [17]. Approximately 20–50% of the survivors have psychoneurological sequelae [25–31]. The availability of medical facilities also affects patient outcomes, with better medical environments contributing to reduced fatality rates, which, in turn, may bring about an increased percentage of sequelae in the survivors [19]. Children and young adults are generally believed to be a high-risk group, and comparative studies have indicated that these populations exhibit more severe manifestations and clinical outcomes [26,32].

## Geographical distribution & epidemiology

Japanese encephalitis virus is distributed in East, South and Southeast Asia, and its distribution continues to expand, although it is not thought to extend towards the north because of the absence of mosquito vectors in northern areas. In 1995, JEV emerged in the Torres Strait Islands, which are northern islands of Australia [33], and JEV activity has been observed annually in this region – with the exception of 1999 – based on serological surveys. JEV has also been isolated from mosquitoes in the Cape York Peninsula on the Australian mainland [34], and JEV activity has been reported in Pakistan [35]. Thus, JEV is expanding to the south and west.

Approximately 3 billion people are at risk of JEV infection [36]. A surveillance system to monitor infectious diseases, including JEV, has been established, and approximately 10,000 cases have been reported via this system [36,201]. Although many cases may remain undetected, 30,000–50,000 cases have been reported worldwide annually; along with 10,000 deaths. There are two patterns of seasonal prevalence [5,36,202]. The first pattern is observed in tropical regions, such as Malaysia, Singapore and Thailand. In these regions, transmission occurs throughout the year, although a peak of cases is observed after the start of the rainy season. The second pattern is observed in temperate/subtropical regions, such as Japan, Korea and China, where the epidemic season starts in May/June and lasts until September/October. This pattern of seasonal prevalence reflects the fact that the density and activity of mosquito vectors increases when rice cultivation starts in these regions.

Based on the nucleotide sequence of the envelope (E) gene, all JEV strains are classified into five genotypes [37]. The geographical distribution of each genotype suggested that JEV originated from the Indonesia–Malaysia region, and was then transported to other regions [38,39]. Although the mechanisms of JEV migration remained uncertain, it is now thought that JEV is transported by migrating birds and wind-blown mosquito vectors [5]. Recently, the dominant genotype changed, and a new genotype emerged in several countries. In Australia, JEV strains, belonging to genotype I, have been isolated since 2000, while all previous isolates (from 1995 to 2000) had belonged to genotype II [40]. Genotype shifts from III to I have been observed in Thailand and Vietnam since the 1980s and the late 1990s, respectively [41,42]. Similarly, JEV strains belonging to genotype I have been isolated in Japan and China since the early to mid-1990s and the 1980s, respectively [43–46]. Although the mechanisms behind these genotype shifts remained unclear, it was clear that they could occur repeatedly and independently in separate regions. Thus, it is important that antiviral drugs and vaccines are developed to be effective against all genotypes.

## Antivirals

There are no specific/nonspecific antiviral reagents available against JE, although a number of researchers have attempted to find/develop such drugs [47,48]. Interferon (IFN) is one of the most well-studied antiviral drugs. IFNs are produced in response to microbial infections, and induce more than 300 IFN-stimulated genes to combat infection [49]. Several of these genes encode proteins, such as Mx1, RNaseL and protein kinase R, which possess direct antiviral activities [50]. As therapeutic drugs against other viral infections, IFN-α2, IFN-β and IFN-γ have been approved by the US FDA. Importantly, IFN-β has been used as an antiviral drug against infections with hepatitis C virus, which belongs to the flavivirus family [51], suggesting that IFN may be effective against JE as well. Thus, trials using IFN to treat JEV infection have been conducted. Treatment with IFN-α effectively inhibited JEV replication *in vitro* [52,53]. However, a randomized double-blind clinical trial in Vietnam revealed that treatment with IFN-α2a did not improve the clinical outcome for JE patients compared with a placebo control [54]. However, only one dose of IFN-α2a via one route was administered in this trial and, thus, different outcomes may be achieved by either using a higher dosage, an alternative route of administration or by combination therapy using other drugs.

Ribavirin (1-β-D-ribofuranosyl-1,2,4-triazole-3-carboxamide) is a synthetic purine nucleoside analogue, first reported in 1972 [55,56], with broad-spectrum antiviral activity. Ribavirin has been tested widely against both DNA and RNA viruses *in vitro* and *in vivo* [55,56]. The mechanisms behind the antiviral effects induced by ribavirin include inhibition of inosine monophosphate dehydrogenase (IMPDH), immunomodulatory effects, inhibition of viral capping, inhibition of viral polymerase activity and induction of error catastrophes [56]. For flaviviruses, ribavirin has been shown to induce antiviral effects, predominantly by depletion of the intracellular GTP pools resulting from the inhibition of IMPDH in a model of yellow fever virus (YFV) [57]. *In vitro* studies have demonstrated that ribavirin treatment is also effective against JE [53]. However, a recent randomized-controlled trial conducted in India revealed that oral intake of ribavirin did not show any efficacy against JE [58]. Thus, further evaluations using a different dosage and/or route are required to determine the effectiveness of ribavirin treatment.

A number of other antiviral compounds have been identified and evaluated against JEV infection. Minocycline, which has been used as an antibiotic drug, has been shown to possess neuroprotective activities, and minocycline treatment has been shown to inhibit West Nile virus (WNV) replication and apoptosis in neuronal cells *in vitro* [59]. More recently, it was reported that minocycline treatment reduced blood–brain barrier damage induced by JEV infection in mice [60]. Furthermore, curcumin, aloe-emodin, 2-(2-Methyl-quinoline-4ylamino)-*N*-(2-chlorophenyl)-acetamide, bovine lactoferrin, α-glucosidase inhibitors, PI-88 and dehydroepiandrosterone have been reported as effective antiviral drugs *in vitro* [61–67]. Also, a pentoxifylline, *N*-methylisatin-β-thiosemicarbazone derivative, as well as arctigenin and rosmarinic acid, have been reported as effective antiviral drugs *in vivo* [68–71].

In recent decades, advances in molecular technology and our understanding of the RNA replication and degradation machinery have led to the emergence of novel nucleic acid-based antiviral drugs. Antisense oligonucleotides and small interfering RNAs (siRNAs) have been a major antiviral strategy against numerous RNA viruses [72]. Antisense peptide nucleic acid is able to suppress viral proliferation *in vitro* [73]. In addition, phosphorodiamidate morpholino oligomers (PMOs) have been demonstrated to be effective against a broad spectrum of flaviviruses *in vitro*, and PMO treatment provided partial protection in a WNV model [74]. siRNA has been shown to be a potent therapeutic drug *in vivo* [75,76], and modification of siRNA with a neurotropic glycoprotein of rabies virus made it possible to deliver siRNA to the brain transvascularly, resulting in effective treatment [77]. This technology demonstrated that the delivery of nucleic acid-based antiviral drugs to specific organs/cells could be achieved by selecting the appropriate modifying proteins.

To be effective *in vivo*, many of the drugs described must be administered soon after, or even before, JEV infection. Therefore, alternative administration methods/routes need to be explored, along with the possibility of antiviral combination therapies.

## Vaccines

Under the current situation in the development of antivirals, protection of humans from JEV infection or disease development is an important element in the effective control of JEV. Although vector control is an effective measure for reducing virus transmission between amplifier hosts, and from amplifiers to humans [5], and the fact

that swine vaccination is also effective at reducing the efficiency of JEV transmission to humans [78], it is generally accepted that vaccination of humans themselves is the most effective measure that to reduce susceptibility to JEV infection.

The introduction of a vaccine against JE for human use in Japan in 1954, and later in other endemic countries, contributed towards a reduction in the number of cases of JEV infection. Currently, Japan, Korea, Nepal, China and Thailand are conducting nationwide routine vaccine programs, and India, Nepal, Sri Lanka, Malaysia and Vietnam have a program operating in certain areas [79,203]. In Japan, Korea and Taiwan, JE has been well controlled as a result of a nationwide vaccination program [5]. JEV infection is not only a threat to people living in endemic areas; since 1945, a total of 179 cases have been reported in travelers and US soldiers [80,81]. Therefore, vaccination has been considered an effective measure to prevent JEV-related diseases in travelers as well. Several JE vaccines have been developed and used for the inhabitants of endemic countries and for travelers from non-endemic countries (Table 1), these include: mouse brain-derived formalin-inactivated vaccine, primary hamster kidney cell (PHK)-derived formalin-inactivated vaccine, live-attenuated vaccine, and Vero cell-derived formalin-inactivated vaccine. Each of these vaccines are discussed in the following sections.

### Mouse brain-derived inactivated vaccine

The mouse brain-derived formalin-inactivated vaccine was developed in 1954 in Japan using the Nakayama strain [82], and was proven to protect humans from disease [83]. The purification protocols were gradually improved, resulting in a highly purified inactivated vaccine [82], which was produced by seven Japanese manufacturers. Since 1989, the Beijing-1 strain has been used for vaccine preparation instead of the Nakayama strain for domestic use [84]. For many years, this mouse brain-derived inactivated vaccine was the only internationally approved vaccine, and the technology was transferred to several countries, including Korea, Taiwan, Thailand, India, Vietnam and Russia [5,36,85,86]. In addition, Connaught Laboratories Inc. in the USA (now known as Sanofi Pasteur) distributed this vaccine for travelers [87]. Generally, two or three doses are administered to induce durable immunity in vaccinees, and a booster immunization is required every 2–3 years [85,88]. Large-scale trials showed that a two-dose vaccination regimen provided 81–95% protective efficacy [89,90], and 97–100% seroconversion rates [91,92]. In these trials, no serious adverse events were observed. Mild (e.g., redness of injection site) and moderate (e.g., fever and headache) side effects were observed in less than 6% of the participants. Systemic reactions to the JE vaccine were thought to be caused by the presence of gelatin in the vaccine, included as a stabilizer [93,94]. However, a similar incidence rate (0.6–0.7 per million doses) was observed after removal of the gelatin components from the JE vaccine [95].

### PHK cell-derived inactivated vaccine

A cell culture-derived formalin-inactivated vaccine, prepared in PHK cells using the Beijing-3 (P-3) strain, was used for many years in China [85]. This PHK cell-derived inactivated vaccine was only licensed in China, and since its development in 1968, approximately 70 million doses have been produced and administrated every year. Despite the widespread use of this vaccine in China and its relatively low cost per dose compared with other vaccines [96], this vaccine has been replaced by the live-attenuated vaccine.

### Live-attenuated vaccine

A live-attenuated vaccine developed using strain SA14-14-2 has been available in China since 1988 [85]. The SA14-14-2 strain was obtained by passing the parental SA14 strain 11 times through mouse brains, followed by over 100 passages through PHK cells, and plaque purifications [97]. Sequence analysis of the SA14 strain and the SA14-14-2 strain revealed that there are 45 nucleotide differences, resulting in 15 amino acid differences, between these strains [98]. Further comparative studies using other attenuated strains derived from strain SA14 indicated that residues E-138, E-176, NS2B-63, NS3–105 and NS4B-106 are the candidate amino acid substitutions responsible for its attenuation [99]. Since 1988, more than 20 million doses of this live-attenuated vaccine, approximately 50% of the global production of JE vaccines, have been administered annually. Field trials in China showed that a one- to three-dose regimen exhibited 95–100% efficacy [100]. Only mild or moderate adverse events were observed, and in less than 5% of the participants [101]. Evaluation of the long-term immunogenicity resulting from immunization with this vaccine showed that a single dose could induce long-lasting immunity (seropositive rate of 89.9% at 4 years and 63.8% at 5 years post-immunization) [102].

Since it is an inexpensive vaccine (US$0.75/

**Table 1. List of vaccines against Japanese encephalitis reviewed.**

| Vaccine | Manufacturer(s) | Strain | Status |
|---|---|---|---|
| Mouse brain-derived formalin-inactivated vaccine | Biken, Denka Seiken, Kaketsu-ken, Chiba, Takeda (Japan), Sanofi Pasteur (USA), Green Cross (South Korea), Central Research Institute (India), National Institute of Preventive Medicine (Taiwan), Government Pharmaceutical Organization (Thailand), National Institute of Hygiene (Vietnam) | Nakayama Beijing-1 | Most manufacturers have ceased production |
| Primary hamster kidney cell-derived formalin-inactivated vaccine | Beijing, Shanghai, Wuhan and Changchun Institute of Biological Products (China) | Beijing-P3 | Replaced by live-attenuated vaccine |
| Live-attenuated vaccine | Chengdu Institute of Biological Products (China) | SA14-14-2 | Approved in China, Nepal, India, Sri Lanka, Korea and Thailand |
| Vero cell-derived formalin-inactivated vaccine | Intercell (Austria), BIKEN (Japan) | SA14-14-2 Beijing-1 | Intercell vaccine was approved in Europe, Australia, USA and Canada, and the BIKEN vaccine was approved in Japan |

dose in Asia) compared with the BIKEN-inactivated vaccine (US$5.00/dose in Asia), and requires only a single dose to induce protective immunity, it appears to be suitable for mass vaccination programs outside of China as well. However, there are theoretical concerns about the acquisition of a back mutation, conferring virulence, since the vaccine is a live virus [103]. In the case of the YFV 17D live-attenuated vaccine, virulent YFV was isolated from a patient who had developed YF vaccine-related encephalitis [104]. Recently, the live-attenuated JE vaccine has been approved in Nepal, India, Sri Lanka, Korea and Thailand [85]. Studies conducted in Nepal and India showed that a single dose of the vaccine exhibited high efficacy (more than 95%), similar to that observed in China [105–107], and the efficacy remained high (96.2%) 5 years after the single-dose vaccination [108].

### Vero cell-derived inactivated vaccine

As an alternative to the mouse brain-derived inactivated vaccine, novel vaccines using large-scale cell-culture technology were developed. In early 2009, Vero cell-derived inactivated vaccines (IC51; Ixiaro® [Intercell AG, Austria] and freeze-dried JE Vaccine [BIKEN, Japan]), were licensed. Although these two vaccines, both prepared from JEV-infected Vero cells, are similar, the IC51 vaccine contains an adjuvant.

The IC51 vaccine has been licensed in Europe, Australia, the USA and, more recently, in Canada. IC51 is a formalin-inactivated vaccine developed using the SA14-14-2 strain of JEV [86]. In preclinical trials, IC51 showed higher immunogenicity than the BIKEN mouse brain-derived inactivated vaccine in mice [109]. In clinical trials, a two-dose immunization with IC51 exhibited a seroconversion rate of 98%, while three doses of the mouse brain-derived inactivated vaccine exhibited 95% seroconversion. Consistently, IC51 caused the development of higher titers of neutralizing antibodies than the mouse brain-derived vaccine (geometric mean titers [GMT] were 1:244 and 1:102, respectively) [110]. Mild and moderate adverse events were observed in approximately 10% of the participants, and no severe or acute allergic reactions were observed [111], which was similar to the frequency of adverse events induced by the mouse brain-derived inactivated vaccine [110]. A long-term evaluation 6 months after the first immunization showed that IC51 vaccination maintained detectable neutralizing antibodies in 95% of the population, whereas the mouse brain-derived inactivated vaccine maintained neutralizing antibodies in only 74% of the population [112]. After 12 months, IC51 vaccination conferred a seropositive rate of 83% [112]. These trials indicated that the IC51 vaccine possessed equivalent or even higher immunogenicity than the mouse brain-derived inactivated vaccine.

Since the two-dose regimen could induce durable immune responses, a single-dose regimen was worth examining. Thus, a trial using a single-dose schedule was conducted. Since 6 µg of viral protein was used in the two-dose regimen, 12 µg was used for the single-dose evaluation. Although the first immunization in the two-dose regimen showed a seroconversion rate of 39.8% with a GMT of 1:11, the single-dose regimen showed a seroconversion rate of 65.8% with a GMT of 1:23, on day 28 [113]. However, the seroconversion rate reached 97.3% after the

second immunization in the two-dose regimen with a GMT of 1:218, while it decreased to 41.2% in the single-dose regimen with a GMT of 1:11, on day 56 [113]. Regardless of the higher immunogenicity of the 12-μg dose than the 6-μg dose, comparable populations showed local and systemic adverse events between these two regimens [113]. The single-dose schedule might be useful for travelers to endemic areas who do not have enough time to receive the vaccine with the two-dose schedule. Since the IC51 vaccine was initially only approved for adults, a trial to examine the IC51 vaccine in children was conducted. In this trial, 3- and 6-μg doses of the IC51 vaccine and the mouse brain-derived inactivated vaccine were compared in a two-dose regimen. Seroconversion rates of 95.7, 95.2 and 90.9% with a GMT of 1:201, 1:218 and 1:230 were shown 56 days after the first immunization, respectively [114]. No severe adverse events were observed in any of the three groups, and no significant differences in the frequency of the adverse events were detected between the three vaccination regimens [114]. Thus, the IC51 vaccine appeared to be safe and effective for children, as well as adults.

At approximately the same time that the IC51 vaccine was approved in Europe, Australia and the USA, the Freeze-dried JE vaccine, produced by BIKEN, which is also developed in Vero cells, obtained licensure in Japan. A Japanese company, Kaketsuken, also developed a Vero cell-derived vaccine independently, which exhibited similar immunological and physicochemical properties to those shown by the mouse brain-derived vaccine [115,116]. In a Phase I clinical trial of the Kaketsuken vaccine, the Vero cell-derived vaccine showed a 96.7% seroconversion rate with a GMT of 1:155, while the mouse brain-derived vaccine showed a 92.9% seroconversion rate with a GMT of 1:123, after the first immunization [117]. Following the second and third immunizations, both vaccines showed a 100% seroconversion rate. During the observation period, only mild adverse events were noted. The adverse event rates observed after the first, second and third immunization with the Vero cell-derived vaccine were 3.3, 3.3 and 6.7%, whereas those with the mouse brain-derived vaccine were 26.7, 13.8 and 10.7%, respectively [117]. Since participants of this clinical trial were 25–30-year-old adults, another trial recruiting 6–90-month-old children was conducted. In this trial, both Vero cell-derived and mouse brain-derived vaccines exhibited 100% seroconversion rates after the third immunization, and the Vero cell-derived vaccine induced neutralizing antibodies with GMTs of 1:309 and 1:9120 after the second and third immunizations, respectively [118]. Although the Vero cell-derived vaccine induced higher neutralizing antibody titers than the mouse brain-derived vaccine, the Vero cell-derived vaccine developed mild and moderate adverse events more frequently than the mouse brain-derived vaccine [118]. This vaccine is currently under review for licensure.

The initial clinical trial of the BIKEN Vero cell-derived vaccine revealed a 100% seroconversion rate following two doses of vaccination in 6–90-month-old children [119]. However, adverse events caused by the Vero cell-derived vaccine were observed at higher rates than with the mouse brain-derived vaccine [119]. Thus, a new vaccine preparation containing a smaller amount of E protein (5, 2.5 and 1.25 μg) was examined next. In this clinical trial, the seroconversion rates following the second and third immunizations with 2.5 μg of the vaccine were 99.2% with a GMT of 1:263 and 100% with a GMT of 1:5834, respectively. Mild and moderate adverse events were observed. For instance, fever was observed in 9.8, 10.7 and 4.9% of participants following the first, second and third immunizations, respectively. However, no severe adverse events were observed [120]. Although immunization with 5 μg of the vaccine could induce 100% seroconversion, which was slightly higher than with the 2.5-μg immunized group, higher adverse event rates were observed in the 5-μg immunized group than in the 2.5-μg immunized group [121,122]. Thus, the lower dose (2.5 μg) of this vaccine was approved for children aged 6–90 months. Based on the results of the ongoing clinical trials, this Vero cell-derived vaccine may also be approved for 9–13-year-old children.

These trials conducted by Intercell, Kaketsuken and BIKEN demonstrated that Vero cell-derived vaccines are an alternative to the mouse brain-derived vaccine. The Kaketsuken and BIKEN Vero cell-derived vaccine does not contain any adjuvant, unlike the IC51 vaccine that contains aluminum hydroxide. The BIKEN Vero cell-derived vaccine requires three doses to induce high, long-lasting immune responses, in contrast to the IC51 vaccine that requires two doses. In addition, the Kaketsuken and BIKEN vaccine is used for those living in endemic countries, whereas the IC51 vaccine is primarily used for travelers who do not need long-lasting immune responses.

### Debate over the necessity for continued JE vaccine use in Japan

In Japan, the number of JE cases has dramatically decreased since 1967, when the use of a well-refined vaccine was introduced nationwide. Prior to that year, more than 1000 cases had been reported annually, and that number decreased to less than ten cases since 1992 [123]. Similarly, the number of JE cases has decreased in Korea to less than ten cases each year since the introduction of an immunization program in 1971 [124]. The incidence rates in Taiwan also decreased from 2.05 per 100,000 in 1967, to 0.03 per 100,000 in 1997, as a result of the introduction of a vaccination program in 1968 [125]. The successes observed in these three countries clearly show that JE is vaccine preventable.

Although no severe adverse events have been reported in the clinical trials of the mouse brain-derived inactivated vaccine [85], post-vaccination acute disseminated encephalomyelitis (ADEM) cases and severe neurological complications have been reported at incidence rates of 0.2 per 100,000 to one per 50,000 [126]. There have been at least 23 cases of ADEM after JE immunization, including probable cases from 1994 to 2007 (~55 million doses) in Japan, according to the adverse event reporting system established in 1994 [127,128]. In July 2004, a 14-year-old girl developed ADEM at 11 days after JE vaccination. Since this was a severe case, and the number of annual ADEM cases had been increasing at that time, the Japanese government (the Ministry of Health, Labour and Welfare) decided to suspend their recommendation of the mouse brain-derived vaccine for routine immunizations in 2005 [128,129,204]. At the same time, the Japanese government stated that there was no scientific evidence to demonstrate the relationship between this case of severe ADEM and JE vaccination. However, there was also insufficient scientific evidence to rule out this possibility, and the Vero cell-derived vaccine was already in the final stages of development. Following the Japanese government's decision, manufacturers anticipated a decrease in demand, and stopped producing the mouse brain-derived vaccine in 2005. The decision also met a large number of responses from other countries. In particular, the Global Advisory Committee on Vaccine Safety (WHO) did not favor this government's decision [130].

Observation of ADEM during a certain post-vaccination period has been reported for a variety of vaccines, including those against JE, rabies and influenza [126]. The substance most likely to induce ADEM is myelin-basic protein or its related proteins derived from the brain tissue [131]. However, a correlation between ADEM and JE vaccination has not been reported. A significant correlation with ADEM had been observed in the cases of a Semple rabies vaccine and a vaccinia virus that had been used as a vaccine against smallpox virus [132]. In these cases, the patients who developed ADEM post-vaccination exhibited high levels of antimyelin-basic protein antibodies [133]. Although the incidence rates of ADEM have been reduced by using neurological tissue-free duck embryo- or human diploid cell-culture-derived rabies vaccines, post-vaccination ADEM is still being reported with low incidence rates (<1/25,000) [132]. Thus, contamination of myelin-basic protein could be one of the potential causative agents of ADEM development, but it was not the sole determinant as far as the rabies vaccine was concerned.

In the case of the JE vaccine, undetectable levels of myelin-basic protein (<2 ng/ml) were detected in the BIKEN mouse brain-derived inactivated vaccine [88]. The threshold amounts of myelin that induce myelitis in guinea pigs and mice are 75 and 400 µg, respectively [134,135]. In addition, the amount of protein derived from tissues or cells in the JEV vaccine preparation is less than 40 µg/ml, as specified by the minimum requirements for biological products, which was recently changed from less than 80 µg/ml [205]. Thus, the amount of myelin contained in the BIKEN vaccine is extremely small and, therefore, unlikely to induce myelitis in humans. Owing to advances in large-scale cell-culture technology, next-generation vaccine development has shifted towards cell-culture-derived vaccines. As expected, there were no significant differences in the induction of expression of inflammation-related genes, such as TNF-α and chemokines, in rats between the Vero cell-derived vaccine and mouse brain-derived JE vaccines [136]. These results imply that both vaccines possess similar reactivity in animals.

In addition, recent surveys revealed that the incidence of ADEM in children in Japan was unaltered by the government's decision to suspend recommendation of the mouse brain-derived vaccine. Specifically, the number of ADEM cases in children (<15 years old) was 0.33 per 100,000 in 2003–2004, before the government's decision, while the frequency in 2005–2006, after the government's decision, was 0.34 per 100,000 [206]. During this period, the vaccination rate in children drastically decreased. The vaccinated population among 3-year-old children was over

80% before 2004 (over 4,000,000 doses), while it decreased to 20% in 2005, and 4% in 2006. If the mouse brain-derived vaccine was the source of ADEM, the frequency of ADEM should have declined as the vaccination rate declined. These surveys, therefore, indicated that the mouse brain-derived vaccine did not correlate with the reported ADEM cases in Japan.

The facts that the number of mosquito vectors and the incidence of JE is declining, and that there is an increasing risk of post-vaccination events, lead us to question the necessity for routine JE vaccination. To address this issue, it is essential to understand the current activity of JEV in Japan. Serological surveys of swine have been conducted as part of a national project to monitor JEV activity in each prefecture. Seroconversion of sentinel swine has been observed in most of the southern and western areas of Japan [123]. High seroconversion rates in swine have also been observed in Taiwan [137]. These surveys indicated that JEV activity still exists in areas where JE-related diseases have been well controlled. Next, it is important to consider the natural infection rate of humans. Natural infection rates in Japan have been evaluated by detecting anti-nonstructural protein (NS)1 antibodies, since individuals who have received the inactivated vaccine fail to develop anti-NS1 immune responses [138]. Surveys utilizing this method revealed that the natural infection rates around the Kobe area (west–central Japan) in the 1980s ranged from 5 to 10% [139]. Similarly, the surveys conducted in other areas in 2001–2004 showed natural infection rates of approximately 2% [140,141]. A more recent survey conducted in 2004–2008 revealed that the average annual infection rates in western and eastern Japan were 1.8 and 1.3%, respectively [142]. In this study, conventional neutralization tests were performed using sera from unvaccinated populations, and a similar prevalence rate of 2.6% was recorded [142]. These data clearly demonstrated that JEV still exists in Japan, and that humans are still at risk of exposure.

The natural infection rates in horses, another incidental dead-end host, have also been tested in Japan. Relatively high natural infection rates (>15%) were observed in several areas [143,144], higher than the rate in humans. This, possibly, reflects the fact that humans live in tightly closed, well-screened houses, while horses are kept in stables under open-air/semi-open-air environments, with greater potential exposure to mosquitoes. Furthermore, the mosquito vector *Cx. tritaeniorhynchus* prefers outdoor environments to rest and feed [145]. These data support the higher natural infection rates in horses compared with humans. For mosquitoes, body temperature, carbon dioxide and odors are key factors in locating potential hosts [146]. The body temperature of horses (37.5–38.5°C) is slightly higher than that of humans, and mosquitoes recognize and prefer specific odors. For example, an anthropophilic mosquito, *Aedes aegypti*, has been known to recognize lactic acid that is produced on human skin, but not on the skin of other mammals [147]. Thus, horses might produce unidentified substances, not produced by humans, which attract *Cx. tritaeniorhynchus*. Such factors may affect the differences in the natural infection rates observed between humans and horses. Nevertheless, this high infection rate in horses supports the natural JEV activity observed in Japan, as demonstrated in swine and humans.

In the past few decades, swine farms have been relocated away from human residential areas. Taking this separation into consideration, the natural infection rate of approximately 2% in humans might be considered high, and suggests that JEV has acquired a new lifecycle that is specific to and maintainable in urban areas. To support this hypothesis, antibodies against JEV have been detected in a variety of mammalian animals, including wild boars, raccoons, raccoon dogs, dogs and bats, all of which live near human residential areas. Recent surveys carried out in Japan showed that 52–86% of wild boars were seropositive [10–12,148]. A more recent survey revealed 66.7% seropositivity in wild boars captured in a small island where there were no swine [149], and JEV has been isolated from a wild boar captured nearby human residential areas [150], suggesting that wild boars could act as an amplifier. JEV has also been isolated from bats [151]. More recently, it was reported that JEV can be transmitted by mosquitoes from bats, which did not exhibit detectable viremia [152]. This finding is important, as it indicates that animals in which JEV has not been isolated could be potential amplifiers. Such animals could be candidate JEV amplifiers in the JEV urban lifecycle.

As described, several animals could play a potential role as an amplifier in an urban lifecycle. However, new mosquito vectors must be involved in this urban lifecycle, since the major mosquito vector species, *Cx. tritaeniorhynchus*, does not generally exist in urban areas. *Aedes albopictus* and *Culex pipiens pallens* have been known to inhabit and breed in urban areas [153–155], and *Culex pipiens molestus* is also known to breed in underground environments, such

as subway tunnels and the basements of houses [156]. In fact, a recent survey conducted in Japan revealed that a larger number of *Ae. albopictus* were caught in urban residential areas than *Cx. tritaeniorhynchus* [207]. These mosquito species have been experimentally confirmed to be capable of transmitting JEV [157,158]. Furthermore, JEV has been isolated from Ae. *albopictus* in nature [159]. Thus, these mosquitoes may play a role as a vector in the urban lifecycle of JEV.

A possible explanation for the dramatic decrease in the number of JE cases observed in Japan, Korea and Taiwan is attenuation of the currently circulating strains of JEV. In fact, the JEV genome has been isolated from meningitis patients in Japan [160], suggesting that there may be more potential cases that have gone unreported owing to their nonencephalitis manifestations. As described previously, the predominant genotype of recent isolates has changed from genotype III to genotype I in Japan [43,45]. If the genotype III viruses were more pathogenic than the genotype I viruses, this could account for the drastic decrease in the number of JE cases. However, there is currently no evidence to suggest pathogenic differences between genotypes [38]. Furthermore, the pathogenicity of JEV varied even among genotype I viruses [161]. Thus, the genotype shift is not likely to be the reason for the dramatic decrease in the number of JE cases in these countries. However, virus strains examined in these studies were mostly isolated from swine and mosquitoes. As suggested, JEV may acquire a new urban lifecycle, utilizing different amplifiers and vector mosquitoes. Thus, it could be speculated that JEV maintained in the new lifecycle is attenuated. This possibility needs to be investigated.

In conclusion, JEV is still circulating in Japan, based on the serological surveys of humans and animals, despite the recommendation for routine vaccination being suspended by the government, and mouse brain-derived vaccine production being stopped in 2005. During the past 5 years, the susceptible population to JEV has increased in Japan, especially among school-aged children (Figure 1). In 1950 (before vaccination was started), approximately 900 cases in 0–4-year-old infants were reported (prevalence 9 per 100,000) [208]. Although environmental and virological factors were changed, vaccine effectiveness has been shown by no reported cases in infants between 1990 and 2006. However, two infant JE cases who did not receive JE vaccination (3 and 1 years old) were reported in 2006 and 2009, respectively [209]. Finally, in 2009, a new JE vaccine derived from Vero cell cultures was approved for use in Japan. Then, in April 2010, the Japanese government (the Ministry of Health, Labour and Welfare) restarted their recommendation for routine immunization of a limited population with the new Vero cell-derived vaccine [209].

## Future perspective

Japanese encephalitis disease, resulting from JEV infection, is characterized by a high mortality rate and a high incidence of neurological sequelae in survivors. Thus, JEV infection is a serious public health threat in endemic countries, and for those traveling to endemic countries. There are currently no approved specific antiviral drugs against JE. Since approval of the mouse brain-derived inactivated vaccine worldwide, and the live-attenuated vaccine in a few countries, JE vaccination has been proven to be a successful strategy in preventing this disease.

Despite many antiviral drug candidates proving effective *in vitro*, several clinical trials have failed to demonstrate their efficacy in humans. Many candidate antiviral drugs, including IFN and ribavirin, are effective against a broad spectrum of viruses. However, the mechanisms behind these antiviral effects remain unclear.

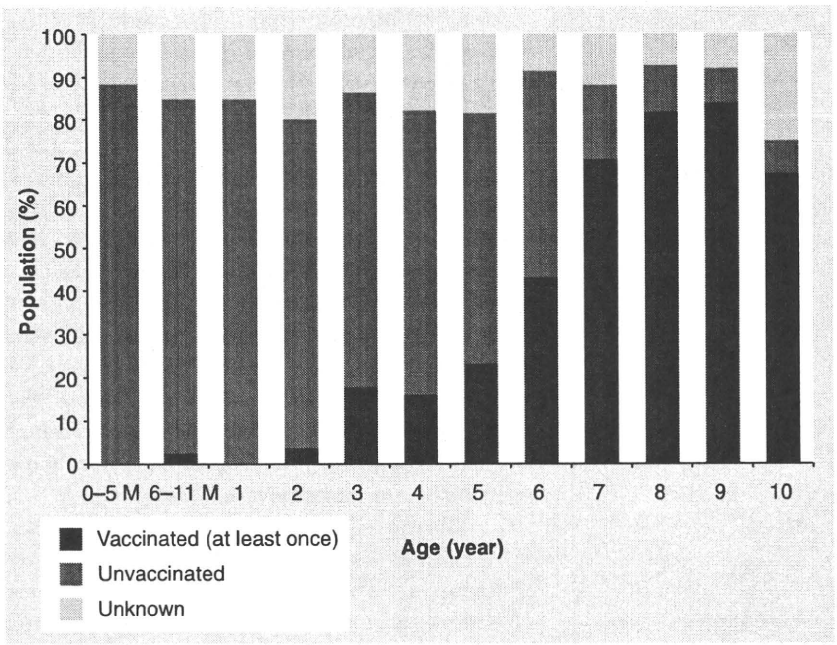


**Figure 1. Accumulation of Japanese encephalitis (JE)-susceptible populations in Japan.** The percentage of the population who received the JE vaccine at least once is shown in red, the percentage that did not receive the vaccine is shown in blue and the percentage whose vaccine history was unknown is shown in gray, for each age group.
Data were modified from the reports available at [210], which represent part of the national surveillance on JE virus activity carried out in 2008.

Recent advances in our understanding of viral replication in host cells, and the mechanisms by which viruses evade the host immune system, will help in the development of new types of antiviral drugs that may be designed to specifically target JEV. Moreover, it may be possible to develop novel technologies to deliver antivirals to specific tissues/cells where JEV replicates.

A new type of inactivated vaccine derived from cell culture has been developed, which is expected to be safer than the mouse brain-derived inactivated vaccine in terms of the associated neurological adverse events. However, a few cases of ADEM following vaccination with the new Vero cell-derived vaccine have already been reported. In Japan, approximately 500,000 doses of the Vero cell-derived vaccine were administered in 2009. Among these, 18 cases of adverse events, including a single meningitis case, were reported [209]. As mentioned, a survey conducted in Japan revealed that vaccination with mouse brain-derived vaccine was not related to the occurrence of ADEM. Since ADEM is induced independently from JE vaccination, severe ADEM might develop coincidently shortly after JE vaccination. This may also be the case with the new Vero cell-derived vaccine. Therefore, vaccinees should be aware of this issue, and the government should not repeat the decision to stop strongly recommending JE vaccination based on the possible occurrence of post-vaccination ADEM cases.

**Financial & competing interests disclosure**

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## Executive summary

### Characteristics of Japanese encephalitis virus (JEV) infection

- High mortality rate (up to 40%).
- High neurological sequelae rate in survivors (up to 50%).
- Children and young adults represent a high-risk group.
- No specific antiviral drugs exist.

### Current status of JEV infection

- JEV distributes throughout Southeast Asia and parts of Australia.
- It is estimated that there are 30,000–50,000 cases worldwide, with 10,000 deaths annually.

### Vaccines to prevent JE

- Mouse brain-derived formalin-inactivated vaccine has been extensively used worldwide; however, its production ceased in 2005.
- Live-attenuated vaccine is mainly used in China.
- Cell culture-derived formalin-inactivated vaccine has been available in Europe, the USA, Australia, Canada and Japan since 2009.

### Recent issues concerning vaccines in Japan

- The Japanese government (Ministry of Health, Labour and Welfare) stopped recommending the use of the mouse brain-derived vaccine for routine vaccination between 2005 and 2010.
- The Japanese government, in 2010, reinstated the strongly recommending the Vero cell-derived vaccine for routine vaccination.
- The frequency of acute disseminated encephalomyelitis (ADEM) in children remained unchanged before and after the recommendation was stopped by the government.

### Necessity for vaccination in Japan

- Less than ten cases per year have been reported since the 1990s.
- Serological surveys of human and horses showed that JEV still circulates in Japan.
- There is no evidence that the currently circulating virus strains are attenuated.
- Vaccination is still an effective method to prevent JEV-related diseases in Japan.

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