VIROLOGY

Sheep serve as amplifying hosts of Japanese encephalitis virus, increasing the risk of human infection

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The transmission cycle of Japanese encephalitis virus (JEV), involving pigs and birds as amplifying hosts and mosquitoes as vectors, was elucidated in the 1950s. However, factors contributing to this cycle remain unclear. Here, sheep were infected with a JEV strain isolated from sheep exhibiting neurological symptoms. The results revealed that sheep are susceptible to JEV infection and develop viremia, with levels and duration comparable to those observed in pigs, a known JEV-amplifying host. Mosquitoes fed viremic sheep blood showed an infection rate of 40.6 to 57.1%. These findings indicate that sheep can serve as amplifying hosts for JEV, potentially contributing to JEV transmission and increasing the public health risk of human infections. We propose an alternative, sheep-associated rural domestic JEV transmission cycle, which may be prevalent in specific regions where sheep are bred but pigs are not. This cycle exists along with the well-known pig-associated rural domestic and bird-associated wild cycles.

INTRODUCTION

Japanese encephalitis virus (JEV, *Orthoflavivirus japonicum*), the causative agent of Japanese encephalitis (JE), was first identified in Japan in 1871. JE affects an estimated 69,000 individuals annually worldwide, with a mortality rate of 20 to 30% (*1*). Although JE is vaccine-preventable, it remains endemic in 24 countries and territories across Asia and Oceania.

JEV, a zoonotic orthoflavivirus within the family Flavivirus, encompasses more than 70 species, including West Nile and Zika viruses (*2*, *3*). Its genome consists of single-stranded, positive-sense RNA encoding a polyprotein that is subsequently cleaved into three structural (C, prM, and E) and seven nonstructural (NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5) proteins (*4*). Phylogenetically, JEV is classified into five genotypes (I to V) based on the E gene nucleotide sequence. Genotypes I (GI) and III (GIII) are the most prevalent across Asia (*5*–*7*); GI is gradually replacing GIII as the dominant genotype in known epidemic areas (*8*). Genotype V (GV) emerged in Korea in 2010 (*9*), whereas genotype IV (GIV) caused an outbreak in mainland Australia in 2022 (*10*). Because of these patterns, JEV is considered an emerging and re-emerging pathogen.

As a zoonotic mosquito-borne virus (*11*), JEV is maintained in a cycle among vertebrate hosts via arthropod vectors. Its transmission cycle, elucidated as early as the 1950s, implicates *Culex* (*Cx.*) mosquitoes as the primary vectors; pigs and ardeid wading birds serve as amplifying hosts (*12*–*14*). Upon infection with JEV, these hosts develop high levels of viremia, facilitating mosquito infection and perpetuating the JEV transmission cycle (*13*).

Viremia and/or seroconversion to JEV have been observed in various mammalian species, including humans, pigs, horses, goats, cattle, dogs, and rodents. Although humans and horses may become infected, they rarely develop sufficient viremia to infect mosquitoes; therefore, they are considered dead-end hosts (*13*). In contrast, pigs consistently exhibit high levels of viremia upon infection, making them a crucial link in the JEV transmission cycle. There remains a lack of clarity concerning the roles of other mammals that demonstrate viremia and/or seroconversion. Preliminary observations suggest that certain wild species (e.g., wild boars, raccoons, flying foxes, and bats) contribute to alternative JEV transmission cycles (*13*).

Intriguingly, sheep have been observed to exhibit JEV seroconversion (*15*), and suspected cases of JEV infection have been sporadically reported on sheep farms in China (*16*). However, the role of sheep in JEV transmission has been unclear. Here, we report the isolation of a JEV strain from the brain tissue of sheep with neurological symptoms. We evaluated the pathogenicity and viremia of this isolated strain in sheep to determine whether sheep contribute to the JEV transmission cycle.

RESULTS

Isolation of JEV from sheep with neurological signs

Brain samples, designated 2201 and 2202, were collected from two sheep exhibiting neurological signs, such as circling and ataxia. These samples were subjected to routine histopathological analysis and molecular diagnostics. Histopathological examination revealed encephalitis lesions, including lymphohistiocytic perivascular cuffing, in both samples (Fig. 1A). Screening for several pathogens that can cause neurological signs in sheep [e.g., JEV, brucella (*17*), pseudorabies virus (*18*), and caprine arthritis encephalitis virus (*19*)] identified JEV as the sole pathogen in both samples (Fig. 1B). Subsequently, Baby Hamster Kidney-21 (BHK-21) cells were used in attempts to isolate the causative virus from both JEV-positive brain samples. After five blind passages, one JEV strain, designated SH2201, was isolated from sample 2201 but not sample 2202. This isolation was confirmed by immunofluorescence assays using JEV E protein-specific antibodies (Fig. 1C) and Western blotting assays using JEV NS1 protein-specific antibodies (Fig. 1D). The replication kinetics of the SH2201 strain in

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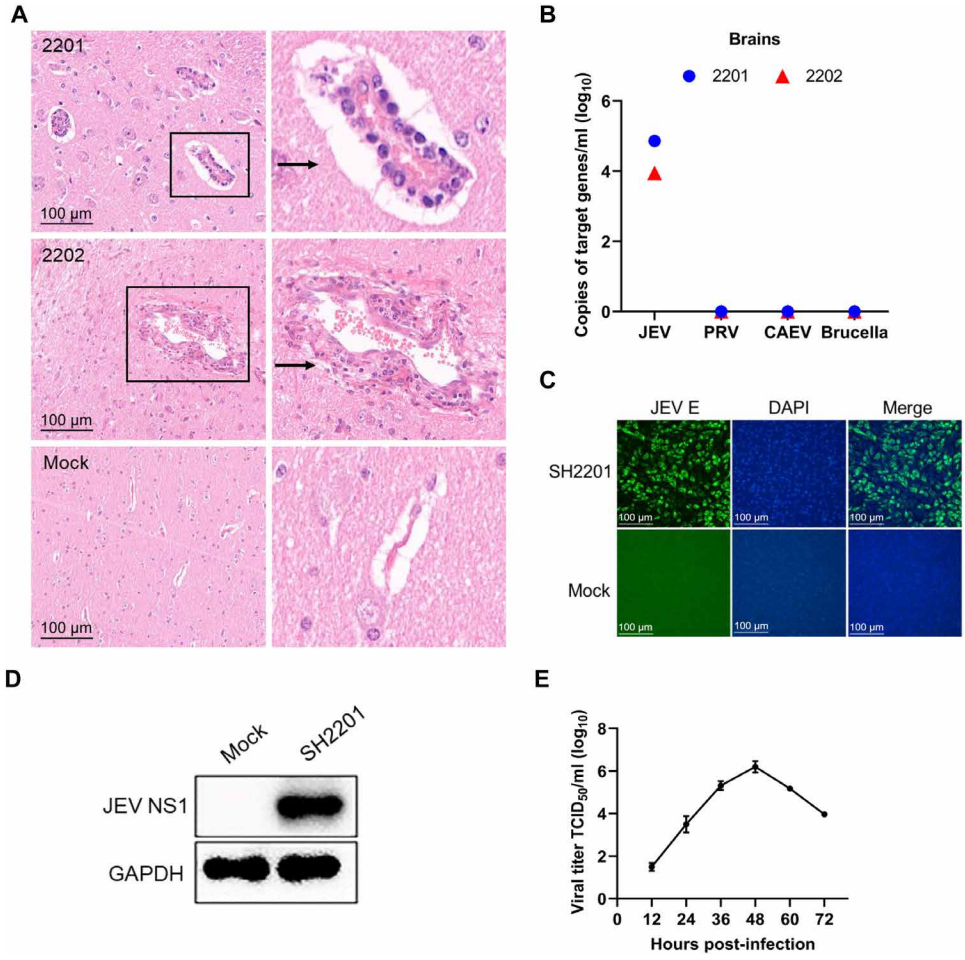


Fig. 1. Isolation of JEV from sheep. (**A**) Histopathological examination of brain tissue from sheep exhibiting neurological signs. Lymphohistiocytic perivascular cuffing is indicated by arrows. (**B**) Detection of pathogens associated with neurological signs using RT-qPCR or qPCR. PRV, pseudorabies virus. CAEV, caprine arthritis encephalitis virus. (**C** and **D**) BHK-21 cells were inoculated with supernatants from cytopathic effect–positive cells and incubated for 36 hours. Immunofluorescence staining was used to detect the JEV E protein (C). Western blotting was used to examine the presence of the JEV NS1 protein (D). (**E**) Growth kinetics of the SH2201 strain in BHK-21 cells. Data are presented as means ± SDs from three independent biological replicates. DAPI, 4′,6-diamidino-2-phenylindole.

BHK-21 cells, determined by the median tissue culture infective dose ($TCID_{50}$) assay, revealed peak viral titers in supernatants at 48 hours post-infection; the maximum titer was 1.4×10^6 $TCID_{50}$/ml (Fig. 1E).

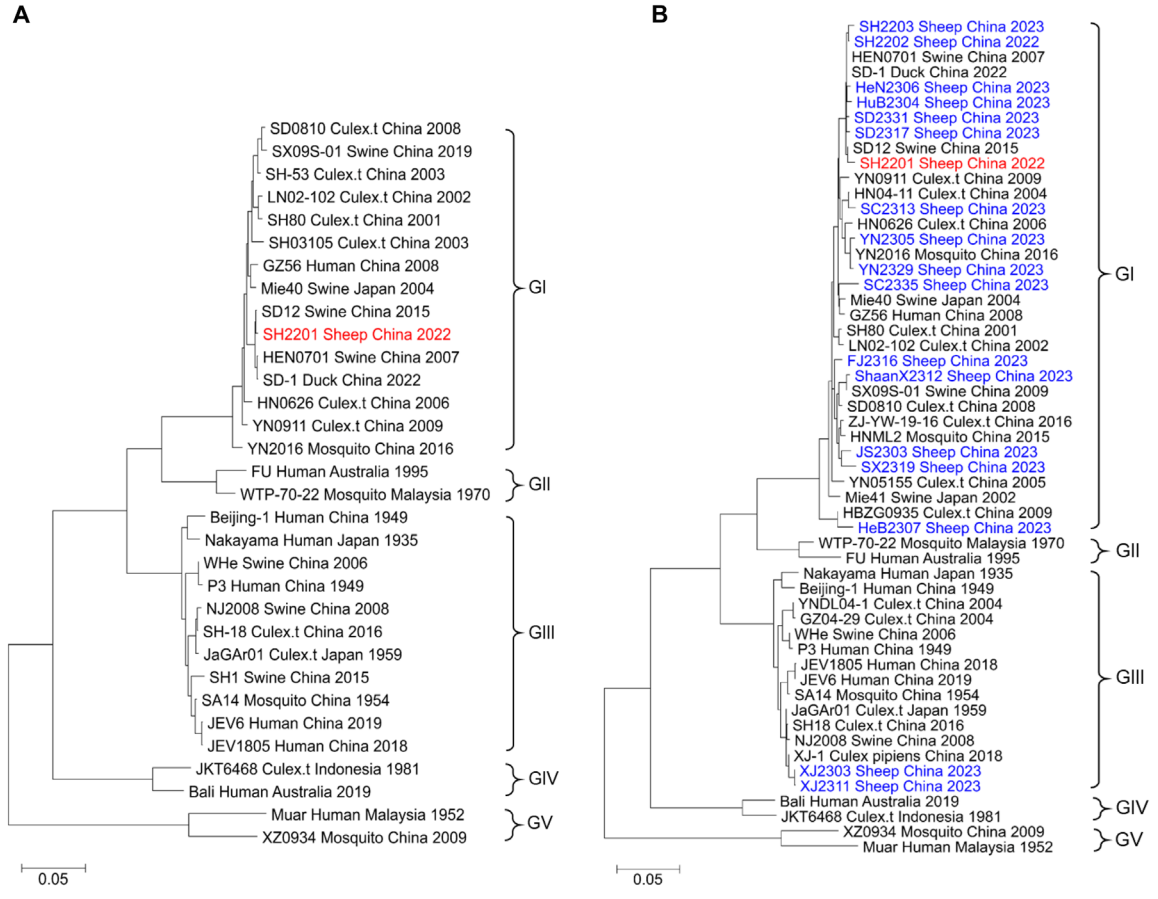
Molecular characterization and phylogenetic analysis of JEV

The complete genome of the SH2201 strain was obtained through whole genome sequencing and has been deposited in the National Center for Biotechnology Information (NCBI) database (GenBank accession number PQ488563). The full-length JEV E gene from the SH2202 strain was also sequenced and deposited in the NCBI database (GenBank accession number PQ488580). Multiple sequence alignments and phylogenetic analyses of the complete genome and the E gene using representative JEV strains (table S1) revealed that the SH2201 and SH2202 strains belong to GI of JEV (Fig. 2, A and B). The complete genome of the SH2201 strain exhibited 99.8% nucleotide sequence identity with the SD12 strain, a local strain isolated from pigs in China (*7*). In addition, sequence alignments of other viral genes (C, prM, NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5) from the SH2201 strain against the reference JEV strains were performed and showed high nucleotide sequence identity (99.6 to 100%) with local strains isolated in China (figs. S1 to S9).

JEV E protein is a major structural protein involved in virulence, pathogenesis, binding to cellular receptors, and the induction of protective immunity against JEV (*20*–*22*). Basic Local Alignment Search Tool (BLAST) analysis of the E gene nucleotide sequence from the SH2201 strain revealed high nucleotide sequence identity (99.3 to 99.5%) with the virulent JEV strains isolated in China, such as SD12 (*7*) and HEN0701 (*23*). Furthermore, no amino acid variations were identified at positions E47, E107, E123, E129, E138, E176, E222, E244, E264, E279, and E315 associated with JEV virulence (*23*–*27*) and between the SH2201 strain and other virulent JEV strains (Fig. 2C). These findings suggest that the viral sequence of SH2201 strain had characteristics in line with other virulent JEV isolates.

Neuroinvasiveness and neurovirulence of the SH2201 strain in mice

Mice are a suitable model for evaluating JEV virulence, which comprises neuroinvasiveness and neurovirulence (*28*). Neuroinvasiveness refers to



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JEV strain	Genotype	Phonotype	E47	E107	E123	E129	E138	E176	E222	E244	E264	E279	E315
SH2201	GI	Virulent	N	L	S	M	E	I	S	E	Q	K	A
SD12	GI	Virulent	N	L	S	M	E	I	S	E	Q	K	A
HEN0701	GI	Virulent	N	L	S	M	E	I	S	E	Q	K	A
SA14	GIII	Virulent	N	L	S	T	E	I	A	E	Q	K	A
NJ2008	GIII	Virulent	N	L	R	T	E	I	A	E	Q	K	A
SD12-F120	GI	Attenuated	N	L	S	M	R	T	S	E	Q	K	A
10S3	GI	Attenuated	N	L	S	M	R	I	S	E	Q	K	A
SA14-14-2	GIII	Attenuated	N	F	S	T	K	V	A	G	H	M	V

Fig. 2. Phylogenetic analysis of JEV strains. Phylogenetic trees were inferred based on nucleotide sequences using the maximum likelihood method. (**A**) Phylogenetic analyses of the full-length genome of JEV. (**B**) Phylogenetic analyses of JEV E gene. The SH2201 strain is highlighted in red, and other strains from sheep are highlighted in blue. (**C**) Comparison of amino acid sites of protein E between JEV strains.

the ability of JEV to enter the central nervous system after intraperitoneal inoculation, whereas neurovirulence reflects its ability to replicate and cause damage within the central nervous system after intracerebral inoculation (*29*). To assess these properties, we subjected mice to intracerebral or intraperitoneal inoculation with the SH2201 strain at doses ranging from 10^0 to 10^4 plaque-forming units (PFU). Mice inoculated via the intracerebral route, even at the lowest dose of 10^0 PFU, developed clinical symptoms of JEV infection with a mortality rate of 25.0%. Mortality rates increased to 62.5, 100.0, 100.0, and 100.0% for intracerebral doses of 10^1 to 10^4 PFU, respectively (Fig. 3A). The 50% lethal dose (LD_{50}) for neurovirulence was calculated as $10^{0.61}$ PFU. Mice inoculated via the intraperitoneal route began to show clinical symptoms with a dose of 10^1 PFU; the mortality rate was 50.0%. Mortality rates for intraperitoneal doses of 10^2 to 10^4 PFU were 62.5, 100.05, and 100.0%, respectively (Fig. 3B). The LD_{50} for neuroinvasiveness was calculated as $10^{1.35}$ PFU. These LD_{50} values for the SH2201 strain were similar to those of known virulent JEV strains (table S2) (*7*, *21*, *30*–*34*). Histopathological analysis of brain tissue from euthanized SH2201-infected mice revealed JEV-induced encephalitis lesions, such as lymphohistiocytic perivascular cuffing and lymphohistiocytic meningitis (Fig. 3, C and D).

The JEV E protein is considered the primary determinant of virulence (*23*, *30*). When the amino acid sequence of the SH2201 E protein

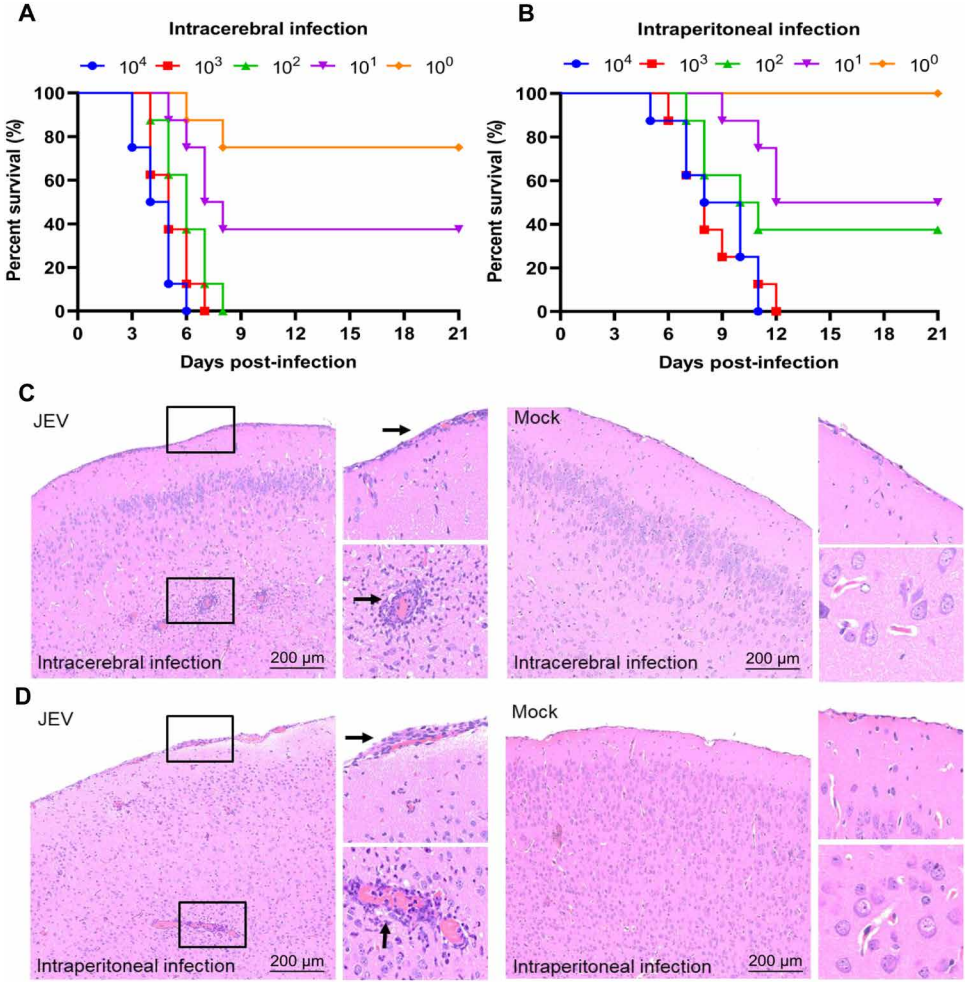

Fig. 3. Virulence of the SH2201 strain in mice. Mice ($n = 8$) were inoculated with the SH2201 strain at doses ranging from 10^0 to 10^4 PFU and then monitored for 21 days. (**A**) Survival curves of mice inoculated via the intracerebral route. (**B**) Survival curves of mice inoculated via the intraperitoneal route. (**C**) Histopathological examination of brain tissue from mice inoculated via the intracerebral route. (**D**) Histopathological examination of brain tissue from mice inoculated via the intraperitoneal route. Lymphohistiocytic perivascular cuffing and lymphohistiocytic meningitis are indicated by arrows.

was aligned with the amino acid sequences of E proteins from virulent and attenuated JEV strains, the residues responsible for virulence—particularly residues 107, 138, and 176 (*21*, *23*, *26*)—were identical to those found in virulent strains (Fig. 2C). Together, these data indicate that the SH2201 strain is virulent, exhibiting neurovirulence and neuroinvasiveness comparable to known virulent JEV strains.

Pathogenicity of the SH2201 strain in sheep

To assess the pathogenicity of the SH2201 strain in sheep, 50-day-old sheep were subjected to intravenous or subcutaneous infection. Mock-infected sheep remained asymptomatic throughout the study. In contrast, sheep in both intravenous and subcutaneous infection groups developed mild, nonspecific clinical signs of varying severity (e.g., reduced appetite and activity, conjunctival redness, moaning, restlessness, and impatience) (Fig. 4, A and B). In the intravenous infection group, three of five sheep developed fever beginning at 2 days post-infection (dpi), with rectal temperatures reaching 40.2° to 40.6°C and persisting for 2 to 3 days (Fig. 4C). One sheep in this group displayed neurological symptoms and was euthanized at 3 dpi (Fig. 4D). Histopathological analysis of its brain revealed JEV-induced encephalitis lesions, such as lymphohistiocytic perivascular cuffing (Fig. 4E). JEV RNA was detected by reverse transcription quantitative polymerase chain reaction (RT-qPCR) in the brain (2.0×10^4 RNA copies/ml), heart (6.6×10^3 RNA copies/ml), and lung (3.2×10^2 RNA copies/ml) of this sheep (Fig. 4F). In the subcutaneous infection group, three of five sheep showed increased rectal temperature beginning at 4 dpi, with peak temperatures reaching 40.2° to 40.4°C and persisting for 2 days (Fig. 4C). No deaths were observed during the 21-day monitoring period (Fig 4D). Overall, these data confirmed the pathogenicity of the SH2201 strain in sheep.

Sheep develop sufficient viremia to infect mosquitoes

Upon infection with JEV, pigs develop high levels of viremia that enable them to infect mosquitoes; thus, they play a crucial role in the JEV transmission cycle (*13*). Here, we sought to determine the levels of viremia in JEV-infected sheep. In the subcutaneous infection group, sheep exhibited viremia from 5 to 9 dpi; the peak level was 3.2×10^4 $TCID_{50}$/ml at 6 dpi (Fig. 5A). This finding was corroborated

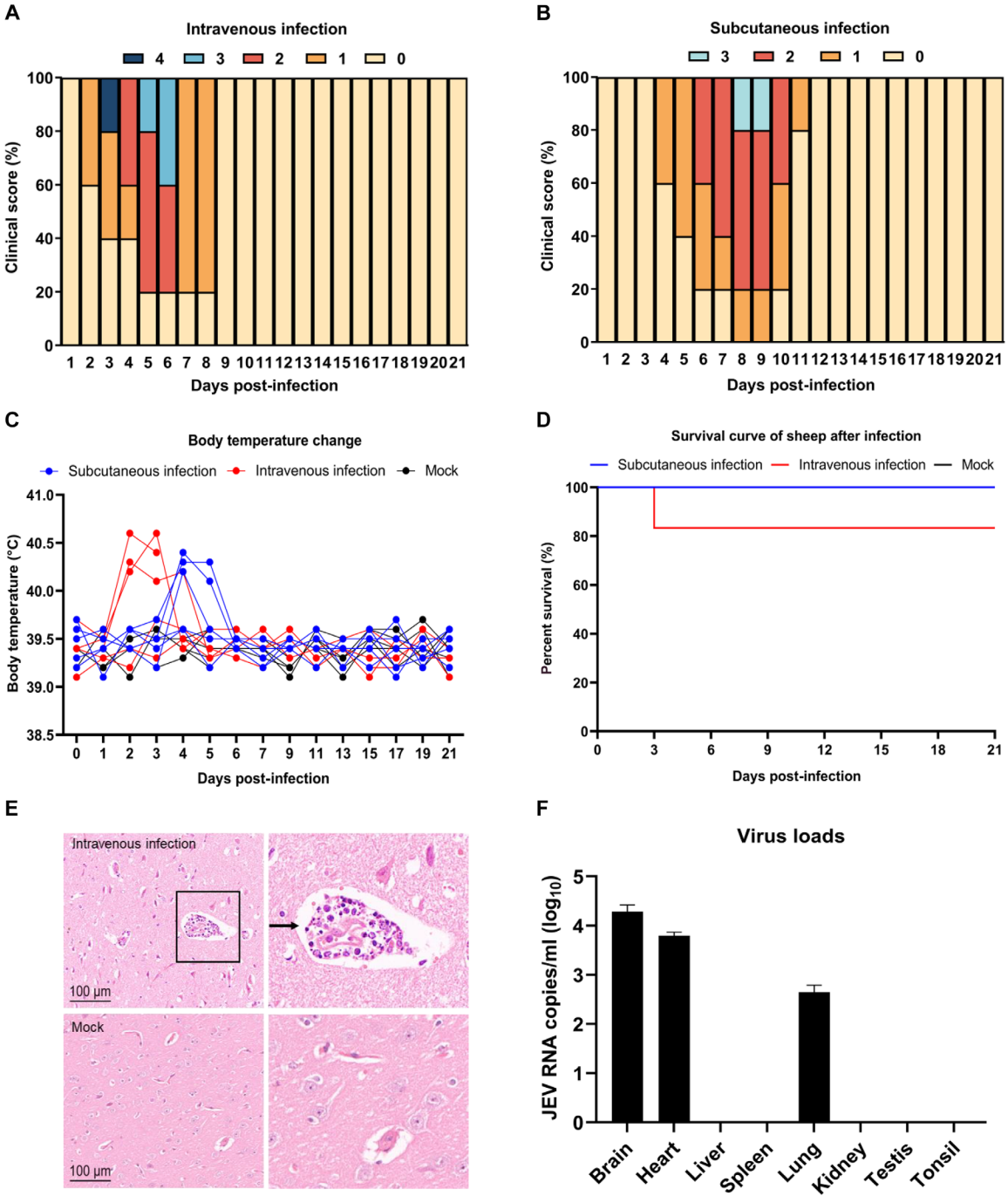

Fig. 4. Pathogenicity of the SH2201 strain in sheep. Fifty-day-old Hu sheep ($n = 5$) were infected with the SH2201 strain at a dose of 10^6 PFU and then monitored for 21 days. Clinical signs were assessed using a clinical scoring system: 0, no restriction in movement, no obvious clinical signs, and normal breathing; 1, reduced appetite, slow movement, and facial flushing; 2, slow movement, conjunctival redness, and shortness of breath; 3, moaning, restlessness, and apparent neurological symptoms (e.g., spinning and difficulty walking); 4, apparent neurological symptoms such as muscle spasms, followed by death. (**A**) Clinical scores of sheep inoculated via the intravenous route. (**B**) Clinical scores of sheep inoculated via the subcutaneous route. (**C**) Changes in rectal temperature. (**D**) Survival curves of sheep inoculated via intravenous and subcutaneous routes. (**E**) Histopathological examination of brain tissue from euthanized sheep. Lymphohistiocytic perivascular cuffing is indicated by arrows. (**F**) Viral loads in tissues collected from euthanized sheep, as measured by RT-qPCR. Data are presented as means ± SDs from three independent biological replicates.

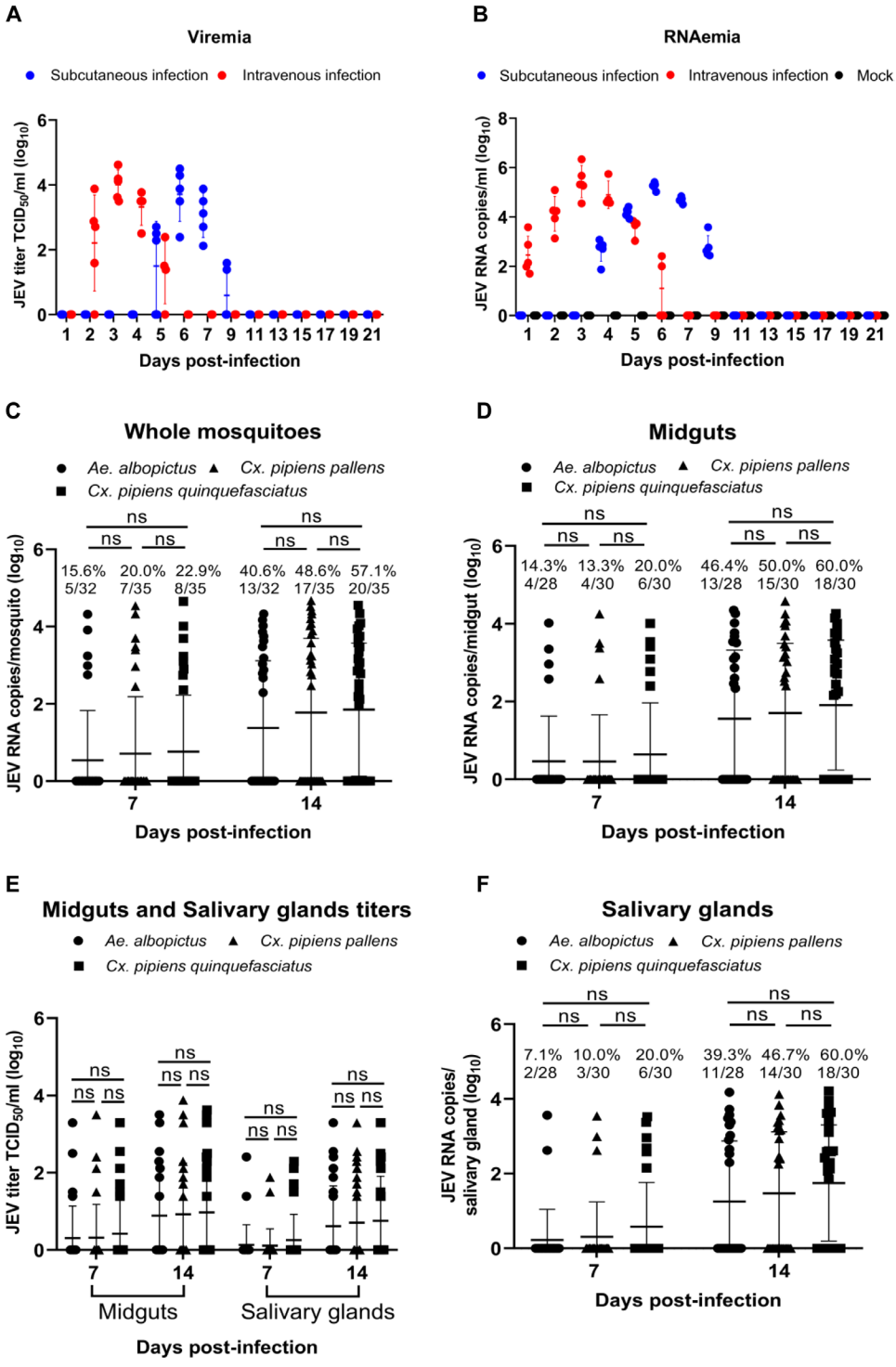

Fig. 5. Viremia levels in sheep infected with the SH2201 strain. Fifty-day-old Hu sheep ($n = 5$) were infected with the SH2201 strain at a dose of 10^6 PFU via subcutaneous and intravenous routes, respectively, and then monitored for 21 days. Blood samples were collected daily to detect viremia. (**A**) Viremia levels measured by $TCID_{50}$ assay. (**B**) RNAemia levels measured by RT-qPCR. (**C** to **F**) *Ae. albopictus*, *Cx. pipiens pallens*, and *Cx. pipiens quinquefasciatus* were fed viremic blood meal collected from SH2201-infected sheep. Engorged mosquitoes were randomly collected at 7 and 14 dpi to measure infection rates and viral loads. JEV NS1 gene copies in whole mosquitoes (C), midguts (D), and salivary glands (F) were measured by RT-qPCR. Viral loads in midguts and salivary glands were determined by $TCID_{50}$ assays (E). Two-tailed Student's *t* test was performed for comparison of variables. Data are presented as means ± SDs.

by the detection of RNAemia (via RT-qPCR), which was present from 4 to 9 dpi; the peak level was 2.6×10^5 RNA copies/ml at 6 dpi (Fig. 5B). In the intravenous infection group, sheep exhibited viremia from 2 to 5 dpi; the peak level was 4.2×10^4 $TCID_{50}$/ml at 3 dpi (Fig. 5A). This finding was also corroborated by the detection of RNAemia, which was present from 1 to 6 dpi; the peak level was 2.2×10^6 RNA copies/ml at 3 dpi (Fig. 5B). Comparison of viremia between sheep and other amplifying hosts revealed that the levels and duration in JEV-infected sheep were similar to those observed in JEV-infected pigs (Table 1) (*35*, *36*), suggesting that sheep can serve as an amplifying host for JEV and might contribute to its transmission.

To determine whether the levels of viremia in sheep were sufficient to infect mosquitoes, one species of *Aedes* (*Ae.*) mosquito (*Ae. albopictus*) and two species of *Cx.* mosquitoes (*Cx. pipiens pallens* and *Cx. pipiens quinquefasciatus*), which are common in China and recognized as competent JEV vectors (*37*, *38*), were fed on the viremic blood meal collected from the SH2201-infected sheep; they were subsequently subjected to measurements of infection rate and viral load. Among 32 *Ae. albopictus* collected at 7 and 14 dpi, 5 and 13 showed JEV positivity, yielding respective infection rates of 15.6 and 40.6% (Fig. 5C). Of 35 *Cx. pipiens pallens* and *Cx. pipiens quinquefasciatus* that were collected at 7 and 14 dpi, respectively, 7 and 17 *Cx. pipiens pallens* showed JEV positivity, indicating respective infection rates of 20.0 and 48.6% (Fig. 5C). Similarly, 8 and 20 *Cx. pipiens quinquefasciatus* showed JEV positivity, with infection rates of 22.9 and 57.1%, respectively (Fig. 5C). JEV infection in the mosquito midgut was further evaluated by RT-qPCR (Fig. 5D) and $TCID_{50}$ assays (Fig. 5E). Among 28 *Ae. albopictus* collected at 7 and 14 dpi, 4 and 13 showed JEV positivity in the midgut; the respective infection rates were 14.3 and 46.4% (Fig. 5D). Of 30 *Cx. pipiens pallens* and *Cx. pipiens quinquefasciatus* collected at 14 dpi, 15 and 18 showed JEV positivity in the midgut, and the respective infection rates were 50.0% and 60.0% (Fig. 5D). The midgut virus titers of the three species of mosquitoes at 14 dpi ranged from 2.5×10^1 to 3.2×10^3 $TCID_{50}$/ml for Ae. *albopictus*, 2.5×10^1 to 7.6×10^3 $TCID_{50}$/ml for *Cx. pipiens pallens*, and 2.5×10^1 to 4.2×10^3 $TCID_{50}$/ml for *Cx. pipiens quinquefasciatus* (Fig. 5E).

Considering the crucial role of saliva in JEV transmission (*38*–*40*), viral loads in mosquito salivary glands were also determined by RT-qPCR (Fig. 5F) and $TCID_{50}$ assays (Fig. 5E). At 7 dpi, two (7.1%) *Ae. albopictus*, three (10.0%) *Cx. pipiens pallens*, and six (20.0%) *Cx. pipiens quinquefasciatus* showed JEV positivity in their salivary glands. These numbers increased to 11 (39.3%), 14 (46.7%), and 18 (60.0%) at 14 dpi (Fig. 5F). No notable differences in viral loads were observed between mosquito species with respect to whole mosquitoes, midguts, or salivary glands. Collectively, these data demonstrate that JEV-infected sheep develop sufficient viremia to infect mosquitoes.

Detection of virus shedding in JEV-infected sheep

Vector-free transmission of JEV has been experimentally observed in pigs, suggesting an alternative transmission route that does not involve arthropod vectors (*41*). Therefore, we examined virus shedding in sheep infected with the SH2201 strain of JEV. Nasal and anal swabs were collected from sheep in the intravenous and subcutaneous infection groups and then analyzed by RT-qPCR to detect virus shedding. In the subcutaneous infection group, virus shedding was observed from 3 to 7 dpi according to nasal swabs; the peak level was 1.1×10^4 RNA copies/ml at 5 dpi (Fig. 6A). According to anal swabs, virus shedding was observed from 4 to 11 dpi; the peak level was 3.8×10^3 RNA copies/ml at 6 dpi (Fig. 6B). Similarly, virus shedding was detected in the intravenous infection group; JEV was evident from 2 to 7 dpi in both nasal and anal swabs (Fig. 6, A and B). The relative levels of infectious virus shed by the infected sheep were calculated using RT-qPCR data and are presented in fig. S11 (A to C). These findings suggest that sheep can shed the virus into the environment, potentially serving as a relevant source of JEV transmission.

JEV can persist in the tonsils of pigs for at least 25 days (*41*). To investigate whether JEV persists in sheep, we collected tissue samples from both intravenous and subcutaneous infection groups at the end of the 21-day experiment. JEV RNA was detected by RT-qPCR only in the tonsils; the peak levels were 7.1×10^4 and 2.5×10^4 RNA copies/ml in the intravenous and subcutaneous infection groups, respectively (Fig. 6C). Further analysis by $TCID_{50}$ assays confirmed the presence of JEV in the tonsil of one sheep from the subcutaneous infection group, with a titer of 3.2×10^3 $TCID_{50}$/ml (Fig. 6D). These results indicate that JEV can persist in sheep tonsils for at least 21 days, mirroring its persistence in pig tonsils (*41*).

Table 1. Levels and duration of viremia in different hosts.

Host	Stains	Infection route	Viremia		RNAemia		Reference
			Virus titration ($TCID_{50}$/ml)	Duration (days)	RNA quantity (copies/ml)	Duration (days)	
Sheep	SH2201	Subcutaneous	2.5×10^1–3.2×10^4	3	7.4×10^1–2.6×10^5	6	This study
		Intravenous	2.5×10^1–4.2×10^4	1–4	5.0×10^1–2.2×10^6	3–6	
Pig	Sw/Mie/40/2004	Subcutaneous	1.8×10^2–1.4×10^5* (1.26×10^2–1.0×10^5 PFU/ml)	4	/	/	(*35*)
	Nakayama	Intradermally	3.2×10^4–3.2×10^5	2–4	/	/	(*41*)
Domestic duck	SD12, SH7, SH15	Subcutaneous	3.2×10^2–3.2×10^6	3–4	/	/	(*69*)
Wild birds	JE-IN, JE-VN	Subcutaneous	7.2×10^1–3.6×10^5 * ($1 \times 10^{1.7}$–$1 \times 10^{5.4}$ PFU/ml)	1–7	/	/	(*14*)

*$TCID_{50}$/ml values were calculated on the basis of PFU/ml values.

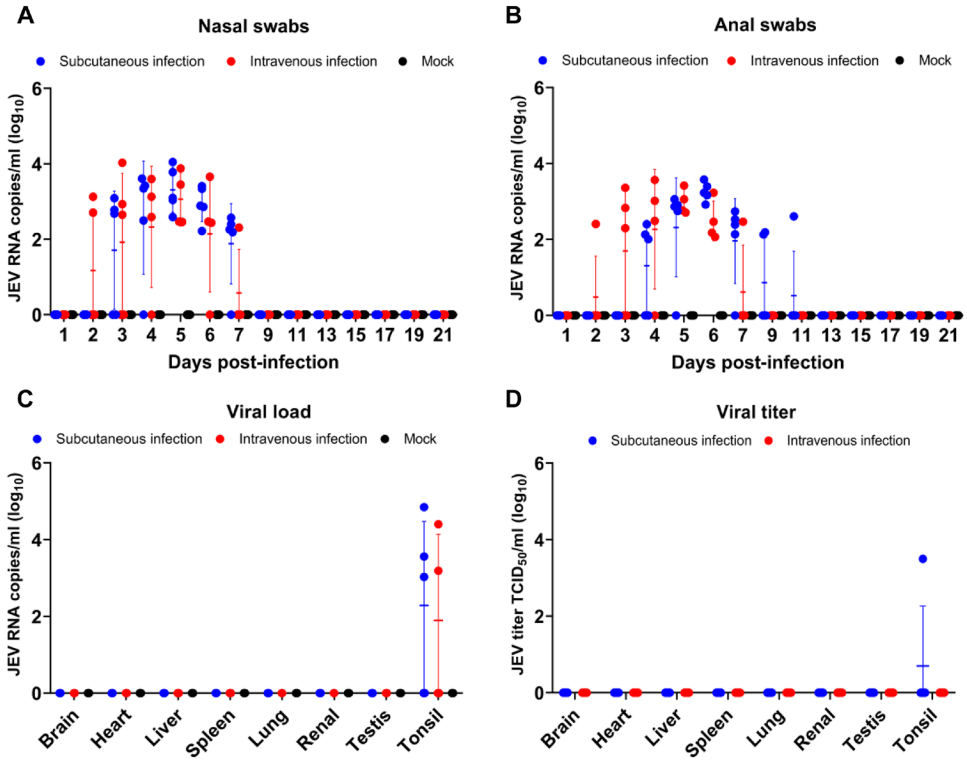

Fig. 6. Detection of virus shedding in JEV-infected sheep. Fifty-day-old Hu sheep (n = 5) were infected with the SH2201 strain at a dose of 10^6 PFU via subcutaneous and intravenous routes, respectively, then monitored for 21 days. Nasal and anal swabs were collected daily to detect virus shedding by RT-qPCR. (**A**) Viral RNA detected in nasal swabs. (**B**) Viral RNA detected in anal swabs. (**C** and **D**) Tissue samples were collected at the end of the experiment (21 dpi) to detect viral loads by RT-qPCR (C) and $TCID_{50}$ assays (D). Data are presented as means ± SDs.

Survey of JEV prevalence in field sheep

In total, 3349 serum samples were collected from sheep farms in 31 provinces across China (table S4) and subjected to JEV testing by RT-qPCR. Among the 31 provinces, 19 showed JEV positivity, with detection rates ranging from 0.7 to 4.6% (Fig. 7A and table S4). Varying levels of RNAemia were detected in JEV-positive serum samples, ranging from 2.3×10^2 to 2.0×10^5 RNA copies/ml (Fig. 7B and tables S3 and S4). The levels of infectious virus in JEV-positive serum samples were further estimated on the basis of the RNAemia values using the standard curve (fig. S11) and shown in table S4. Most samples exhibited RNAemia levels similar to those observed in SH2201-infected sheep. These findings suggest that JEV is prevalent in the surveyed sheep, with sufficient viremia for mosquito infection.

Sixteen full-length JEV E gene sequences were amplified from 48 JEV-positive serum samples (table S4) and deposited in the NCBI database. Detailed information, including GenBank accession number, is provided in table S4. Phylogenetic analysis of these sequences revealed that most JEV strains belonged to GI, although two strains from Xinjiang were classified as GIII (Fig. 2B and table S4). All strains were genetically related to local JEV strains from other hosts. For example, the SH2202 and SH2303 strains from Shanghai were closely related to the HEN0701 strain isolated from pigs (*23*), with 99.6 to 99.9% homology. The YN2305 and YN2329 strains from Yunnan shared 99.7% homology with the YN2016 strain isolated from mosquitoes in Yunnan (*42*). These results indicated that the JEV strains in sheep are primarily related to the strains circulating in nearby areas.

A total of 3349 serum samples were collected from sheep farms in 31 provinces across China (table S5) and tested for JEV seropositivity using the 50% plaque reduction neutralization test ($PRNT_{50}$). Among the 31 provinces, 20 showed JEV seropositivity, with rates ranging from 2.0 to 26.7% (Fig. 7C and table S5). Neutralizing antibody titers in JEV-positive serum samples varied from 1:20 to 1:160 (table S5). These results suggest that JEV is prevalent in the investigated sheep populations.

DISCUSSION

JEV, a leading cause of viral encephalitis in humans, continues to spread throughout South, East, and Southeast Asia, as well as Australasia (*43*, *44*). Although the JEV transmission cycle (with pigs and wild birds as amplifying hosts and mosquitoes as vectors) was elucidated in the 1950s (*13*), the factors contributing to its transmission, geographic emergence, and spread are not fully understood. This lack of understanding is at least partially due to the wide range of mammals that exhibit viremia and/or seroconversion after JEV infection (*43*). In this study, we isolated a JEV strain, SH2201, from sheep displaying neurological symptoms similar to those of JE. We characterized the viremia and pathogenicity of SH2201 in sheep, revealing that they are susceptible to JEV infection and develop sufficient viremia to infect mosquitoes. These findings suggest that, in addition to pigs, sheep can act as amplifying hosts for JEV and contribute to its transmission cycle.

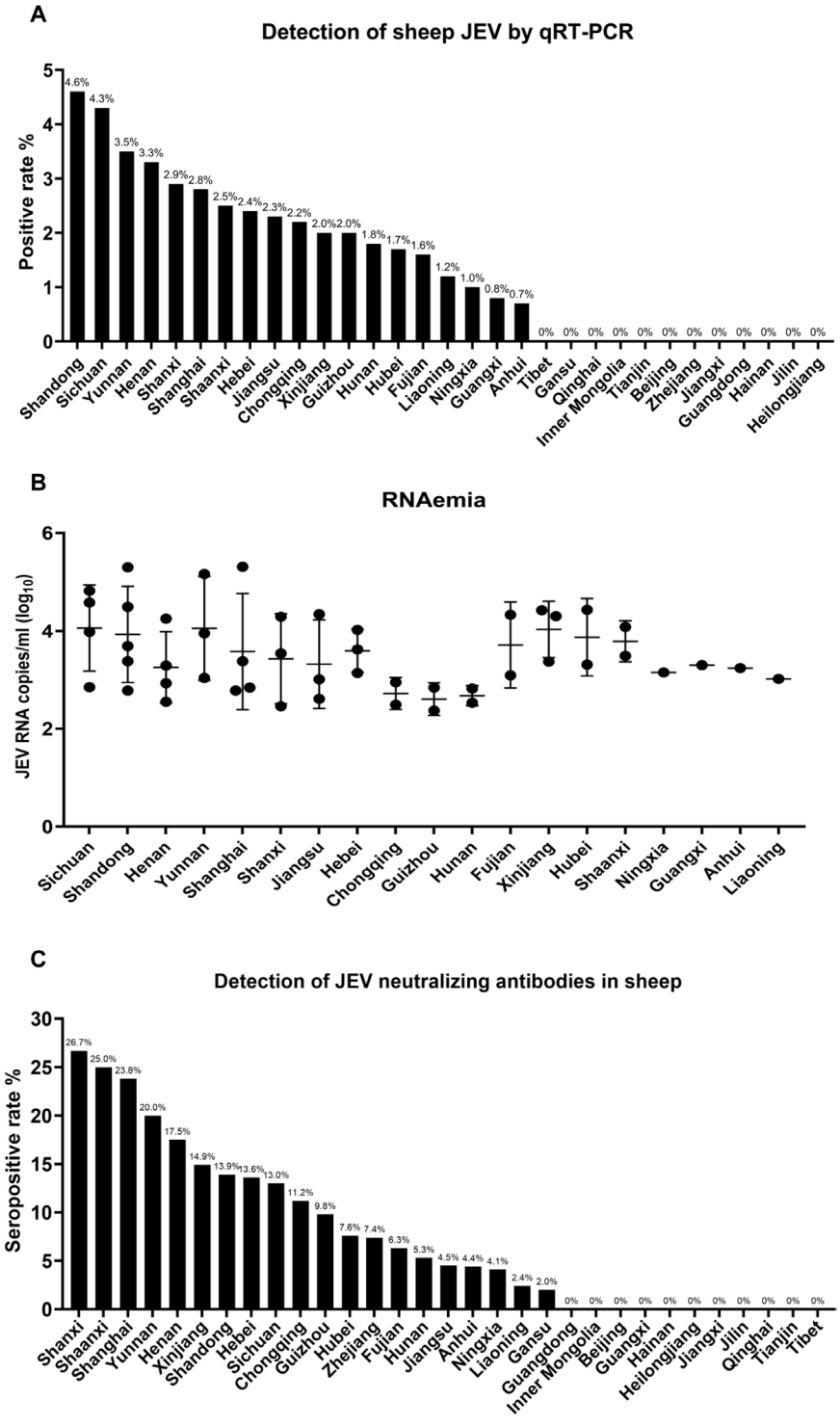

Fig. 7. Prevalence of JEV in field sheep. A total of 3349 serum samples were collected from sheep farms located in 31 provinces in China to detect the presence of JEV and seropositivity rate by RT-qPCR and $PRNT_{50}$. (**A**) Positivity rates of JEV. (**B**) RNAemia levels. (**C**) Seropositivity rates of JEV. Data are presented as means ± SDs.

To our knowledge, the SH2201 strain is the first JEV strain isolated from sheep. It belongs to GI and is most closely related to the local SD12 strain, which was isolated from an aborted swine fetus in China (*7*). Analyses in mice revealed that SH2201 exhibits virulence at levels similar to those of the SD12 strain (*30*).

Despite high seroprevalence rates of JEV infection in numerous mammal and bird species (*13*), clinical symptoms primarily develop in humans, horses, and pigs, with occasional occurrences in other mammals and birds (*2*, *45*). Neurological symptoms resembling JEV infection have been sporadically observed in sheep on farms in China (*16*), but further diagnostic testing has not been conducted. In addition, JEV-induced neurological symptoms may be misdiagnosed or ignored in the field. Therefore, it is difficult to determine the frequency of JEV-induced neurological signs on sheep farms. Our analysis of SH2201 pathogenicity in sheep revealed that all sheep infected with SH2201 at a dose of 10^6 PFU that was similar to the doses used for experimental infection of pigs (*41*, *46*) developed high levels of viremia. Most displayed mild, nonspecific clinical signs (e.g., fever, reduced appetite, and decreased activity), similar to the signs present in experimentally infected pigs (*36*). In addition, brain samples from a euthanized SH2201-infected sheep showed JEV-induced encephalitis lesions, such as lymphohistiocytic perivascular cuffing, which are frequently observed in the brains of JEV-infected humans and pigs (*46*). These findings demonstrate that the SH2201 strain is pathogenic to sheep, highlighting the potential impact of JEV infection on the sheep industry.

Pigs and ardeid wading birds are recognized as JEV-amplifying hosts due to their high levels of viremia after infection (*13*). Notably, we observed high levels of viremia in SH2201-infected sheep, with peak levels reaching 3.2×10^4 to 4.2×10^4 $TCID_{50}$/ml in the subcutaneous and intravenous infection groups; we also observed high levels of RNAemia in serum samples collected from field sheep (peak level: 2.0×10^5 RNA copies/ml). These levels of viremia and RNAemia in JEV-infected sheep were similar to the levels observed in JEV-infected pigs (*36*). In addition, JEV loads detected in the brain (2.0×10^4 RNA copies/ml) and tonsils (3.2×10^3 $TCID_{50}$/ml) of SH2201-infected sheep were comparable to the viral loads observed in JEV-infected pigs (*36*). These data suggest that, similar to pigs, sheep can act as mammalian amplifying hosts for JEV.

To determine whether viremia levels in JEV-infected sheep were sufficient to infect mosquitoes, we utilized *Ae. Albopictus*, *Cx. pipiens pallens*, and *Cx. pipiens quinquefasciatus* in this study. These mosquito species are common in China and are competent JEV vectors capable of transmission (*47*). For example, *Ae. albopictus* mosquitoes can be infected by feeding on viremic blood with a titer of $10^{3.5}$ $TCID_{50}$ per mosquito (*48*) or on viremic chickens displaying titers of $10^{3.7}$ to $10^{4.7}$ PFU/ml (*49*). In the present study, *Ae. Albopictus*, *Cx. pipiens pallens*, and *Cx. pipiens quinquefasciatus* fed viremic blood collected from SH2201-infected sheep exhibited infection rates of 40.6% (13 of 32), 48.6% (17 of 35), and 57.1% (20 of 35) at 14 dpi, respectively, indicating that viremia levels in infected sheep were sufficient for mosquito infection. Furthermore, 39.3% (11 of 28) of *Ae. Albopictus*, 46.7% (14 of 30) of *Cx. pipiens pallens*, and 60.0% (18 of 30) of *Cx. pipiens quinquefasciatus* showed JEV positivity in their salivary glands. Noticeably, the value of RNAemia in the pooled blood meal for experimental infection of mosquitoes was 6.9×10^4 RNA copies/ml that was in the range of 2.3×10^2 to 2.0×10^5 RNA copies/ml detected in the field sheep, suggesting that a high level of viremia developed in field sheep with natural infection of JEV would be sufficient to infect mosquitoes in nature. These findings suggest that JEV-infected sheep can contribute to JEV transmission; along with pigs, sheep may be another important mammalian species involved in the JEV transmission cycle.

In addition to vector-mediated spread, JEV can be transmitted via the oronasal route in pigs (*41*), monkeys (*50*), and mice (*51*). In experimental pigs, a dose as low as 10 $TCID_{50}$ per animal is sufficient for widespread infection (*41*). Analysis of virus shedding in nasal and anal secretions of SH2201-infected sheep revealed high levels of JEV shedding in both sample types, which persisted for 6 to 8 days. These results suggest that sheep can shed the virus into their surroundings, potentially acting as an important source of JEV transmission.

As a zoonotic pathogen, JEV displays two main transmission cycles: a pig-associated rural domestic cycle involving pigs as amplifying hosts and a bird-associated wild cycle involving wading birds as amplifying hosts (*43*). These cycles can exist together or in isolation, depending on ecological factors present in endemic areas. Our study demonstrated that sheep develop viremia levels and durations similar to those observed in pigs, a known amplifying host (*36*). Furthermore, 40.6 to 57.1% of mosquitoes became infected after feeding on viremic blood from SH2201-infected sheep. A survey of JEV prevalence revealed that strains isolated from field sheep share high homology with local JEV strains from other hosts, suggesting transmission between sheep and other species. On the basis of these findings, we propose an alternative, sheep-associated rural domestic cycle for JEV transmission. This cycle may be prevalent in specific ecological niches where sheep are bred but pigs are not. JEV has been detected in mosquitoes (*52*) and sheep (this study) in Kashi, Xinjiang, China, an area with sheep breeding but not pig breeding. This sheep-associated cycle could coexist with the pig-associated and bird-associated cycles in regions where sheep, pigs, and birds cohabitate. However, further validation of this proposed cycle is needed through epidemiological, virological, and ecological investigations.

Serological survey of JEV prevalence in field sheep revealed that among the 31 provinces, 20 showed JEV seropositivity, with rates ranging from 2.0 to 26.7%. It is known that flavivirus diagnostics are complicated by substantial cross-reactivity of antibodies between different flavivirus species (*53*, *54*). Several flavivirus species—such as West Nile virus, dengue virus, and Zika virus—have been detected serologically and/or virologically in certain regions of China (*55*–*57*). It cannot exclude a positivity that sheep might be susceptible to a species of these flaviviruses and produce antibodies against the infected flavivirus despite no serological or virological evidences of sheep being infected with these flaviviruses in China. Therefore, it should notice a possibility that the seroprevalence data of JEV obtained in the filed sheep in this study might be interfered by potential cross-reactivity of antibodies against other species of flaviviruses. Further serological studies are needed to determine the potential cross-reactivity of antibodies between JEV and other species of flaviviruses in sheep.

Despite these findings, the present study had two limitations. The first limitation involves the methods used for sheep infection, which consisted of intravenous and subcutaneous injection. This artificial approach may not fully replicate natural JEV infection through mosquito bites. A sheep infection model using bites from JEV-infected mosquitoes would provide more accurate and comprehensive insights into the role of sheep in the JEV transmission cycle. However, such a model is currently unavailable due to the lack of

information needed to establish this type of experimental system. For example, it is unclear which mosquito species are competent vectors for the sheep JEV strain and preferentially bite sheep under experimental conditions or what JEV titer in experimentally infected mosquito saliva is sufficient to transmit the virus to sheep and cause infection. Therefore, we used an artificial infection method in this study. This approach has been widely implemented to study JEV infection in other hosts (*7*, *41*, *49*). Our findings showed that viremia levels in sheep infected subcutaneously and intravenously were comparable to those observed in field-collected sheep sera and in other amplifying hosts (*7*, *41*, *49*). These results support the conclusion that sheep can serve as amplifying hosts for JEV.

The second limitation is the methods for infection of mosquitoes, in which the mosquitoes were infected by feeding on the viremic blood meal from JEV-infected sheep, not by biting JEV-viremic sheep, because of a lack of the information on the mosquito species feeding preferentially on sheep. The species of mosquitoes that play major role in maintaining the sheep-associated rural domestic cycle for JEV transmission are unknown at present. Further studies on identification of the mosquito species contributing to the sheep-associated rural domestic cycle are needed.

In conclusion, we isolated the SH2201 strain of JEV from an infected sheep. Experimental infection with the SH2201 strain revealed that sheep are susceptible to JEV; they develop levels and durations of viremia similar to those observed in pigs, suggesting that sheep can act as another mammalian amplifying host for JEV. The use of mosquitoes fed viremic blood from SH2201-infected sheep resulted in 40.6 to 57.1% JEV infection rate, and 39.3 to 60.0% of the mosquitoes showed JEV positivity in their salivary glands. These findings indicate that sheep can serve as an important mammalian species contributing to JEV transmission. Accordingly, we propose an alternative, sheep-associated rural domestic cycle for JEV transmission, which may be prevalent in specific ecological niches where sheep are bred but pigs are not.

MATERIALS AND METHODS

Ethics statement

All animal experiments were approved by the Institutional Animal Care and Use Committee (IACUC) of Shanghai Veterinary Research Institute, China (IACUC no: Shvri-20230113-02 and Shvri-20230414-05) and conducted in accordance with the Guidelines on the Humane Treatment of Laboratory Animals (policy no. 398 of the Ministry of Science and Technology of the People's Republic of China, 2006).

Sample collection and detection

In August 2022, a sheep farm in Shanghai, China, experienced a severe disease outbreak among approximately 1-month-old Hu sheep. Eleven of 200 sheep exhibited neurological signs resembling JE, the morbidity rate was 5.5% (11 of 200). Brain tissue samples were collected from two euthanized sheep that showed significant neurological symptoms for routine diagnosis using RT-qPCR or quantitative PCR with specific primers (*58*, *59*) (table S6). Serum samples were collected from sheep farms in 31 provinces across China between July and August 2023 by the China Animal Health and Epidemiology Center (table S3). These samples were tested for JEV using RT-qPCR. Briefly, total RNA was extracted from the samples using TRIzol reagent (Thermo Fisher Scientific, USA, catalog no. 10296010CN) and subsequently subjected to RT-qPCR analysis using a CFX96 Real-Time System (Bio-Rad). The primers targeting JEV NS1 gene were designed using SnapGene software (version 6.0.2, SnapGene, USA) (table S6). To generate JEV standard curve, RNA was in vitro transcribed from a plasmid harboring JEV NS1 gene using MegaScript T7 transcription kit (Thermo Fisher Scientific, USA, catalog no.AM1333) and quantified using a NanoDrop (Thermo Fisher Scientific) to estimate copy number. The quantified RNA was diluted in 10-fold serial dilutions and used for generation of linear regression equation. The resulting linear regression equation for JEV standard curve was $y = -3.168X + 40.86$ ($R^2 = 0.999$) (fig. S10A); this equation was used to quantify genomic copies of JEV in the samples. The estimated detection limit was 10 RNA copies, with a threshold (*Ct*) of 38 (fig. S10B). In addition, RT-qPCR results were negative for other sheep pathogens, including BTV, PRV, FMDV, and PPRV (fig. S10C).

Detection of JEV seropositivity rate in sheep

In total, 3349 serum samples were collected from sheep farms in 31 provinces across China (table S5) between July and August 2023 to detect neutralizing antibodies against JEV using the $PRNT_{50}$, as previously described (*27*). Briefly, serum samples were inactivated in a water bath at 56°C for 30 min and then serially diluted twofold, beginning at a dilution of 1:5 and reaching 1:320. The diluted serum samples were mixed with an equal volume of JEV SH2201 strain at a concentration of 200 PFU/0.1 ml and incubated at 37°C for 1 hour. The mixture was then added to BHK-21 cells (obtained from the American Type Culture Collection) grown in six-well plates (Corning, USA, catalog no. 3506) and incubated at 37°C with 5% CO_2 for 2 hours. After the adsorption period, the cells were overlaid with 1.2% methylcellulose (Merck, Germany, catalog no. M0512) in Dulbecco's modified Eagle's medium (DMEM; Gibco Invitrogen, catalog no. 11995065) containing 2% fetal bovine serum (FBS; Gibco Invitrogen, catalog no. 10099141C) and incubated at 37°C for 3 to 5 days until extensive viral plaques appeared. The plaques were stained with 0.5% crystal violet; neutralizing antibody titers and JEV seropositivity rates were calculated. $PRNT_{50}$ titers below 1:10 were considered indicative of neutralizing antibody negativity.

JEV isolation

The supernatants of sheep brain homogenates were filter-sterilized and inoculated onto BHK-21 cells (baby hamster Syrian kidney) grown to 90% confluence in a monolayer in 24-well plates (Corning, USA, catalog no. 3526). After 1 hour of incubation at 37°C, the cells were supplemented with DMEM containing 2% FBS and subsequently cultured at 37°C with 5% CO_2. The cells were monitored daily for cytopathic effects (CPEs). If no CPEs were observed, the cells were harvested at 5 days post-inoculation and subjected to three freeze-thaw cycles. The supernatants were collected by centrifuging the freeze-thawed samples at 4°C and inoculated into BHK-21 cells to observe CPE. This process was repeated three to five times, depending on the sample. If no CPEs were observed after five passages, the samples were considered negative for infectious virus and discarded. If CPEs were observed, the CPE-positive cells were harvested and analyzed for JEV presence using Western blotting with JEV NS1–specific antibodies (GeneTex, USA, catalog no. GTX633820) and immunofluorescence assays with JEV E–specific antibodies (GeneTex, USA, catalog no. GTX125867) (*60*, *61*). The supernatants (passage 5 stock) collected from cells with complete CPE, which tested positive for JEV by Western blotting and immunofluorescence assays, were stored at −80°C and used for JEV

isolation. These samples were also used for Western blotting with NS1-specific antibodies and immunofluorescence assays with E-specific antibodies.

Viral replication kinetics was determined in BHK-21 cells. Briefly, BHK-21 cells were infected with JEV SH2201 at a multiplicity of infection of 0.01 and subsequently cultured in DMEM containing 2% FBS at 37°C with 5% CO_2 after 1-hour incubation. The supernatants of JEV-infected cells were collected at different time points and titrated using $TCID_{50}$ assays on BHK-21 cells (*21*). The E gene nucleotide sequence of the isolated strain was compared with the sequence amplified from the sheep brain sample to confirm the absence of mutations in the isolated strain.

Whole-genome sequencing and assembly of the JEV SH2201 strain

Viral RNA was extracted from the passage 5 stock of the JEV SH2201 strain using Trizol reagent (Thermo Fisher Scientific, USA, catalog no.15596018CN) in accordance with the manufacturer's instructions for whole-genome sequencing. The cDNA synthesis was performed using the SuperScript III First-Strand Synthesis SuperMix kit in according with the manufacturer's protocol (Thermo Fisher Scientific, USA, catalog no.18080400). The resulting cDNA was subsequently subjected to Illumina pair-end sequencing (Tgene Biotech, Shanghai, China). The paired-end library with insert sizes of ~400 base pair was constructed using at least 1 μg of cDNA of JEV SH2201 strain following the Illumina's standard genomic library preparation procedure. Briefly, the cDNA was sheared into smaller fragments with a desired size and the blunt ends were generated by using T4 DNA polymerase. After adding an "A" base to the 3′ end of the blunt phosphorylated cDNA fragments, the adapters were ligated to the ends of the fragments. The desired fragments were purified through gel-electrophoresis and subsequently selectively enriched and amplified by PCR. The index tag was introduced into the adapter at the PCR stage. Following a library quality test, the qualified samples were sequenced to a coverage depth of 100× by the Illumina NovaSeq6000 PE150 sequencer (Illumina, USA). For sequence assembly, the raw paired-end reads were trimmed and quality controlled by Trimmomatic (*62*) with parameters (SLIDINGWINDOW:4:15MINLEN:75) (v0.36, http://www.usadellab.org/cms/?page=trimmomatic) to obtain the clean data. The clean reads were assembled using the ABySS v2.2.0 (*63*) with multiple-Kmer parameters (http://www.bcgsc.ca/platform/bioinfo/software/abyss). The remaining local inner gap was filled up and the single base polymorphism was corrected using the GapCloser (*64*) (https://sourceforge.net/projects/soapdenovo2/files/GapCloser/) to finalize the sequence assembly. The obtained whole-genome sequence of JEV SH2201 strain was deposited in the NCBI database and used for phylogenetic analysis.

Phylogenetic analysis

The full-length sequence of the JEV E gene was amplified from sheep sera and brain tissues using RT-PCR with specific primers (table S6), as previously described (*59*). The amplified sequences were sequenced and deposited in the NCBI database. Multiple sequence alignment and phylogenetic analysis of the SH2201 whole-genome nucleotide sequence and E gene nucleotide sequences were performed against reference JEV strains (table S1) using MEGA software (version 11.0.13, Mega Limited, New Zealand). The phylogenetic tree was constructed using the maximum likelihood method, as previously described (*65*–*67*).

Neuroinvasiveness and neurovirulence assays in mice

Three-week-old female C57BL/6 mice (Jiangsu Huachuang Sino Pharmaceutical Technology Co.) ($n = 8$) were intraperitoneally or intracerebrally inoculated with the SH2201 strain at doses ranging from 10^0 to 10^4 PFU; they were subsequently monitored for 21 days to determine neuroinvasiveness and neurovirulence, respectively, as previously described (*21*). Infected mice exhibiting neurological symptoms, such as paralysis or tremors, were euthanized by CO_2 asphyxiation in accordance with the Guidelines for the Humane Treatment of Laboratory Animals (policy no. 2006398).

Sheep infection

Infection experiments were conducted in 50-day-old, average body weight of 13.12 ± 0.75 kg, clinically healthy Hu sheep with confirmed negativity for JEV, *Brucella*, pseudorabies virus, caprine arthritis encephalitis virus, and JEV antibodies. Sheep ($n = 5$) were intravenously or subcutaneously inoculated with 10^6 PFU of the SH2201 strain prepared in BHK-21 cells as described previously (*41*, *46*). Clinical signs and rectal temperatures were monitored daily for 21 days and scored accordingly (table S7) (*68*). Blood, anal swabs, and nasal swabs were collected for analyses of viremia and viral load by RT-qPCR and $TCID_{50}$ assays, as previously described (*58*, *69*). Sheep displaying neurological symptoms were euthanized by intravenous injection of pentobarbital anesthesia in accordance with the Guidelines for the Humane Treatment of Laboratory Animals (policy no. 2006398). Viral loads in tissues collected from the euthanized sheep were measured by RT-qPCR. All remaining animals were euthanized at 21 dpi by intravenous injection of pentobarbital anesthesia.

Mosquito infection

Ae. albopictus (Jiangsu strain) and *Cx. pipiens pallens* (Jiangsu strain) were provided by the Second Military Medical University, Shanghai, China. *Cx. pipiens quinquefasciatus* (Guangzhou strain) was obtained from Southern Medical University, Guangzhou, China. All mosquitoes were reared at 28°C under 80% relative humidity in accordance with standard rearing procedures. Blood samples were collected from three sheep randomly selected from the group subcutaneously infected with the SH2201 strain at 6 dpi and mixed equally at a 1:1:1 ratio. The JEV titer of the pooled blood meal was 4.8×10^3 $TCID_{50}$/ml (6.9×10^4 RNA copies/ml). Female mosquitoes were starved for 24 hours and then fed the pooled blood meal for 1 hour using a Hemotek membrane feeder (6W1, Hemotek Limited, Blackburn, UK). Engorged mosquitoes were transferred to new containers and maintained under standard conditions for subsequent detection of JEV infection, as previously described (*70*, *71*). Mosquitoes were randomly sampled at 7 and 14 dpi to detect JEV in whole mosquitoes, midguts, and salivary glands using RT-qPCR or $TCID_{50}$ assay, as previously described (*38*).

Supplementary Materials

The PDF file includes:

Figs. S1 to S11
Tables S1 to S7
Legend for data file S1

Other Supplementary Material for this manuscript includes the following:

Data file S1

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