Surveillance of Japanese Encephalitis Virus in Piggery Effluent and Environmental Samples: A Complementary Tool for Outbreak Detection

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Abstract:

Japanese encephalitis virus (JEV) is an emerging public health and biosecurity concern in Australia, with recent human cases and detections in mosquitoes and pigs across multiple states highlights the risk to susceptible human and animal populations. While traditional surveillance relies on mosquito trapping, sentinel chicken surveillance and direct testing of samples collected from pigs, monitoring effluent provides a complementary approach for detecting infections within animal populations. This study presents the first evidence of JEV in Australian piggery effluents/environmental waters, demonstrating the feasibility of effluent and environmental water surveillance for JEV monitoring. Effluent/environmental samples from multiple piggery sites were analyzed using real-time reverse transcription (RT)-PCR, revealing the presence of JEV genetic fragments in solid and liquid effluents at three farms, with corresponding clinical cases in some herds. Viral RNA was detected more frequently in solid effluent samples, aligning with previous findings on the partitioning behaviour of mosquito-borne viruses. The detection of JEV in the borrow pit (i.e., a man-made excavation that holds water) water sample highlights potential transmission pathways via mosquito vectors. These findings demonstrate the value of effluent monitoring as an additional tool for JEV surveillance in piggery settings, supporting potential early warning systems and mitigation strategies. Integrating effluent-based monitoring with traditional surveillance approaches could improve livestock industry related disease detection, risk assessments, and response efforts for human and animal health in endemic and emerging regions. Wastewater/effluent surveillance may have important applications for the management of a wide range of emerging animal diseases.

Keywords: Japanese encephalitis virus (JEV); Wastewater surveillance; Piggery effluents; Early detection; Viral pathogen monitoring; Animal livestock industries.

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Introduction

Japanese encephalitis virus (JEV), a member of the genus *Orthoflavivirus* in the family *Flaviviridae*, represents a major etiological agent of epidemic encephalitis throughout the Asia and western Pacific nations (Mackenzie and Williams 2009; Mackenzie et al. 2022a; Liu et al. 2024). Following the emergence of JEV in south-eastern Australia in 2022, there is significant concern about continued re-emergence in seasons where favorable climatic conditions exist (Mackenzie et al. 2022a; Mackenzie et al. 2022b). JEV is maintained in an enzootic transmission cycle primarily involving ardeid wading birds and *Culex* mosquitoes (van den Hurk et al. 2009; Hameed et al. 2021; Moore et al. 2025). Wild birds and pigs serve as important amplifying hosts for JEV and pigs experience direct health impacts (i.e., reproductive disease) from the virus (Park et al. 2022; Moore et al. 2025). The risk of epizootic spillover to humans has been associated with the farming domestic pigs in endemic regions of southeast Asia (Park et al. 2022).

To mitigate the growing impact of JEV transmission and its spread to new regions, disease management strategies include human and pig vaccination programs, mosquito control and bite mitigation measures, and public awareness and education (Mackenzie et al. 2022b; Khan et al. 2024). However, these combined measures may still be insufficient to fully control the spread, particularly in regions favouring mosquito proliferation with gaps in vaccination coverage. Many JEV endemic countries also do not routinely vaccinate domestic pigs. In Australia, there is currently no swine JEV vaccine approved for use. Continued research and enhanced surveillance efforts are therefore essential to effectively mitigate the risk of JEV. In this context, urban (human) wastewater surveillance can provide a population-level assessment of infection burden and has proven to be effective in detecting both symptomatic and asymptomatic cases of disease in communities across a range of settings (Chen et al. 2024; https://data.wastewaterscan.org/). As viral shedding often precedes clinical symptoms, it enables earlier outbreak detection than traditional methods (Hellmér et al. 2014; Gupta et al. 2023). Wastewater/effluent surveillance can be equally effective for detecting viral diseases in animal industry settings, where livestock population-level samples may be collected from waste streams in animal housing areas, wastewater/effluent management

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facilities (e.g., effluent pits and ponds), and environmental waters (Xiao and Zhang 2023; Ahmed et al. 2024).

A case study of JEV detection in municipal wastewater during an outbreak highlights the potential of wastewater surveillance as a complementary tool to existing monitoring efforts (Fanok et al. 2023). Expanding the scope of wastewater surveillance to include livestock effluents aligns with the principles of "One Health" and "Planetary Health," providing valuable insights into the health of animal populations and connections between human, animal and environmental settings (Lee et al. 2022). Monitoring of viral pathogens like JEV in livestock population waste streams may contribute to early detection systems, which not only help manage human and animal population health but may also support economic stability where certain disease outbreaks threaten domestic and international trade resilience (Ahmed et al. 2024).

Experimental and field evidence has shown that domestic pigs shed JEV following infection (Ricklin et al. 2016; Lyons et al. 2018; Chiou et al. 2021; Hick et al. 2024). Detection of JEV nucleic acid and infectious virus has been reported in oronasal swabs for up to six and five days, respectively (Ricklin et al. 2016), and JEV RNA was detected in swine oral fluids for up to 11-24 days after inoculation (Lyons et al. 2018; Hick et al. 2024). JEV genome has also been detected in the urine and feces of experimentally infected pigs (Ricklin et al. 2016; Cool 2020). Virus could be detected in fecal swabs from 3 to 7 days post inoculation, with viral loads ranging from 32 to 213 genome equivalents per mL in fecal swabs (Cool 2020). These findings suggest that effluent surveillance in piggery environments could be effective, potentially offering early detection of JEV circulation and potentially identifying outbreaks before they escalate. This approach follows the successful approach of human wastewater surveillance in detecting viral pathogens.

No studies have yet detected JEV in piggery effluents, but the 2022 outbreak of JEV, together with the re-emergence in south-eastern Australia in 2025 (ProMED 2025) highlight the increasing risk of infection to human and animal health. Here, we present the first evidence of JEV in Australian piggery effluents. Our findings show that effluent and environmental monitoring can be a complementary tool for JEV surveillance in piggery settings, offering detection and monitoring at the animal population level. This proactive

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surveillance approach could complement traditional methods like mosquito monitoring, chicken sentinel surveillance and direct pig surveillance, enhancing overall detection, mitigation, and response strategies. The approach may have applications for the management of a wide range of emerging animal diseases.

Materials and Methods

Effluent sampling and concentration of viruses

Piggeries typically have a range of sheds designated for different pig categories or activities, e.g., sow, breeding, farrowing, gestating, and nursery etc. These provide opportunities for effluent monitoring before the effluent stream combine and mix within pits and larger effluent ponds (Fig. 1). Aggregated effluent samples (50 to 200 mL) were collected from five pig farms (Farm 1, 3, 4, 8, and 10) in Victoria and one farm (Farm 11) in Queensland, Australia over a period of two months during the summer of 2024-2025. At Farm 1, four effluent grab samples were collected (on different days over the duration of the study) from a pit that receives effluent from flush channels. A pit is an in-ground tank designed to store at least one day of effluent production from multiple sheds. Five, two and one effluent samples were collected from effluent ponds at Farm 3, 4, and 8, respectively. Effluent ponds are commonly located within several 100 m of pig housing sheds. At Farm 10, a total of four grab samples were collected from a pit, an effluent pond, farrowing shed, and breeding shed. In addition, a solid effluent sample was collected from a sow shed. At Farm 11, eight effluent grab samples were collected, four from a gestating shed and another four from an effluent pond on 12/02/2025, 21/02/205, 24/02/2025 and 27/02/2025. In addition, three grab environmental water samples were collected from a borrow pit on 21/02/2025, 24/02/2025, and 27/02/2025. A borrow pit is a man-made excavation that holds water (no pig effluent) and was assumed to be a potential habitat for mosquito larvae. Samples were stored at 4°C up to 48 h prior to further analysis.

Viral DNA/RNA concentration from wastewater and nucleic acid extraction

An adsorption-extraction (AE) concentration method, with minor modifications for effluent liquid and solid components, was used to concentrate viruses from effluent samples (Akter et al. 2024). The AE workflow started with centrifuging 50 mL of an effluent sample at 4,750 *g*

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for 20 min. The supernatant was filtered through a 0.80-µm pore-size, electronegative HA membrane (47-mm diameter, AAWG04700) using a magnetic filter funnel (Pall Corporation, Port Washington, New York, USA) and a filter flask (Merck Millipore Ltd.), following the method described by Akter et al. (2024). After filtration, the membrane was aseptically removed from the filter funnel, rolled, and placed into a 7-mL bead-beating tube with 1 g of 0.5 mm diameter Zirconium Oxide beads (Next Advance, Inc, NY, USA) for nucleic acid extraction. The pellet resulting from the centrifugation step was collected from the 50-mL tube and transferred into a 7-mL bead-beating tube for nucleic acid extraction. Water samples (~50 to 100 mL) collected from the borrow pit were directly processed using the AE method without any centrifugation (Akter et al. 2024).

Nucleic acid extraction from the membrane (effluent liquid) and the pellet (effluent solid) was performed separately using the RNeasy PowerWater Kit (Cat. No. 14700–50-NF, Qiagen). For each bead-beating tube containing the membrane or pellet, 990 µL of lysis buffer PM1 and 10 µL of β-mercaptoethanol (Cat. No. M6250–10 mL, Sigma-Aldrich) were added. The tube contents were then homogenized using a Precellys 24 tissue homogenizer (Bertin Technologies) at 9,000 rpm for three 15 s cycles, with a 10 s interval between cycles. After homogenization, the tubes were centrifuged at 4000 *g* for 5 min to pellet the filter debris, solids, and beads. Nucleic acid was eluted in 150 µL of DNase- and RNase-free water and stored at -20°C prior to RT-PCR analysis.

RT-PCR assays

The detection of JEV was carried out using RT-PCR assays targeting the NS-1 and M genes of JEV (Shao et al. 2018), performed in two separate laboratories (Lab 1 and Lab 2). When a nucleic acid sample tested positive in Lab 1 (CSIRO Environment RU BC2 laboratory), it was then sent to Lab 2 (Australian Centre for Disease Preparedness) for further verification. This is due to JEV being classified as Category 1 restricted matter under the Queensland Biosecurity Act and consequently subject to certain testing and reporting requirements. The NS-1 assay enables the detection of all JEV genotypes (i.e., a universal assay), while the M gene assay specifically detects JEV genotype 4 (JEV-G4), the genotype currently circulating in Australia (Mackenzie et al. 2022a). Initially, universal JEV RT-PCR was conducted in Lab

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1, but it was later replaced with the G4 RT-qPCR assay due to the latter's increased sensitivity.

In Lab 1, positive controls were prepared from nucleic acid extracts of gamma irradiated JEV-G4. In Lab 1, Universal JEV RT-PCR amplifications were performed with a 20 µL reaction mixture containing 5 µL of TaqMan™ Fast Virus 1-Step Master Mix (Applied Biosystems, Thermo Fisher Scientific, MA, USA), 400 nM of forward primer (GCC ACC CAG GAG GTC CTT), 400 nM of reverse primer (CTT CCT CTC AGA ACC CCT ATC C) and 400 nM of probe (FAM-CTA ATG GAA CGC ATC CC-MGB). A 5 µL nucleic acid sample was used in each reaction. JEV G4 RT-PCR amplifications were performed with a 20 µL reaction mixture containing 5 µL of TaqMan™ Fast Virus 1-Step Master Mix (Applied Biosystems), 900 nM of forward primer (TTC AAT ATG GAC GGT GCA CAA), 900 nM of reverse primer (CCA TGC GTG TGG ACA GAC A) and 250 nM of probe (FAM-AAC CTC ACA CTC CCA GAC AAG CAG GAG ATC-MGB). A 5 µL nucleic acid sample was used in each reaction. Each run included triplicate positive controls and triplicate no-template controls. The RT-PCR assays were performed on a Bio-Rad CFX96 thermal cycler (Bio-Rad Laboratories, Hercules, California, USA) with manual settings for threshold and baseline (40 to 100 RFU). The RT-PCR cycling parameters were 10 min at 50°C, 10 min at 95°C, 45 cycles of 30 s at 95°C, and 45 s at 56°C for Universal JEV RT-PCR and 10 min at 50°C, 10 min at 95°C, 45 cycles of 30 s at 95°C, and 45 s at 60°C for G4 JEV RT-PCR. All RT-PCR reactions were performed in triplicate.

In Lab 2, JEV G4 RT-PCR amplifications were performed with a 15 µL reaction mixture containing 7.5 µL of AgPath-ID™ One-Step RT-PCR Reagents (Applied Biosystems), 900 nM of forward primer (TTC AAT ATG GAC GGT GCA CAA), 900 nM of reverse primer (CCA TGC GTG TGG ACA GAC A) and 250 nM of probe (FAM-AAC CTC ACA CTC CCA GAC AAG CAG GAG ATC-BHQ1). A 5 µL nucleic acid sample was used in each reaction. Each run included one positive control, one negative extraction control and one no-template control, each tested in duplicate. The RT-PCR assays were performed on a QuantStudio 5 real-time PCR systems (Thermo Fisher Scientific) with manual settings for threshold (0.1) and baseline (3-15). The RT-PCR cycling parameters were 10 min at 45°C, 10 min at 95°C, 45 cycles of 15

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s at 95°C, and 45 s at 60°C for the G4 assay. All RT-PCR reactions were performed in duplicate.

Quality assurance/controls

A SARS-CoV-2 RT-PCR assay was applied to determine PCR inhibition in extracted nucleic acid samples from effluents by seeding known copy number (10^4) of gamma-irradiated SARS-CoV-2 (CDC 2019-novel coronavirus (2019-nCoV) real-time RT-PCR diagnostic panel). The reference quantification cycle (Cq) value was determined from triplicate RT-PCR reactions containing only the positive control and compared with the Cq values obtained from all effluent nucleic acid samples. Samples were considered to have no PCR inhibition when the Cq values of effluent nucleic acid samples were within 2 Cq values of the reference Cq value (Staley et al. 2012). Nucleic acid extraction and RT-PCR setup were performed in separate laboratories to minimize potential contamination.

Sequencing

RT-PCR product (Farm 10 effluent sample collected on 18/02/2025) was sequenced using a Sanger (Applied Biosystems, Foster City, CA, USA) sequencing platform. For Sanger sequencing, 5 µL of RT-PCR reaction was electrophoresed in a E-Gel™ SizeSelect™ II Agarose Gel, 2% and the E-Gel Power Snap Plus Electrophoresis System (Invitrogen™) following the manufacturers protocol for collecting size fragments. Target bands of expected size were excised from the gel and submitted to a Sanger sequencing service provider along with forward and reverse primers (AGRF, Brisbane, Australia).

Data interpretation

In Lab 1, samples were classified as positive for JEV by RT-PCR if amplification was detected in at least one out of three replicates within 40 cycles, whereas in Lab 2, samples were considered positive if amplification was detected in at least one of two replicates within the same cycle threshold. Samples were considered indeterminate when the Cq value was between 40 to 45. A sample was considered not-detected (ND) or negative when none of the three or two replicates showed amplification.

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Results and Discussion

All RT-PCR negative controls consistently returned negative results, confirming the absence of contamination, while positive controls reliably tested positive for each run, ensuring RT-PCR assay validity. For RT-PCR inhibition assay, the reference Cq value was 30.8, and the Cq values for effluent nucleic acid samples ranged from 30.1 to 31.2. These values were well within the acceptable ±2 Cq value, indicating no PCR inhibition in the nucleic acid samples. The piggery effluent samples were analyzed for the presence of JEV nucleic acid across various sampling locations within three farms (1, 10, and 11) over multiple sampling dates (Table 1).

JEV nucleic acid was not detected in any effluent samples from Farm 3, Farm 4, and Farm 8, and no clinical cases were reported in the herds on these farms. The absence of JEV nucleic acid in effluent samples suggests that the JEV was either not present or present at levels below the assay limit of detection (ALOD). The former scenario aligns with the lack of clinical cases, suggesting that JEV transmission did not occur on these farms during the sampling period. At Farm 1, JEV was not detected in either the liquid or solid effluent matrices on the first pit sampling day, 17/12/2024. However, the effluent collected from the same pit on 14/01/2025 was positive for JEV in both matrices, with all replicates (3/3) testing positive at Lab 1. The mean Cq values were 35.9 ± 1.1 for liquid samples and 33.8 ± 0.43 for solid samples, indicating a higher viral load in the solid sample. This could suggest that viral particles are more effectively retained in solid waste compared to liquid effluent. Our findings are in accordance with Roldan-Hernandez et al. (2025) who reported that seeded mosquito borne viruses may partition several orders of magnitudes higher in solids than in liquids in wastewater samples collected from the primary sludge line of wastewater treatment plants in the USA.

Continued monitoring of Farm 1 on 21/01/2025 showed that JEV was only detected in the solid matrix effluent (3/3 replicates positive, Cq = 39.7 ± 0.69) but not in the liquid phase, and on 04/02/2025 was RT-PCR negative for either matrix. These combined results suggest a possible shift to detectable JEV levels only in solids, followed by a potential decline in shedding or an effect of effluent dilution or decay of JEV genome in the sampling environments. It is also possible that the infected pigs stopped shedding or were shedding at

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levels that were below the ALOD. In this regard, PCR detection and virus isolation studies indicated oronasal shedding of JEV can persist for between 5 to 6 days and can be detected from pen-based chew rope samples 11 to 24 days after virus inoculation (Hick et al. 2004; Ricklin et al. 2016; Lyons et al. 2018). Fecal shedding of JEV in experimentally infected swine appeared from day three and persisted for three to seven days, with the average viral load ranging from 32 to 213 genome equivalents per mL (Cool 2020). These findings suggest that the transient nature of JEV shedding in pigs may explain the decline in detection over time observed in this study. Interestingly, Farm 1 reported no clinical cases of JE, despite the detection of viral nucleic acid in effluent samples, indicating that asymptomatic pigs were contributing to viral shedding. Farm 1 is a breeding farm comprising sows and newborn piglets (up to 3-4 weeks old). Natural infection of pigs is usually clinically inapparent, and typically manifests as reproductive disease in pregnant sows, leading to abortion, stillbirths and mummified fetuses, which becomes apparent at farrowing (Williams et al. 2019; Park et al. 2022). Infection of young pigs can also occasionally lead to a wasting syndrome with neurological signs. While no evidence of clinical JE has been confirmed on Farm 1, at the time of writing not all sows pregnant when positive samples were collected had farrowed, and we cannot rule out future cases from infections that may have occurred at that time.

Effluent samples collected from Farm 10 on 11/02/2025 were JEV positive, with effluent from the pit showing 2/3 positive replicates for the liquid samples (Cq = 35.2) and 3/3 positive replicates for the solid samples (Cq = 33.6 ± 0.19). A second effluent sample collected on the same day from an effluent pond also yielded positive results, albeit at lower detection levels, with liquid samples showing 1/3 positive replicates (Cq = 36.6) and solid samples 2/3 positive replicates (Cq = 36.5). Farm 10 had a confirmed JE clinical case, with a litter of stillborn and mummified piglets born 02/02/2025 testing PCR-positive for JEV in clinical samples (i.e., cerebrospinal fluid and placenta), aligning with the effluent RT-PCR detection results.

On 18 February 2025, three additional samples were collected from Farm 10 from separate breeding, farrowing and sow sheds. A solid effluent sample from the sow shed tested positive in all three replicates (Cq: 34.7 ± 0.50), Lab 2 also confirmed the sample as positive for JEV in 2/2 replicates (Cq: 38.8). Effluent from the farrowing shed showed no detection in either liquid or solid samples. However, effluent sample collected from the

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breeding shed on the same date revealed detection in the liquid fraction (3/3 positive with mean Cq: 34.6 ± 0.77) and 3/3 solid replicates (Cq: 30.2 ± 0.08). Lab 2 also confirmed both samples as positive.

On 12 February 2025, effluent samples were collected from two locations (gestating shed and effluent pond) at Farm 11. At the outlet from the gestating shed, 1/3 liquid samples tested positive (Cq: 37.2), but no detection was recorded in Lab 2. In the solid samples from the gestating shed, 3/3 replicates were positive with a mean Cq value of 35.5 ± 0.40. Lab 2 confirmed the presence of JEV in these solid samples, with 1/2 replicates positive (Cq 38.8). In the effluent from the pond, no JEV was detected in the liquid samples, while the solid samples showed a positive result in 2/3 replicates with a Cq value of 37.1. However, Lab 2 did not detect JEV in these solid samples. Clinical cases in the herd were confirmed from samples that were collected from piglets on 31/01/2025, 12 days prior to the collection of effluent samples. Affected sows experienced abortion, stillbirth, fetal mummification, or gave birth to weak piglets. Reproductive failure can occur in sows infected with JEV up to approximately 60–70 days of gestation, while infection at later stages does not appear to affect piglets (Platt and Joo 2006; Chapagain et al., 2022). Therefore, affected sows were likely infected approximately 6-8 weeks or more earlier, and were unlikely to be shedding at the time of effluent sampling, based on existing knowledge of virus shedding dynamics in pigs (Ricklin et al., 2016; Lyons et al., 2018; Cool 2020; Hick et al., 2024). This suggests that the source of the positive effluent was residual virus shed during farrowing (e.g., from infected piglets and placental fluids), or from other infected sows experiencing an acute stage of infection.

Subsequent effluent samples collected from the gestating shed and effluent pond from Farm 11 on 21/02/2025, 24/02/2025, and 27/02/2025 were also JEV positive in Lab 1. Lab 2 detected JEV in samples from the gestating shed and effluent pond samples from 21/02/2025 and from the effluent pond from 24/02/2025. Effluent sample (solid fraction) collected from Farm 11 gestating shed on 27/02/2025 were positive (1/3 replicates; Cq = 37.8), but liquid fraction was negative. Effluent sample (both liquid and solid fractions) from the pond was also positive by Lab 1. However, Lab 2 could not detect JEV in the effluent pond liquid sample collected on 27/02/2025.

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Interestingly, of the three environmental water samples collected from the borrow pit, one sample collected on 24/02/2025 was positive for JEV by both Lab 1 and 2. This detection is significant because a borrow pit can collect stagnant water, creating an ideal breeding ground for mosquitoes, particularly *Culex* species, which are the primary vectors of JEV. Virus present in or shed from infected larvae or present in infected mosquito eggs that were collected in these water samples may have been the source of the positive sample from the borrow pit. Subsequent environmental sample collected from the borrow pit was negative.

The RT-PCR results from Lab 1 and 2 revealed both agreements and discrepancies across different sample matrices and farm sampling locations (Table 1). For positive results in agreement, Cq values from Lab 1 testing were either comparable or lower than those from Lab 2. Ten samples also tested positive at Lab 1 but were negative (not detected) at Lab 2. The Cq values for these samples were all ≥36.6 and, for seven samples, only detected in 1/3 or 2/3 replicates at Lab 1. These discrepancies were attributed to a combination of differences in RT-PCR assay chemistry and sensitivity between the two laboratories, sub-sampling variability, samples with high Cq values at or near the ALOD, RNA degradation from freeze-thaw cycles, or sample degradation during shipment to Lab 2. The aligned forward and reverse Sanger sequences confirmed a 100% match to the JEV G4 isolate, further validating the RT-PCR findings.

An important finding of this study is that solid effluent matrices consistently yielded lower Cq values and a higher frequency of positive detections than liquid effluent. This suggests that solid effluent may act as a more stable reservoir for JEV, supporting previous research indicating that viral particles preferentially adsorb to organic matter. Processing 50 mL of effluent samples was challenging due to high turbidity and the presence of suspended solids. To address this, we centrifuged the samples to separate the solid fraction from the liquid phase before RNA extraction. The results suggest that analyzing only the solid fraction may be sufficient for JEV detection, potentially streamlining future surveillance efforts by reducing sample processing complexity.

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Conclusions

This study highlights the potential utility of effluent and environmental water surveillance for detecting JEV in piggery settings, with potential applications for emerging animal disease surveillance in a range of livestock industries. These findings provide a foundation for improving JEV monitoring strategies and supporting its mitigation in piggery operations. Further research is needed to understand the dynamics and mode of virus shedding in infected pigs at different stages of production, particularly for infected pregnant sows for which there is a paucity of information reported. It is also important to investigate environmental factors such as temperature, pH, and effluent management practices that may influence the persistence of JEV in effluents. Additionally, assessing the correlation between viral detection in effluent compared to trapped mosquito pools, and oral fluids collected from piggeries could provide valuable insights into the most sensitive method or optimal combination of methods for cost-effective and efficient piggery-based surveillance for JEV. Continuous monitoring of farms is essential especially in summer (when environmental and ecological factors favour the transmission mosquito-borne viruses) to determine whether virus is shed early before JEV outbreaks occur. In this study, we collected grab samples which were taken at a specific time. If JEV from infected pigs is intermittently excreted into effluents at a farm or shed level, a grab sample may not contain detectable levels of virus, leading to false negatives. Passive sampling may provide a more effective approach by continuous collection of samples over time, potentially increasing the sensitivity of viral detection and improving surveillance efficiency. Future studies should evaluate the feasibility and effectiveness of different passive sampling regimens for JEV monitoring using piggery effluent.

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Table 1: Detection of JEV in piggery effluent samples and environmental samples.

Farm ID (Sampling date)	Sampling locations (sample type)	Sample matrices	No. of RT-PCR replicate positive/No. of RT-PCR replicates tested in Lab 1	Lab 1 Cq values for JEV RT-PCR replicates (mean ± SD)	No. of RT-PCR replicate positive/No. of RT-PCR replicates tested in Lab 2	Lab 2 Cq values for JEV RT-PCR replicates (mean)	Confirmed clinical cases in herd (date)
Farm 1 (17/12/2024)	Pit (effluent)	Liquid	0/3	ND	N/A	N/A	No
		Solid	0/3	ND	N/A	N/A	
Farm 1 (14/01/2025)		Liquid	3/3	35.9 ± 1.07[a]	2/2	36.5[b]	
		Solid	3/3	33.8 ± 0.43[a]	2/2	38.5[b]	
Farm 1 (21/01/2025)		Liquid	0/3	ND	N/A	N/A	
		Solid	3/3	39.7 ± 0.69[a]	0/2	N/A	
Farm 1 (04/02/2025)		Liquid	0/3	ND	N/A	N/A	
		Solid	0/3	ND	N/A	N/A	
Farm 10 (11/02/2025)	Pit (effluent)	Liquid	2/3	35.2[a]	2/2	37.4[b]	Yes (on 06/02/2025, samples were taken from piglets born on 02/02/2025)
		Solid	3/3	33.6 ± 0.19[a]	2/2	35.7[b]	
	Effluent pond (effluent)	Liquid	1/3	36.6[a]	0/2	ND	
		Solid	2/3	36.5[a]	0/2	ND	
Farm 10 (18/02/2025)	Sow shed (solid effluent)	Solid	3/3	34.7 ± 0.50[b]	2/2	38.8[b]	
	Farrowing shed (effluent)	Liquid	0/3	ND	N/A	N/A	
		Solid	0/3	ND	N/A	N/A	
	Breeding shed (Effluent)	Liquid	3/3	34.6 ± 0.77[a]	2/2	36.5[b]	
		Solid	3/3	30.2 ± 0.08[a]	2/2	31.3[b]	
Farm 11 (12/02/2025)	Gestating shed (effluent)	Liquid	1/3	37.2[a]	0/2	ND	
		Solid	3/3	35.5 ± 0.40[a]	2/2	38.8[b]	
	Effluent pond (effluent)	Liquid	0/3	ND	N/A	N/A	
		Solid	2/3	37.1[a]	0/2	ND	
Farm 11 (21/02/2025)	Borrow pit (water sample)	Liquid	0/3	ND	N/A	N/A	
	Gestating shed (effluent)	Liquid	1/3	37.5[b]	2/2	38.5[b]	
		Solid	3/3	37.5 ± 0.15[b]	0/2	ND	
	Effluent pond (effluent)	Liquid	3/3	37.7 ± 0.09[b]	0/2	ND	

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		Solid	2/3	38.0[b]	1/2	38.8[b]	Yes (on 06/02/2025, samples were taken from piglets born on 31/01/2025)
Farm 11 (24/02/2025)	Borrow pit (water sample)	Liquid	2/3	36.1[b]	1/2	38.8[b]	
	Gestating shed (effluent)	Liquid	1/3	37.7[b]	0/2	ND	
		Solid	3/3	36.0 ± 0.66[b]	0/2	ND	
	Effluent pond (effluent)	Liquid	2/3	37.4[b]	0/2	ND	
		Solid	3/3	37.1 ± 1.13[b]	1/2	38.4[b]	
Farm 11 (27/02/2025)	Borrow pit (water sample)	Liquid	0/3	ND	N/A	N/A	
	Gestating shed (effluent)	Liquid	0/3	ND	N/A	N/A	
		Solid	1/3	37.8[b]	NT	NT	
	Effluent pond (effluent)	Liquid	1/3	38.2[b]	0/2	ND	
		Solid	2/3	37.8[b]	NT	NT	

SD: Standard deviation; ND: Not detected; N/A: Not applicable; NT: Not tested; [a]: Universal JEV RT-PCR; [b]: G4 JEV RT-PCR.

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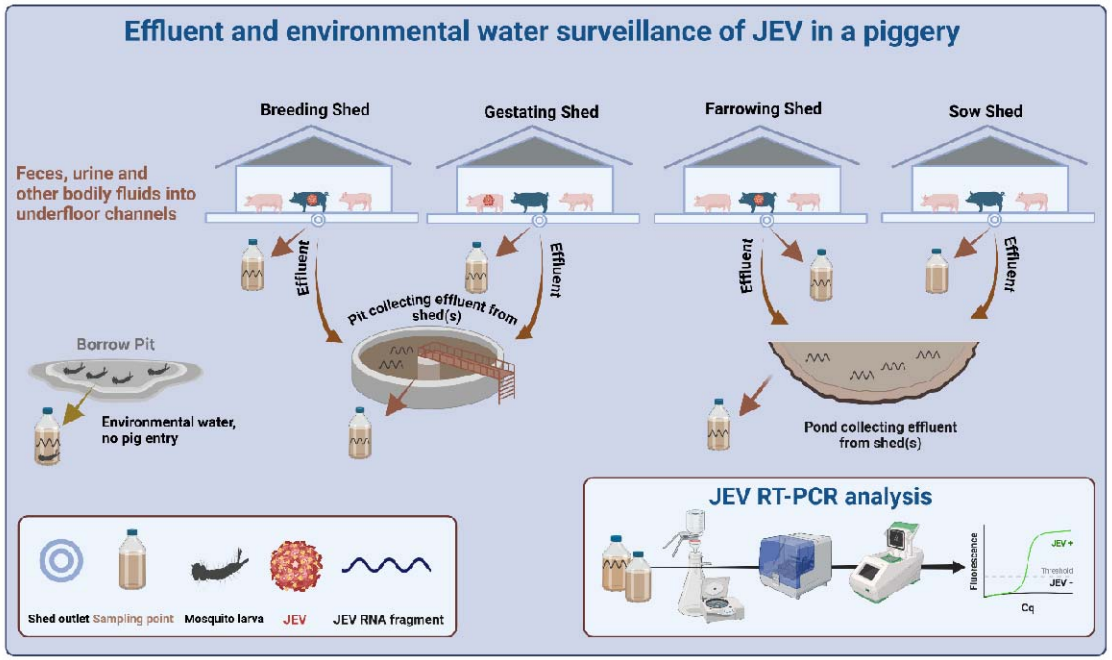

Fig. 1. A typical piggery showing various sheds for pig housing, effluent systems and sampling points.

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