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Research article

Detection of Japanese encephalitis virus and its specific antibody in abnormal swine litters in Vietnam

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Abstract

Japanese encephalitis is considered an essential disease-causing swine reproductive failure in Asian countries; however, significant knowledge gaps remain about this problem in actual cases. This study examined 55 cases of reproductive failure in sows, including one abortion and 54 full-term farrowing litters, to find the Japanese encephalitis virus (JEV) and specific antibodies against JEV. Haemagglutination test was used to detect specific antibodies against JEV from 63 samples of fetal thoracic fluids and newborn un-suckling piglet sera (54 thoracic fluid samples of dead fetuses, two thoracic fluid samples of abortion fetuses, seven sera samples of weakly newborn un-suckling piglets), viral isolation and RT- PCR technique was attempted from 60 brains of fetuses and newborn un-suckling piglets, histopathology sections of fetal brains which were positive with JEV confirmed by HI test and RT-PCR were also made. The results showed that 17.46% (11/63) of thoracic fluids and sera were positive for JEV. No JEV isolation was found from 60 brain samples of dead fetuses and piglets, but RNA of JEV were detected from 5 of them (8.33%). Our results suggest that JEV should be considered the important cause of swine reproductive failure in Viet Nam.

Keywords: Japanese encephalitis virus, Pigs, Reproductive failure, Viet Nam

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INTRODUCTION

Japanese encephalitis (JE) is an arthropod-borne zoonosis caused by a flavivirus transmitted by mosquitoes, especially Culex species. Japanese encephalitis virus (JEV) is the leading cause of viral encephalitis in Asian countries, with an estimated 68,000 clinical cases of clinical JE each year and approximately 13 600 to 20 400 deaths yearly (Burns, 1950). The disease is a significant threat to public health, especially in East Asia and West Pacific. In nature, many animal species are susceptible to Japanese encephalitis viruses (JEV), such as pigs, horses, cattle, sheep, and wild birds. Swine are the most susceptible and considered amplifying host for JEV. Piglets infected with JEV may develop clinical signs of encephalitis (Clarke and Casals, 1958; Desingu et al., 2016) and die in severe cases. Although infection in adult pigs does not result in clinical disease, it can cause significant reproductive failure (Clarke and Casals, 1958; Endy and Nisalak, 2002). Innate gilts and sows infected by JEV may show reproductive failure manifested by abortion and abnormal farrowing. Infected litters commonly contain a varying number of stillborn, mummy fetuses and weak piglets with nervous signs and normal piglets (Gao et al., 2013). The first association between JEV and reproductive failure in pigs was observed in 1947-1948 in Japan when there were large outbreaks in humans and horses, simultaneously with outbreaks of stillbirths in pigs, and JEV was subsequently isolated from stillborn piglets (Hosoya et al., 1950). Experimented induction of reproductive failure by infection with JEV also caused similar damage to litters (Khan et al., 2014), and several other reports proved the impacts of JEV on swine productivity (Lindahl et al., 2012; Lindahl et al., 2013; Mansfield et al., 2017).

Mekong delta, located in the West South of Vietnam, was reported as an endemic region of JE (Morimoto, 1969; Mulvey et al., 2021), and high seropositive JEV prevalence has been demonstrated in pigs (Ogasa et al., 1977; OIE, 2021) and JEV was isolated from mosquitoes collected in this region (Sherer et al., 1959). Consequently, swine reproductive failure caused by JEV is inevitable, but it is scarce literature on this field. In this study, we investigated the association between Japanese encephalitis virus infection and reproductive failure in sows by detecting specific antibodies and JEV from the litters with reproductive failure.

MATERIALS AND METHODS

Research subject

Fifty-five cases of reproductive failure in sows from two big farms in An Giang and Vinh Long provinces of the Mekong delta, including one abortion and 54 full-term litters, were examined in this study. Pigs in these farms were routinely vaccinated against food and mouth disease, classical swine fever and Aujeszky's disease, and porcine parvovirus.

Materials

Inactivated JEV antigen from Nakayama strain, positive and negative serum (Pasteur institute in HCM city, Viet Nam), goose erythrocyte 0.33%, and other chemicals used in hemagglutination inhibition (HI) test.

C6/36 cell culture, total RNA isolation kit (Promega, USA), Titan one tube RT-PCR (Promega, USA), primers: JEM-1 and M-99; 100bp size marker, agarose, ethidium bromide, mouse monoantibody against JE, FITC conjugated antimouse IgG (Paris Pasteur institute, French).

Collection of dead fetuses and weak piglets

All of the reproductive litters that happened at the two biggest farms of An Giang and Vinh Long province from August to October were examined. Fifty-four abnormal full-term litters and one abortion case were examined. One to 3 dead fetuses (or newborn un-suckling weak piglets) were collected per litter. Sixty dead fetuses and weak piglets from 55 abnormal litters were used for JE diagnosis by determination of JEV and its specific antibodies.

Collection of neonatal piglet sera and fetal thoracic fluids

Sera from 7 unsuckled weak piglets from abnormal litters were collected by taking its blood from the heart, and 56 samples of fetal thoracic fluids were collected from dead fetuses. Piglet serum and fetal thoracic fluids were stored in a -20^{o}C freezer in the laboratory of the Veterinary Medicine department, College of Agriculture, Can Tho University for examination of specific antibodies against JEV.

Collection of piglet and fetal brains

For isolation and RT-PCR technique, 2 grams of each brain (including the cerebrum, cerebellum, and medulla oblongata) was collected and stored in a 30 ml plastic cone tube containing glycerin 50% in distilled water. Brain samples were kept at -80^{o}C for viral isolation and RT- PCR performance.

In addition, some presentative brains from fetuses that showed transparent fluid accumulation in the thoracic and abdominal cavity were also kept in 10% formalin solution bottles and transported to the laboratory the of Veterinary Medicine department, College of Agriculture, Can Tho University for preparation of histopathological sections. The information on the litters was collected simultaneously with collecting the samples from abnormal litters such as identification of the sow, total alive piglets, number of mummies, stillbirths, and weak piglets per sow. We also measured the crown-to-rump length of each examined dead fetus to estimate the fetal age at death.

Detecting specific antibodies against JEV from dead fetuses and weak piglets

The haemagglutination inhibition (HI) test was used to detect specific antibodies against JEV from thoracic fluids of dead fetuses and serum samples of weak piglets (Shimizu et al., 1954). For this test, the sera were first treated with kaolin, then adsorbed with homotypic RBCs to remove any nonspecific haemagglutinins in the test sera or thoracic fluids. The test was conducted with

the treated sera and eight units of standard antigen at pH=6.2 (Straw et al., 1999). The samples with HI titers were higher than or equal to 20, considered positive, and geometric mean titer (GMT) was calculated using the reciprocal of antibody titer (Thrusfield, 2005).

Virus isolation and identification

Viral isolation and RT- PCR technique were performed at Pasteur Institute in Ho Chi Minh city. There were 60 brains of dead fetuses, and weak piglets examined. Two grams of brain sample (including the cerebrum, cerebellum, and medulla) were homogenized with 5ml of MEM (Minimum essential medium) containing 2% of fetal calf serum, 50µg of gentamycin, 50UI of penicillin, 50µg of streptomycin and 50µg of amphotericin per ml and then, centrifuged at 3,000rpm for 15 minutes at 40°C. The brain homogenate supernatant was passed through a 0.2µm millipore filter, aliquoted, and stored at –70°C until testing. Brain homogenate supernatants were inoculated into 25cm^2 plastic flasks of Aedes alpopictus clone C6/36 cell monolayer (initial cell concentration of 10^5 cells/mL). Infected cells were harvested after incubation at 28°C for seven days. Virus identifications were determined by indirect immunofluorescent assay (IFA). Each cell culture harvested was placed on two wells of a 12-well plate, dried on a clean bench, and fixed in chilled acetone for 10 minutes. The fixed cells were incubated with specific monoclonal mouse antibodies against JE at 37°C for 30 mins in a humid chamber. After washing in PBS, the cells were stained with fluorescein isothiocyanate (FITC) conjugated antimouse IgG at 37°C for 30 mins. After washing out excess unbound reagent with PBS, the cells were mountewithby buffered glycerol and observed by fluorescent microscope at 40X lens. Cell culture was confirmed positive with green fluorescent foci in the cytoplasm; meanwhile, negative one was all red.

Reverse transcription- polymerase chain reaction (RT-PCR)

Genomic RNA of JEV was extracted from infected cell culture fluid or brain homogenate supernatant using ARN isolation reagent of Qiagen or Promega Kit according to the manufacturer's instructions. The precipitated RNA was dissolved in distilled water and stored at –70°C until use. The Titan one-step kit was used for RT-PCR with specific primers (Table 1) to detect the pre-M gene of JEV (Thu et al., 2006). The reversed reaction was set at 55°C for 30 mins, inactivation of reverse reaction for 2 mins at 94°C, followed by 35 cycles of PCR, each consisting of denaturation at 94°C for the 30s, annealing at 55°C for 30s, and elongation at 68°C for 1 min and extension at 68°C for 7 mins, using a thermal cycler (Applied Biosystem, Singapore). The PCR products were detected by electrophoresis in 2.0% low melting temperature agarose gels (Rockland, ME, USA) containing 5% ethidium bromide.

Table 1 Nucleotide sequences of primers are used to imply the prM gene.

Primer	Oligonucleotide sequence (5'-3')	Expected size (bp)
JEM-1	GGA AAT GAA GGC TCA ATC ATG TG	326
M99	TTG GAA TGC CTG GTC CG	

Histopathological examination

The formalin-fixed brains were processed to prepare hematoxylin-eosin-stained sections as standard procedure. Histopathological changes were examined under a light microscope at 100X, 200X, and 400X high–powered fields.

RESULTS

The results in Table 2 showed that 17.46% (11/63) of tested samples (54 thoracic fluid samples of dead fetuses, two thoracic fluid samples of abortion fetuses, seven sera samples of weakly newborn unsuckled piglets) were positive for specific antibody tests of HI, the positive rate of piglet sera was (28.57%) higher than that of thoracic fluids of dead fetuses (16.67%), and antibody titers ranged from 20-40, and GMT was 24.16.

Table 2 The results of specific JEV antibody examination from dead fetuses and weak piglets.

Kind of samples	No. of samples	Positive	(%)	Titer range	GMT
The thoracic fluid of dead fetuses	54	9	16.67	1/20- 1/40	25.20
Thoracic fluids of abortion fetuses	2	0	0.00	-	-
Sera* of weak piglets	7	2	28.57	1/20	20
Total	63	11	17.46	1/20- 1/40	24.16

Sera: Piglet sera collected from colostrums of newborn unsuckled pig*

The results in Table 3 showed that no JEV was isolated from all brain samples, but there were 5/60 (8.33%) of brain samples were positive with JEV by RT-PCR; the highest positive rate was reported in abortion fetuses (100%) followed by weak piglets (11.0%), and the lowest in dead fetuses (4.8%).

Table 3 Results of JEV isolation and RT-PCR from brains of fetuses and weak piglets.

Brains of	No. of samples	Results of viral isolation		Results of RT-PCR	
		Positive	%	Positive	%
Weak piglets	9	0	0.00	1	11.11
Abortion fetuses	2	0	0.00	2	100.0
Dead fetuses	49	0	0.00	2	4.08
Total	60	0	0.00	5	8.33

Table 4 Confirmation of JEV positive cases from examined reproductive failure sows.

Abortion/Full term farrowing	No. of sows	The number of sows had positive fetuses or piglets with			Total	%
		HI	RT-PCR	RT-PCR and HI		
Abortion	1	0	1	0	1	100.00
Full-term	54	8	2	1	11	20.37
Total	55	8	3	1	12	21.82

Twelve sows had positive fetuses or piglets confirmed by HI or RT-PCR (Table 4). The information on sows and their positive fetuses or piglets was presented in Table 5, which showed that reproductive failure caused by JEV could happen at any litter of the sows (from 1st- to 9th litter). The chance of detecting JEV antibodies by HI (11/15) from fetuses and the newborn unsuckled piglet was higher than that of detecting JEV by RT-PCR (5/15). Only one fetus (fetus of the sow 979) was positive with both HI and RT-PCR. JEV antibodies could not be detected in pig fetuses younger than 70 days old, but JEV was detected in fetuses older than 70 days.

Table 5 The information about sows and their positive fetuses (or piglets).

No.	Information of examination sows				Information on tested fetuses and piglets				
	Sow identification	Parity	No. newborns	No. Mummies and stillborns	Full-term /aborted	Fetal measure (mm)	The estimated age of the fetus (day)	RT-PCR result	HI result
1	53	1	12	2	Full-term	250	85-110	-	+
2	356	9	11	2	Full-term	250	85-110	-	+
3	746	9	5	7	Full-term		Piglet	-	+
					Full-term		Piglet	-	+
4	797	2	10	2	Full-term	240	85-110	+	+
5	870	7	12	2	Full-term	240	85-110	-	+
6	882	8	0	4	Full-term	240	85-110	+	-
7	1063	7	0	2	Full-term	260	85-110	-	+
8	1806	4	13	3	Full-term	250	85-110	-	+
9	1922	4	14	1	Full-term		Piglet	+	-
10	2066	4	12	1	Full-term	280	>110	-	+
11	2806	6	0	6	aborted	190	70-85	+	-
					aborted	130	50	+	-
12	12485	2	6	5	Full-term	190	70-85	-	+
					Full-term	180	70-85	-	+

The results in Table 6 reported that 13/15 (86,67%) JEV positive fetuses and piglets were from different size litters, and the macro-lesion observation of JE-positive dead fetuses or weak piglets revealed some typical macro-lesions, the most common lesion was hydrothorax (Figure 1) and ascites (73.33%), followed by hydrocephalus (Figure 2) and subcutaneous edema (66.67%).

Table 6 Lesion frequency of positive JEV dead fetuses and weak piglets (n*=15).

Lesion	Frequency	%
The fetus (piglet) belongs to a different size litter	13	86.67
Hydrothorax	11	73.33
Ascites	11	73.33
Subcutaneous edema	10	66.67
Hydrocephalus	10	66.67
Brain hemorrhage	6	40.00
Brain congestion	3	20.00
Necrotic liver	9	60.00
Necrotic spleen	3	20.00
Congested lymph nodes	3	20.00
Skin hemorrhage	1	6.67

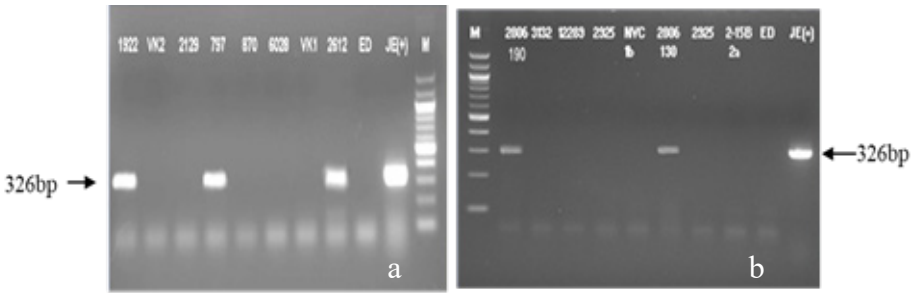

Figure 1 RT-PCR products with JEM-1 and M-99 primers in agarose 2%; ED: distilled water, JE (+): positive control, M: 100bp DNA marker; 1922, 797, 2612: positive brain samples (a); RT-PCR products with JEM-1 and M-99 primers in agarose 2%; ED: distilled water, JE (+): positive control, M: 100bp DNA marker; 2806-190, 2806-130: positive brain samples (b).

Figure 2 Litter with different sizes in dead fetuses.

Histopathology examination of the brain section showed vesicular congestion and several vacuoles (Figure 3), diffuse nonsuppurative with proliferating lymphocytes, and vesicular cuffing with lymphocytes (Figure 4).

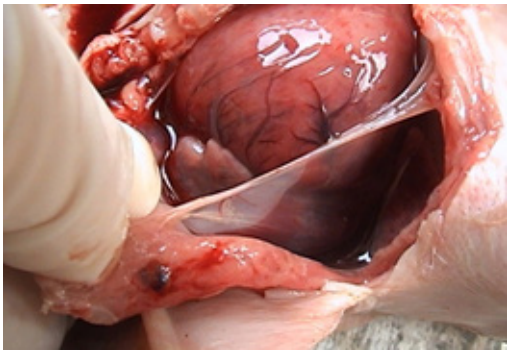

Figure 3 Hydrothorax in dead fetuses.

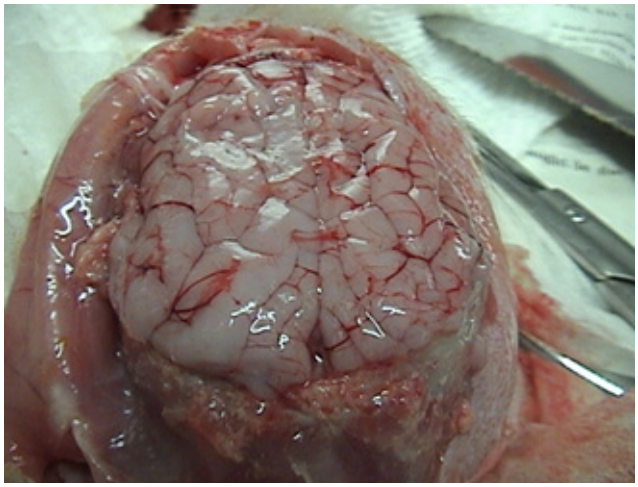

Figure 4 Edematous brain of dead fetuses.

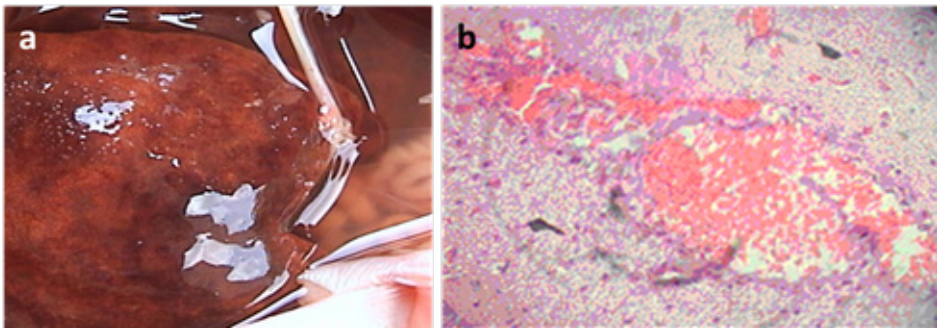

Figure 4 Necrotic liver of dead fetuses (a); Vesicular congestion and edema in the brain with several vacuoles (400X) (b).

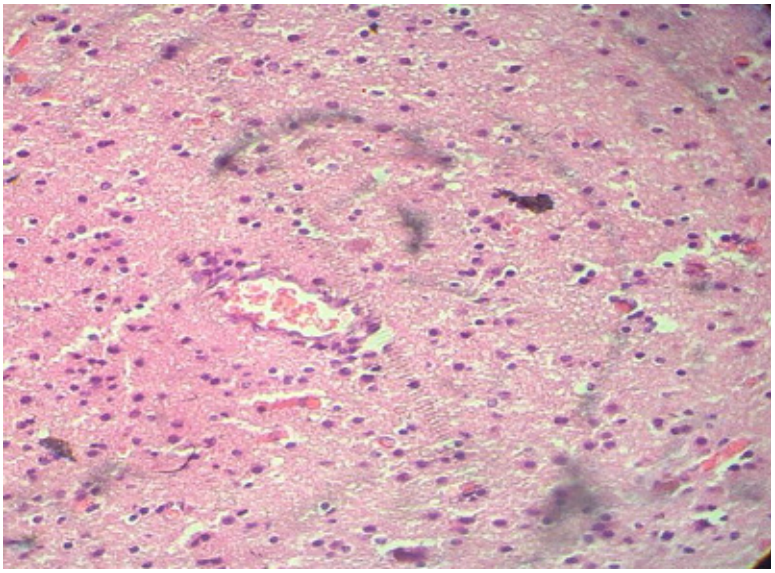

Figure 6 Proliferating lymphocytes in brain tissue and perivascular cuffing lymphocytes (100X).

DISCUSSION

Diagnosis of the cause of reproductive disorder in swine is seldom straightforward and successful due to the agent that produced death in the fetuses, and abortion is no longer present when the problem is recognized clinically. So serology is often used to diagnose swine reproductive failure by measuring levels of specific immunoglobulins in porcine fetuses. Table 2 showed that JEV-specific antibodies were found in 17.46% (11/63) tested samples (sera of unsuckled neonatal piglets and fetal thoracic fluids), providing indirect evidence of transplacental infection of JEV. Porcine fetuses are generally considered at least partially immunocompetent by 70 days of gestation (Straw et al., 1999). In a normal pregnancy, the uterine environment is sterile, no maternal antibodies transfer across the placenta, and piglets are born without immunoglobulins. Therefore, the finding of specific antibodies in fetuses indicates fetal infection.

However, the negative antibody result doesn't prove that there is no prior infection. Antibodies might be present at levels below the test's threshold for the detection or in cases of fetal dead before the day of immunocompetent. Therefore, determining infectious agents from fetuses and neonatal sick piglets is also necessary for screening more cases of fetal JEV infection. The results in Table 3 showed that no JEV was isolated from all brain samples, but 5/60 (8.33%) of brain samples were positive with JEV by RT-PCR. Although JEV was affirmed as one of the important causes of reproductive failure in pigs, the attempts of JEV isolation from reproductive failure litters seldom succeed (Tomiak et al., 1972). Furthermore, the agents that produce death in fetuses or abortion are no longer present when the problem is recognized clinically (Straw et al., 1999). However, there were five positive samples by RT-PCR (Figure 5). This can be explained by the PCR technique detecting nucleotides from degraded material, including dead virion. The results in Table 4 expressed the

numbers of sows infected by JEV when pregnancy based on detecting JEVgene or JEV-specific antibodies from their dead fetuses or neonatal unsuckled piglets. Twelve out of 55 (21.82%) examined sows were diagnosed that JEV infected their litters. These results showed the important impact of JEV on sow reproductive performance. This finding was different from the conclusion of Lindahl et al. (2012) that JEV caused a minor effect on reproductive performance in sows in the Mekong delta when analyzing the difference in reproductive performance between JEV seronegative and seropositive sows; this survey could not demonstrate the exact result because JEV positive antibody titer from sow serum only indicate that sow was infected with this virus, it did not affirm whether that JEV was the cause of the reproductive failure or not.

The results in Table 5 showed that reproductive failure caused by JEV could happen at any parturition of the sows (from 1st- to 9th parturition); this can be explained by the fact that antibodies triggered by natural JEV infection provide short-term protection (Sherer, 1959) so that sows become susceptible again when their JEV antibody level dropdown. In nature, JEV is maintained in a cycle between mosquitoes and vertebrate hosts, primary pigs, and wading birds. Mekong delta is the largest rice region and an important pig production area in Viet Nam. Water rice cultivation combined with pig production in a hot and humid climate in the Mekong delta favored the transmission of JEV in nature. The presence of JEV in the pig population was a risk for swine production and public health due to the tradition of growing pigs in close contact with humans and mosquito abundance environment. Hence, it is necessary to be concerned about preventing JEV infection in pigs, especially JE vaccination. Immunization of pigs is also potentially beneficial in reducing JEV infection in humans (Khan et al., 2014).

The results in Table 6 reported that 13/15 (86,67%) JEV-positive fetuses and piglets were from different size litters. This figure is typical for most viral infections such as pseudorabies, porcine parvovirus, and JEV infections, the other size litter, including mummies and stillbirths, is likely caused by an infectious agent. The smaller fetus died sometime earlier than the bigger ones because of the gradual transmission of infectious agents among fetuses in the sow uterus. Experimental induction of reproductive failure by JEV caused no clinical sign in dams but abnormal farrowing with varying numbers of mummified fetuses of different sizes and stillborn and weak piglets by Shimizu et al. (1954).

Besides, gross lesion examination of JEV-infected fetuses in Table 6 and Figure 6 showed the common lesions in positive fetuses with similar changes as findings of Morimoto (1969) when observing the stillborns and weak neonatal piglets from an epizootic swine stillbirth caused by Japanese encephalitis virus. In addition, microlesions from representatively positive fetuses were resembling with reports of Straw et al. (1999) and the findings of Yamada et al. (2004) from an experiment on Japanese encephalitis virus infection in piglets.

CONCLUSIONS

The presence of JEV and its specific antibodies in dead fetuses and neonatal piglets, as well as gross and microlesions, indicated that their mother sows were infected with JEV during the pregnancy period, and JEV should be considered to be the important cause of swine reproductive failure in Viet Nam.

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AUTHOR CONTRIBUTIONS

HTVT and HNT collected samples, analyzed and interpreted the data. HTVT and NDH designed, and wrote the manuscript. All authors approved the final manuscript.

CONFLICT OF INTEREST

We have no conflict of interest.

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