Vector-free transmission and persistence of Japanese encephalitis virus in pigs

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Japanese encephalitis virus (JEV), a main cause of severe viral encephalitis in humans, has a complex ecology, composed of a cycle involving primarily waterbirds and mosquitoes, as well as a cycle involving pigs as amplifying hosts. To date, JEV transmission has been exclusively described as being mosquito-mediated. Here we demonstrate that JEV can be transmitted between pigs in the absence of arthropod vectors. Pigs shed virus in oronasal secretions and are highly susceptible to oronasal infection. Clinical symptoms, virus tropism and central nervous system histological lesions are similar in pigs infected through needle, contact or oronasal inoculation. In all cases, a particularly important site of replication are the tonsils, in which JEV is found to persist for at least 25 days despite the presence of high levels of neutralizing antibodies. Our findings could have a major impact on the ecology of JEV in temperate regions with short mosquito seasons.

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Japanese encephalitis virus (JEV) causes an important zoonotic vector-borne disease first isolated from a human in Japan in 1935 (ref. 1). It is currently present in East and Southeast Asia and Australia2,3. The annual incidence of human cases is reported to be in the range of 50,000–175,000 (refs 2–4). During an epidemic, roughly 0.1–4% of infected individuals develop clinically apparent encephalitis. The mortality associated with JEV encephalitis is as high as 25–30%, and ~50% of surviving patients suffer neuropsychiatric sequelae2,3. JEV is considered to be the most frequent viral encephalitis associated with fatal or severe outcomes2,3.

JEV is vector-borne with *Culex* mosquitoes as its main vectors, and with waterbirds such as egrets and herons as reservoirs. However, it has been reported that pigs serve as amplifying hosts in human epidemics2,3,6–10. As early as the 1950s, studies found that pigs are readily infected with JEV and develop viraemia for several days5,8,11. While clinical symptoms in pigs are mild, humans and horses can develop severe disease with encephalitis. Factors favouring pigs as being the main amplifying host for JEV include a high birth rate and a rapid population turnover, resulting in constant generation of an immunologically naive population. Furthermore, an important JEV vector *Culex tritaeniorhynchus* preferentially feeds on pigs2,3,9,12,13. Fortunately, viraemia in humans and horses is probably insufficient to infect mosquitoes, and they are considered to be dead-end hosts2.

In the past, the temperate northern Japanese Island Hokkaido was affected by JEV epidemics, and the virus was shown to hibernate and re-emerge in the same local region14. Some ecological and epidemiological aspects of these outbreaks remained enigmatic. First, during several Japanese encephalitis outbreaks, no virus was isolated from locally collected mosquitoes15,16. Second, two geographically isolated distinct outbreaks, no virus was isolated from locally collected mosquitoes15,16. Fortunately, viraemia in humans and horses is probably insufficient to infect mosquitoes, and they are considered to be dead-end hosts2.

In the past, the temperate northern Japanese Island Hokkaido was affected by JEV epidemics, and the virus was shown to hibernate and re-emerge in the same local region14. Some ecological and epidemiological aspects of these outbreaks remained enigmatic. First, during several Japanese encephalitis outbreaks, no virus was isolated from locally collected mosquitoes15,16. Second, two geographically isolated distinct outbreaks were identified on pig farms in Hokkaido over at least 3 years, demonstrating that the virus can hibernate locally14. However, the underlying mechanisms were not clarified. Also, during epidemiological investigations in Taiwan, no viraemic mosquitoes were found in the period before JEV outbreaks in pigs17.

We questioned if vector-free transmission might be possible and, if so, could help explain some of the observations made in temperate regions. Therefore, in the frame of a pathogenesis study with pigs, we placed sentinels with intravenously (i.v.) infected pigs and found vector-free transmission of JEV in pigs. This finding was confirmed and further supported by demonstrating efficient oronasal infection with low doses of virus. Moreover, tonsils appear to play a prominent role as a source of virus replication and persistence.

**Results**

**JEV can transmit between pigs in the absence of vectors.** We observed JEV transmission from needle-infected pigs to uninfected naive pigs when three infected pigs were housed with two uninfected animals. Before infection, all piglets were healthy and alert, with normal body temperatures of 38.7–39.4°C. Body temperature in the needle-infected animals increased after 24 h, with readings up to 40.6°C; fever lasted for 4–5 days before dropping to pre-infection levels (Fig. 1a). The two contact animals developed fever 6 and 9 days after needle inoculation of the other three animals. In all but one animal, fever curves were double-peaked. Appetite was reduced in all animals. They produced less manure and were reluctant to move for 3–6 days. When body temperature normalized, clinical symptoms declined and finally disappeared.

Viraemia in needle-infected animals lasted 3 days and reached maximum values in the range of 10^4 RNA U ml^-1 (Fig. 1b); viral titres were 3.2×10^4 (2 animals)–3.2×10^5 (1 animal) tissue culture infectious dose 50 (TCID50) per ml. Viraemia was found in both contact pigs for 2 and 4 days, with maximum values around one to two orders of magnitude lower than the maximum in the needle-infected animals (Fig. 1b). Both sera were positive for virus isolation by cell culture, but their infectious titres were close to the detection limit of the assay (50 TCID50 per ml). To verify this unexpected and to our knowledge previously not described observation of viral transmission in a vector-free environment, we conducted a second experiment. Two animals were needle-infected and six healthy animals were kept in the same stable to act as sentinels. Clinical outcomes in the needle-infected animals were as described above, with one pig’s body temperature passing 41°C. Both became viraemic both in terms of viral RNA (Fig. 1c,d) and live virus detection (1.5 and 3.2×10^4 TCID50 per ml). Body temperature increased over 40°C in two of the sentinels (Fig. 1c). However, real-time reverse transcription–quantitative PCR (RT–qPCR) revealed viraemia in only one. Viraemia lasted for 3 days, and values were close to 10^4 RNA U ml^-1, similar to the needle-infected pigs (Fig. 1d). The other five sentinel animals did not develop detectable viraemia or seroconversion, and RT–qPCR-positive organs were not detected during the 11-day observational period.

**JEV organ tropism is independent of mode of infection.** Necropsy of needle-infected animals was performed at day 11 (first experiment) or 7 (second experiment), and necropsy for sentinels was performed at day 10 or 11, which was 6–8 days after estimated transmission (post transmission). Figure 2 shows relative RNA quantities for both needle-infected and sentinel animals. Relative RNA quantities were comparable between all animals, independent of mode of infection. The lymph nodes, the ileum with its continuous Peyer’s patches, and parts of the nasal cavity were positive for viral RNA. Interestingly, relative RNA levels of up to 10^5 U g^-1 were found in the tonsils. These values were 1–2 orders of magnitude higher than in the other organs (Fig. 2a). In the brain tissues, values were also comparable between the needle- and contact-infected pigs. All examined regions remained positive until the end of the study period, with highest levels of up to 10^5–10^6 RNA U g^-1 in the (frontal) neocortex, thalamus and basal nuclei. In the brain stem and olfactory bulb, we found roughly 10 times less viral RNA compared with the other regions (Fig. 2b). By titrating lysed material from the tonsils, we confirmed the presence of live virus in all tonsils of infected pigs (Table 1).

**Oronasal virus shedding by JEV-infected pigs.** RT–qPCR indicated that needle-infected animals started to shed virus oronasally as early as two days post infection (p.i.) for a period of ~4 days. Animals infected by contact first shed virus 5 days after first contact with the needle-infected animals. In two of them, viral RNA in oronasal swabs was detected for 1 day only. The third animal shed virus for 3 days (Fig. 3). Swabs from the eyes, rectum and vagina/preputium, and the urine were negative, with the exception of one animal in which a foreskin swab was RT–qPCR positive at 5 days p.i. (0.6 RNA U ml^-1). We used cell culture to confirm that the oronasal swabs contained live virus. Most swabs collected at 4–5 days p.i. were positive (Table 2, second column). Similarly, pigs infected by contact shed live virus 6–10 days after contact, depending on the animal (Table 2, third column).

**Pigs are highly susceptible to oronasal JEV infection.** Considering that three out of eight in-contact animals became ill and shed virus oronasally for 1–4 days, we tested the oronasal...
route as a means of infection by JEV. As described in Methods, nine animals were infected oronasally using three different doses of virus. In all nine pigs, body temperatures raised after 4–9 days, reaching 41.5°C in some animals. Interestingly, two animals infected with the lowest dose (10^3 TCID_{50}) developed the highest body temperatures. By day 10 p.i., body temperatures of all animals returned to normal levels (Fig. 4a). Viraemia in all three groups was comparable, although two animals infected with the lowest dose developed viraemia 1–2 days later than the other pigs (Fig. 4b). In all pigs, viraemia lasted for 4 days. One animal, infected with the highest dose, suffered from rebound viraemia on day 16 p.i., with viral RNA detected in the serum. Only one blood sample per week was taken, and we cannot determine the duration of this second viraemia.

Animals infected via the oronasal route were also positive for JEV RNA in oronasal swabs (Fig. 4c). Swabs were positive in pigs infected with the highest dose at day 1 p.i., possibly representing input virus. Thereafter, most pigs shed virus between day 4 and 7 p.i., with relative RNA levels reaching 100 U ml^{-1}. Nevertheless, some animals had RNA-positive swabs up to 9 days p.i., which

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**Figure 1 | Body temperature and viraemia of pigs infected by needle or contact.** (a, b) Three needle-infected pigs (10^7 TCID_{50} per pig, black lines) were housed with two naive sentinel animals (red lines). (c, d) Two animals were needle-infected (black) and housed with six sentinel pigs (red and blue). Viraemia is shown as viral RNA loads determined by real-time RT-PCR, and expressed as U ml^{-1} (1 U corresponding to the RNA quantity found in 1 TCID_{50} of a virus stock).

**Figure 2 | Viral RNA loads in peripheral and CNS tissues.** Viral RNA loads in peripheral (a) and CNS (b) tissues were determined at necropsy by real-time RT-PCR and expressed as U g^{-1} (1 U corresponding to the RNA quantity found in 1 TCID_{50} of a virus stock). Solid symbols represent needle-infected pigs (n = 5) killed at 7 (circles) and 11 (squares) days p.i. Open symbols represent pigs infected by contact (n = 3). Two animals, corresponding to those shown in Fig. 1b, were killed at day 11, which was 4 and 7 days after the peak of viraemia, respectively. One animal, corresponding to Fig. 1d, was killed at day 10, which was 7 days after peak viraemia. Asterisks (*) indicate significant differences calculated with a nonparametric two-tailed Mann-Whitney U-test (P < 0.05).
was 2 days beyond the end of the viraemic phase (Fig. 4c). Between 3 and 6 days p.i., the majority of swabs were also positive for virus isolation (Table 2).

Considering the relatively low levels of virus in oronasal swabs, we decided to perform a follow-up oronasal experimental infection with doses of 10, 100 and 1,000 TCID\textsubscript{50} per pig (Fig. 4d,e). Strikingly, all animals again became infected, with incubation times of 2–3 days and viraemia lasting 5–6 days. At 4 and 5 days p.i., body temperature in all pigs was above 39.5°C (Fig. 4d). Again, as early as 3 days p.i., some pigs had viral RNA-positive oronasal swabs; by day 7 all swabs were positive (Fig. 4f). In most pigs, oronasal virus excretion lasted 5–6 days.

Viral tropism for non-central nervous system (CNS) tissue was similar in needle and oronasally infected pigs. At necropsy 10 days p.i., lymph nodes, ileum and tonsils from the lowest-dose-infected pigs were positive for viral RNA (Fig. 5a). In the lymph nodes and ileum, only 100–1,000 RNA U g\textsuperscript{-1} were detected, while almost 100,000 RNA U g\textsuperscript{-1} were found in the tonsils. No difference was observed between the 10\textsuperscript{2} and 10\textsuperscript{3} dose in the lymph nodes and tonsils. The trachea and nasal cavity were negative for viral RNA except in one pig (Fig. 5a).

Urine samples were collected on the day of slaughter; we found one positive sample (0.5 RNA U ml\textsuperscript{-1}). RNA levels in the brain were comparable in tissues isolated from pigs infected with the low and middle doses. Thalamus and basal nuclei reached the highest levels of around 1,000–10,000 RNA U g\textsuperscript{-1} (Fig. 5b).

**Histopathological lesions.** Regardless of mode of infection, JEV induced histopathological CNS lesions typical of a viral meningoencephalomyelitis. Lesions were characterized by multifocal lymphohistiocytic perivascular cuffs, affecting mainly the grey matter, and to a lesser degree, the white matter. They were associated with glial nodules and evidence of neuronal degeneration and necrosis. Frequently, few neutrophils were present in the areas of neuronal necrosis. In addition, multifocal mild lymphohistiocytic meningitis was present. Scoring the lesions in the brain stem, cerebellum, midbrain, thalamus, hippocampus, basal nuclei, neocortex and the bulbus olfactorius indicated that the mode of infection did not fundamentally influence virus-induced pathology and distribution of CNS lesions (Fig. 6). Note that the time after the virus had reached the CNS was unequal between the groups as the animals were not slaughtered the same day, the incubation period differed (Figs 1 and 4) or the time of infection was unknown. Nevertheless, the overall score calculated as an average score of the CNS tissue analysed was similar for all modes of infections. Lymphatic tissues including tonsils showed slight follicular hyperplasia, which is indicative of activation but otherwise no pathological alterations.

**JEV can persist in the tonsils for at least 25 days.** The levels of viral RNA were always highest in the tonsils in all animals, independent of the route and dose of infection (Figs 2 and 5). Given that the longest observational time in our initial experiment was 11 days p.i., we decided to keep a new group of animals longer to examine the potential persistence of JEV. Strikingly, on day 21 after oronasal infection with the Nakayama strain, the peripheral organs and CNS for two animals were negative for viral RNA, but 10\textsuperscript{3}–10\textsuperscript{4} U g\textsuperscript{-1} remained in the tonsils. These values were comparable to those at 7 and 11 days p.i. The lymph nodes, jejunum, trachea, olfactory bulb, neocortex and basal nuclei were positive in one animal (Fig. 7a, red squares). This animal had a second viraemia at 17 days p.i. (Fig. 4b). RT–PCR was negative in urine samples collected at 21 days p.i.

To determine if persistence of virus in the tonsils is unique to the Nakayama strain or if it can result from infection with other genotypes, we analysed six pigs infected with Laos strain, a genotype I JEV. At necropsy on day 11, the highest RNA values were in the tonsils, whereas the CNS samples were negative or roughly two orders of magnitude lower (Fig. 7b). Strikingly, at 25

### Table 1 | Virological data from tonsils.

| Pig No. | Infection | Virus titration* (TCID\textsubscript{50} per g) | RNA quantity (RNA U per g) |
|---------|-----------|---------------------------------|--------------------------|
| 1395    | i.v./i.d. | 3.16 × 10\textsuperscript{3} | 9.4 × 10\textsuperscript{4} |
| 1401    | i.v./i.d. | 4.39 × 10\textsuperscript{2} | 6.0 × 10\textsuperscript{4} |
| 1402    | i.v./i.d. | 6.81 × 10\textsuperscript{4} | 6.6 × 10\textsuperscript{4} |
| 1415    | i.v./i.d. | 3.16 × 10\textsuperscript{4} | 2.5 × 10\textsuperscript{4} |
| 1420    | i.v./i.d. | >5 × 10\textsuperscript{1} | 1.0 × 10\textsuperscript{5} |
| 1403    | Contact   | 6.81 × 10\textsuperscript{3} | 6.6 × 10\textsuperscript{4} |
| 1411    | Contact   | 4.39 × 10\textsuperscript{2} | 1.3 × 10\textsuperscript{4} |
| 1416\textsuperscript{f} | Contact   | Negative | Negative |
| 1417\textsuperscript{f} | Contact   | Negative | Negative |
| 1418\textsuperscript{f} | Contact   | Negative | Negative |
| 1421\textsuperscript{f} | Contact   | Negative | Negative |
| 1422    | Contact   | >5 × 10\textsuperscript{1} | 1.4 × 10\textsuperscript{4} |
| 1423    | Contact   | Negative | Negative |

*In some samples toxic effects of the lysates reduced sensitivity (theoretically 50 TCID\textsubscript{50} per g).

†These animals did not get infected.

### Table 2 | Virus isolation from oronasal swabs*†.

| Days p.i. | Mode of infection |
|-----------|-----------------|
| i.v./i.d. | Contact | Oronasal |
| 1         | 0/5\textsuperscript{z} | 0/3 | 0/9 |
| 2         | 4/11 | 0/3 | 0/9 |
| 3         | 3/10 | 0/3 | 6/9 |
| 4         | 6/7 | 0/3 | 5/9 |
| 5         | 8/9 | 0/3 | 5/9 |
| 6         | 2/3 | 2/2 | 6/6 |
| 7         | 0/3 | 1/2 | 2/9 |
| 8         | 0/3 | 1/2 | 0/9 |
| 9         | — | 1/2 | — |
| 10        | — | 1/2 | — |
| 11        | — | 0/2 | — |

*Theoretical sensitivity 50 TCID\textsubscript{50} per ml.
†Results for some of the swabs were lost due to cell culture contamination and loss of material.

(Number of virus-positive swabs/number of virus-negative swabs.)
p.i., all organs were negative for viral RNA except the tonsils, confirming JEV’s ability to persist in this organ for over 3 weeks (Fig. 7c). All tonsils of infected pigs were also positive for virus isolation.

**Immune response.** All infected animals mounted a rapid immune response in terms of JEV-neutralizing antibodies (Fig. 8). At 7–10 days p.i. (6–9 days after first viraemia), all animals had titres of 40–80 TCID$_{50}$ per ml. Similar titres were
found in pigs infected by contact. This finding was confirmed in the oronasally infected pigs. The levels of neutralizing antibodies increased with time after infection, but they did not differ by mode of infection or JEV genotype. These results demonstrate that JEV persists in the tonsils despite the presence of an efficient humoral immune response.

Discussion

This study describes two findings concerning JEV infection in pigs, both of which may have a significant impact on our understanding of JEV’s ecology, epidemiology and on approaches to controlling it. First, vector-free transmission between pigs can occur via direct contact, with animals being highly susceptible to oronasal infection. Second, the tonsils are a primary replication site of JEV, regardless of mode of infection, and JEV can persist in them for at least 25 days despite the presence of neutralizing antibodies.

Textbooks and published scientific articles describe Japanese encephalitis as being exclusively mosquito-borne, with Culex species as the main vectors (reviewed in refs 2,3). In our first experimental infection, both sentinel animals became ill, but only one out of six was infected in the second. This difference could be due to the fact that in the second experiment only two animals were needle-infected, which could have reduced the chances of contact. In fact, our facility’s efficient ventilation system and low stocking density (>3 m² per animal) support transmission by contact-dependent route rather than by aerosols. Although our study had too few animals to estimate the reproduction value of transmission, it indicates that this process is not as efficacious as with viruses that have adapted to enter through the mucosal surfaces of the airways, such as influenza virus. This possibility is understandable, given that mosquitoes are clearly the main transmission mode of JEV. Nevertheless, it is possible that under field conditions with a dense pig population and other pathogens, the rate of vector-free transmission could be higher compared with experimental conditions with clean stables, controlled temperature and humidity, high ventilation and no crowding.
oronasal inoculation also did not appear to have a major impact on oronasal/contact transmission. The virus doses employed for and antibody response. If anything, viraemia lasted longer after respiratory tract, virus tropism in the lymphoid and CNS tissues, differences in viraemia, virus excretion through the upper respiratory tract could be a possible source of virus leading to oronasal infection. Also, the peak of viral RNA in oronasal swabs was found around 6 days p.i., which was 2–3 days after the peak of viraemia. In addition, virus-positive swabs were still found after the viraemic phase.

Several reports have demonstrated oral or nasal infection of West Nile virus (WNV), a closely related flavivirus, in a wide range of different species, including mice, wild birds, hamsters and alligators26–29. In humans, there is evidence for transmission via breastfeeding; this evidence is supported by data in hamsters27,30. Furthermore, laboratory infections may have occurred through aerosol transmission21,32. Although data supporting oronasal JEV infection species other than pigs is rare, our data should be taken as a warning that infection via the oronasal route might be possible, and direct pig–human and bird–bird transmissions cannot be excluded. Clearly, these possibilities require future investigation.

Our second important observation was JEV’s tropism for the tonsils, where viral loads were 2–3 orders of magnitude higher than in other organs. Furthermore, JEV can persist in the tonsils for at least 3 weeks. To our knowledge, JEV infection of the tonsils in other species has not been described, although in one study, the tonsils were used as a source of virus isolation in pigs33. We found that high viral load in the tonsils persisted well beyond the viraemic phase of infection, despite the presence of neutralizing antibodies. Even by week 3 p.i., high RNA and live virus levels were detected in tonsil homogenates. All other organs tested were negative by then.

As this finding indicates a possible persistence for more than 1 month, future studies are required to determine the occurrence and duration of JEV persistence in porcine tonsils under field conditions. Persistence may be associated with reactivation and oronasal transmission events to naive pigs, thereby affecting the epidemiology of Japanese encephalitis.

A recent review34 notes that WNV virus persists in several mammalian species, including rhesus monkeys, hamsters, mice and humans. In monkeys, hamsters and mice, virus can persist for several weeks to months in the CNS and peripheral tissues, including lymphoid tissues and kidney. Kidney targeting by WNV is related to viruria and renal pathology in human WNV-infected patients. We did not observe renal targeting by JEV, and positive RT–PCR in urine was rare. These findings indicate tropism differences between WNV and JEV.

In fact, for recurrent JEV outbreaks in temperate regions such as Hokkaido, the mechanisms of JEV hibernation are still unexplained14,35. In tropical regions, Japanese encephalitis is endemic throughout the year, as are mosquito vectors, and disease occurrence is clearly related to vector-borne transmission. In contrast, in temperate regions, Japanese encephalitis cases occur only in the warm season. Therefore, JEV re-emergence would require either reintroduction of JEV by migrating birds or a mechanism of virus overwintering in unknown hosts36,37. The re-emergence of porcine Japanese encephalitis cases in Hokkaido at the same locations indicates that JEV can overwinter locally14. In fact, more recent molecular analyses of JEV isolates from several genotypes present in temperate regions showed an important relationship between phylogeny and sampling location, favouring the concept of local overwintering. These studies indicate that genetic diversity of JEV isolates is driven by local virus transmission cycles rather than virus introduction from distant regions for instance by migratory birds36,37. Researchers have proposed that overwintering occurs in vertebrate hosts such as bats, in cold-blooded species or in invertebrates such as mosquitoes and ticks, the latter involving vertical virus transmission35–37. Indeed *Culex* species can overwinter locally35 and can transmit JEV following experimental hibernation38. Vertical transmission of JEV has
been demonstrated experimentally in mosquitoes39–41. Nevertheless, the winter host has not been identified, despite significant effort42. For example, only one JEV-positive larva was found in 382,000 larvae over a period of 3.5 years in Taiwan, a fact that questions the importance of vertical transmission as an overwintering mechanism42. In the abovementioned areas of Hokkaido Island, in which JEV remained endemic in pigs kept in distinct areas for several years, early JEV-induced abortions were observed before mosquito season43. Thus, alternative transmission pathways for JEV in pigs might exist, as well as vector-free transmission. Future studies are now urgently required to define the impact of our findings in the light of the One Health Initiative. Despite answering questions on the occurrence of vector-free transmission and virus persistence in pigs under field situations, the cellular target of virus replication and persistence in the tonsils, the swine immune response and the impact of persistence and vector-free transmission on virus adaptation and evolution need to be investigated.

Methods

Animal experiment. Five animal experiments were performed under biosafety level 3 conditions and approved by the Cantonal Ethical Committee for animal experiments (BE 118-13). In total, 28 healthy 7-week-old Swiss Large White pigs (15 castrated males and 13 females) from our specific-pathogen-free breeding facility of the IVI, representing a BSL3-Ag facility. Before use after two passages on Vero cells (ATCC, Manassas, VA, USA). Two naive animals were kept together with the needle-infected animals to determine a possible vector-free transmission. All animals underwent necropsy at day 7 p.i. In a second experiment, we confirmed the ability of JEV to transmit in the absence of vector by infecting two animals i.v. and i.d. as described above, and adding six naive pigs to the same pen after 24 h. The needle-infected animals underwent necropsy at day 7 p.i. and the contact pigs 9 days after being in contact with the needle-infected pigs. In a third experiment, we determined the efficacy of oronasal infection. Nine pigs were housed separately in groups of three. Each group was infected oronasally with either 10^3, 10^4 or 10^5 TCID_50 of JEV (Nakayama strain). Six animals underwent necropsy at day 10 p.i., and those infected with the highest dose were kept until day 21 p.i. In a fourth experiment, nine pigs were again housed separately in groups of three and infected oronasally with a lower dose (10^2, 10^2 or 10^3 TCID_50 of JEV Nakayama strain). For both experiments, the needle solution was injected carefully into different parts of the mouth without pressure using a needle-free syringe, and 1 ml was applied dropwise into the nose while using a needle-free syringe. After 4 h of incubation, the inoculum was removed and replaced with fresh medium. The cells were incubated for 72 h before fixing with 100 µl of 0.4% Acroledehyde (Polysciences, Warrington, PA, USA) for 10 min and staining with anti-flavivirus E protein monoclonal antibody 4G2 (HB-112, ATCC) diluted in a saponin–PBS buffer (Sigma-Aldrich Chemie GmbH, Buchs, Switzerland), followed by horse-radish peroxidase-conjugated goat-anti mouse antibody (Dako, Baar, Switzerland) and a final colour reaction with 3,3′-diaminobenzidine (Sigma-Aldrich). Titres were calculated using the Reed and Muench formula46.

Histopathology. Samples were embedded in paraffin, cut to 4-µm thickness and stained with haematoxylin and eosin. Lesions in the CNS system were semiquantitatively scored from 0 to 4 (0: no lesions; 1: minimal lesions; 2: mild lesions; 3: moderate lesions; 4: severe lesions). Scoring was performed by a blinded histopathologist and based on neurophagocytic perivascular cuffs, neuronal necrosis, glial nodules and parenchymal infiltration by inflammatory cells.

Antibody responses. For plaque reduction neutralization tests (PRNT), sera were serially diluted twofold in medium in triplicate, starting at a 1:5 dilution in medium. One hundred plaque forming units per well of homologous virus were added to each well, and the serum–virus mix was gently agitated and incubated at 37°C for 30 min. Confluent Vero cells were then incubated with the serum–virus mix for 1 h at 37°C before incubation at 37°C and addition of 200 µl 1% methylcellulose medium (Sigma-Aldrich) supplemented with 100 IU penicillin and 100 µg ml^-1 streptomycin per well. After incubation for 48 h at 37°C, the cells were fixed and stained as described above. As a secondary antibody, horse-radish peroxidase-conjugated goat-anti pig was used at 1:500 (Bethyl, Montgomery, TX, USA). PRNT titres were read as the last serum dilution that showed a 50% plaque forming unit reduction.

Statistical analysis. For statistical analysis, GraphPad Prism (GraphPad Software Inc., La Jolla, CA, USA) was used. To determine differences, groups were compared using a nonparametric two-tailed Mann–Whitney U-test setting significance to 5%.

References

1. Solomon, T. et al. Japanese encephalitis. J. Neurol. Neurosurg. Psychiatry 68, 405–415 (2000).
2. Impoinvil, D. E., Baylis, M. & Solomon, T. Japanese encephalitis: on the One Health agenda. Curr. Top. Microbiol. Immunol. 365, 205–247 (2013).
3. van den Hurk, A. F., Ritchie, S. A. & Mackenzie, J. S. Ecology and geographical expansion of Japanese encephalitis virus. Annu. Rev. Entomol. 54, 17–35 (2009).
4. Campbell, G. L. et al. Estimated global incidence of Japanese encephalitis: a systematic review. Bull. World Health Organ. 89, 766–774, 774a–774e (2011).
5. Watanabe, T. C. & Barrett, A. D. Transmission cycles, host range, evolution and emergence of arboviral disease. Nat. Rev. Microbiol. 2, 789–801 (2004).
6. Greiser, I., Hardy, J. L., Hu, S. M. & Scherer, W. F. Factors influencing transmission of Japanese B encephalitis virus by a colonized strain of Culex tritaeniorhynchus Giles, from infected pigs and chicks to susceptible pigs and birds. Am. J. Trop. Med. Hyg. 7, 365–373 (1939).
7. Scherer, W. F., Moyer, J. T. & Irimia, T. Immunologic studies of Japanese encephalitis virus in Japan. V. Maternal antibodies, antibody responses and viremia following infection of swine. J. Immunol. 83, 620–626 (1959).
19. Raengsakulrach, B.  
20. Myint, K. S.  
17. Okuno, T., Mitchell, C. J., Chen, P. S., Wang, J. S. & Lin, S. Y. Seasonal infection  
16. Takashima, I., Hashimoto, N., Watanabe, T. & Rosen, L. Mosquito collection in  
13. Bendell, P. J. Japanese encephalitis in Sarawak: studies on mosquito  
10. Konno, J., Endo, K., Agatsuma, H. & Ishida, N. Cyclic outbreaks of Japanese  
11. Hurlbut, H. S. & Thomas, J. I. Observations on the experimental transmission  
22. Sasaki, O.  
24. Konishi, E.  
25. Takahashi, M. The effects of environmental and physiological conditions of  
immunisation of mice with live Japanese encephalitis virus induces a protective  
production of lethal infection that resembles fatal human  
30. Centers for Disease Control and Prevention (CDC). Possible West Nile virus  
31. Nir, Y. D. Airborne West Nile virus infection.  
27. Reagan, R. L., Yancey, F. S., Chang, S. C. & Brueckner, A. L. Transmission  
26. Nir, Y., Beemer, A. & Goldwasser, R. A. West Nile Virus infection in mice  
21. Yang, D. K. et al. TaqMan reverse transcription polymerase chain reaction for  
the detection of Japanese encephalitis virus. J. Vet. Sci. 5, 345–351 (2004).  
46. Reed, L. J. & Muench, H. A simple method of estimating fifty per cent  
endpoints. Am. J. Epidemiol. 27, 493–497 (1938).  

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