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Protocols

Comparative analysis of RT-qPCR assay sensitivity and process limit of detection for Japanese encephalitis virus (JEV) detection in piggery wastewater

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ABSTRACT

Japanese encephalitis virus (JEV) poses a significant public health threat in Asia, the Western Pacific, and Australia, necessitating robust surveillance and management strategies. This study evaluates three RT-qPCR assays (Universal JEV, ACDP JEV G4, and VIDRL2 JEV G4) for detecting JEV in piggery wastewater, a promising approach for early outbreak detection. We assessed assay limit of detection (ALOD), process limit of detection (PLOD), and recovery efficiency using gamma-irradiated JEV seeded into wastewater samples, alongside field-derived samples from an Australian piggery. The ACDP JEV G4 assay demonstrated superior sensitivity, with an ALOD of 2.20–5.70 copies/reaction and PLOD of 72–282 copies/10 mL of piggery wastewater, detecting JEV in 23/30 field samples compared to 17/30 for Universal JEV and 0/30 for VIDRL2 JEV G4 assays. Recovery efficiencies varied, with ACDP JEV G4 showing consistent performance (14.9–26.6 %) across concentrations. McNemar's test confirmed ACDP JEV G4's higher sensitivity ($p < 0.05$). Based on the results obtained in this study, the ACDP JEV G4 assay is recommended for wastewater surveillance in genotype 4 regions, with a dual-assay approach suggested for broader genotype coverage. These findings enhance JEV surveillance strategies, supporting early detection and control.

1. Introduction

Japanese encephalitis virus (JEV) is a mosquito-borne flavivirus that causes Japanese encephalitis, a severe neurological disease posing significant public health challenges across Asia, the Western Pacific, and parts of Australia (Mackenzie et al., 2004). Transmitted primarily by *Culex tritaeniorhynchus* mosquitoes, JEV relies on pigs and ardeid waterbirds as amplifying hosts, facilitating its enzootic cycle (Erlanger et al., 2009; van den Hurk et al. 2009). Infection by JEV can cause significant reproductive disorders in pigs, such as abortions and stillbirths, and severe neurological disease in humans, including encephalitis with potential long-term sequelae (Campbell et al., 2011; Park et al., 2022). Since the emergence of JEV in southeastern Australia in 2022, there is growing apprehension regarding its potential re-emergence during seasons with favourable climatic conditions (Mackenzie et al., 2022a; Mackenzie et al., 2022b).

To address the increasing threat of JEV transmission and its expansion to new regions, disease management strategies encompass human and pig vaccination, mosquito control, bite prevention, and public education (Mackenzie et al., 2022b; Khan et al., 2024). However, these measures may not fully curb the spread in areas with high mosquito activity and incomplete vaccination coverage. Sustained research and improved surveillance are critical to effectively manage JEV risks. Clinical surveillance for JEV in pigs and humans primarily involves serological testing and molecular methods (Duong et al., 2011; Kumar et al., 2020). In piggeries, enzyme-linked immunosorbent assays (ELISA), such as IgM-capture ELISA, detect JEV-specific antibodies in pig serum, while RT-qPCR is used to identify viral RNA in tissues, oral fluids and saliva collected via chewing rope, or stillborn piglets (Dhanze et al., 2020; Chiou et al., 2021). In humans, RT-qPCR on cerebrospinal

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fluid or blood confirms acute infections, and serological tests like ELISA and plaque reduction neutralization tests (PRNT) detect antibodies to diagnose recent or past infections (Campbell et al., 2011). These tests, while generally effective, are limited by cross-reactivity with other flaviviruses in serology and the need for invasive sampling and some methods are time-consuming, limiting its applicability in routine diagnostics.

JEV has been detected in the urine and feces of experimentally infected pigs (Ricklin et al., 2016; Cool, 2020). Monitoring viral pathogens like JEV in livestock waste streams offers a promising strategy for early detection, supporting the management of animal population health while bolstering economic stability by mitigating disease outbreaks that threaten domestic and international trade (Ahmed et al., 2024). Wastewater surveillance provides a cost-effective method to track JEV circulation in piggeries, enabling early intervention to prevent outbreaks. A case study during a JEV outbreak demonstrated the potential of wastewater surveillance as a complementary tool to existing monitoring systems (Fanok et al., 2023). A recent study provided the first evidence of JEV in Australian piggery effluents and environmental waters, and alignment with clinical cases in pigs confirmed the feasibility of using wastewater surveillance for effective JEV monitoring (Ahmed et al., 2025).

The success of wastewater surveillance for JEV depends on the availability of sensitive and reliable diagnostic assays, capable of detecting low viral loads in complex wastewater matrices. Reverse transcription quantitative polymerase chain reaction (RT-qPCR) is the gold standard for detecting JEV nucleic acid due to its high sensitivity, specificity, and ability to quantify viral loads (Santhosh et al. 2007; Ahmed et al., 2025). RT-qPCR assay performance is influenced by multiple factors, including the design of the assay, the specificity of primers and probes, and the presence of PCR inhibitors in environmental samples (Mikeska and Dobrovic, 2009; Schrader et al., 2012).

Several RT-qPCR assays have been developed for JEV detection, targeting different genomic regions and genotypes (Pyke et al., 2004; Santosh et al., 2007; Bharucha et al., 2018; Shao et al., 2018; Martin et al. 2023). The Universal JEV assay, designed to detect all JEV genotypes, offers broad applicability but may have reduced sensitivity for specific genotypes due to its non-specific targeting (Shao et al., 2018). In contrast, genotype-specific assays, such as those targeting JEV genotype 4 (G4), which was associated with the 2022 Australian outbreaks, may provide enhanced sensitivity for regionally relevant strains. The VIDRL2 JEV G4 assay, developed by the Victorian Infectious Diseases Reference Laboratory (VIDRL), is reported to exhibit sensitivity similar to that of the Universal JEV assay (Shao et al., 2018; Martin et al., 2023). Although JEV RT-qPCR assay comparisons have been conducted for clinical samples (Bharucha et al., 2018; Shao et al., 2018; Martin et al., 2023), their performance in environmental samples remains largely underexplored. Variability in assay performance, particularly regarding the assay limit of detection (ALOD) and process limit of detection (PLOD), significantly affects the reliability of JEV detection in wastewater. Detecting JEV in piggery wastewater can be more challenging than clinical samples due to lower viral concentrations and therefore an effective sample concentration method is essential to concentrate viral particles prior to nucleic acid extraction (Ahmed et al., 2025). While Ahmed and colleagues (Ahmed et al., 2025) successfully detected JEV in piggery wastewater samples, data on percent recoveries remain unavailable.

The objectives were to: (i) determine the ALOD and PLOD for three JEV RT-qPCR assays using gamma-irradiated JEV control materials seeded into piggery wastewater samples; (ii) assess the recovery efficiency of JEV from piggery wastewater samples using an adsorption-extraction (AE) method; and (iii) compare the sensitivity of the three assays in field-derived piggery wastewater samples. By systematically evaluating these parameters, this study aimed to identify the most sensitive RT-qPCR assay for JEV detection in piggery wastewater samples.

2. Materials and methods

2.1. JEV seeding materials

The gamma-irradiated JEV (strain JEV/sw-22–00722–11/Qld/2022; GenBank ON624132.1) was provided by the Australian Centre for Disease Preparedness (ACDP), CSIRO. Gamma radiation was performed using an MDS Nordion Irradiator at a dose of 50 kilogray (5 Mrad) dose using an MDS. Gamma irradiation was required to mitigate the risk of infection associated with handling infectious JEV in a biosecurity containment level 2 (BC2) laboratory, where this study was conducted. Upon receiving the JEV from ACDP stock, it was stored at −80°C.

2.2. Optimization of JEV RT-qPCR assays

The Universal JEV RT-qPCR assay was optimized in our recent study (Liu et al., 2024). The JEV G4 (designed by Australian Centre for Disease Preparedness (ACDP), CSIRO based on M gene sequences of JEV G4) and VIDRL2 JEV G4 RT-qPCR (Martin et al., 2023) assays were independently optimized in this study through primer/probe titration and gradient PCR to determine the optimal assay conditions (Supplementary Tables ST1 and ST2). RT-qPCR amplifications were carried out in 20-μL reaction volumes using 5 μL of TaqMan™ Fast Virus 1-Step Master Mix (Applied Biosystem, California, USA) and 3 μL of control nucleic acid (~1×10^2–10^4 copies/μL). The threshold and baseline for each assay were set manually (110 for Universal JEV, 200 for ACDP JEV G4 and 50 for VIDRL2 JEV G4).

2.3. JEV RT-qPCR assays

The optimized RT-qPCR assay conditions, including primer and probe concentrations and annealing temperatures, and cycling parameters are summarized in Table 1. All reactions used a 20 μL mixture with 5 μL TaqMan™ Fast Virus 1-Step Master Mix (Applied Biosystems, Thermo Fisher Scientific, MA, USA) and 3 μL nucleic acid sample. Specific conditions are:

- **Universal JEV RT-qPCR**: 400 nM forward primer, 400 nM reverse primer, and 400 nM probe.
- **ACDP JEV G4 RT-qPCR**: 800 nM forward primer, 800 nM reverse primer, and 400 nM probe.
- **VIDRL2 JEV G4 RT-qPCR**: 800 nM forward primer, 800 nM reverse primer, and 400 nM probe.

Each assay run included triplicate positive controls and triplicate no-template controls. All RT-qPCR assays were performed on a Bio-Rad CFX96 thermal cycler (Bio-Rad Laboratories, Hercules, California, USA) with manual settings for threshold and baseline (110 for Universal JEV, 200 for ACDP JEV G4 and 50 for VIDRL2 JEV G4).

2.4. RT-qPCR assay limit of detection (ALOD)

To determine the RT-qPCR assay limit of detection (ALOD), gamma-irradiated JEV was diluted in DNase and RNase free water to ~800, 80, 8, 4, and 0.8 GC/reaction) and analyzed using RT-qPCR. At each dilution, 12 replicates were analyzed using RT-qPCR. The ALOD was determined by probit analysis, which allow the calculation of an ALOD at a desired level of certainty (e.g., 95 %) (Stokdyk et al., 2016).

2.5. RT-qPCR process limit of detection (PLOD)

Immediately prior to seeding experiments, the mean concentration and standard deviation of the gamma-irradiated JEV stock (~$5.86 \times 10^4 \pm 1.05 \times 10^4$ GC/μL) were determined in duplicate using both the undiluted stock suspension and a 10-fold dilution, employing the QIAcuity digital PCR (dPCR). The Universal JEV assay was conducted in 40-μL

Table 1
RT-qPCR assays for Japanese encephalitis virus (JEV) detection including primer and probe sequences and cycling parameters.

RT-qPCR assays	Primers and probes (5'–3')	Primer and probe concentrations	Cycling parameters	References
Universal JEV	F: GCC ACC CAG GAG GTC CTT	400	10 min at 50°C,10 min at 95 °C, 45 cycles of 30 s at 95°C, and 45 s at 56°C	Shao et al. 2018
	R: CCC CAA AAC CGC AGG AAT	400		
	P: FAM-CAA GAG GTG GAC GGC C	400		
ACDP JEV G4	F: AAG AAG CCT GGC TGG ATT CA	800	10 min at 50°C,10 min at 95 °C, 45 cycles of 30 s at 95°C, and 45 s at 60°C	ACDP (in house)
	R: GGA TTC CTT ATG ATC CAA TTT TCA G	800		
	P: FAM-CGA AAG CCA CCC GGT ATC TCA-BHQ1	400		
VIDRL2 JEV G4	F: GGC CTT CTG GTG ATG TTT C	800	10 min at 50°C,10 min at 95 °C, 45 cycles of 30 s at 95°C, and 45 s at 60.0°C	Martin et al. 2023
	R: TAG CAC TAC GTA CCT CRC CAR AT	800		
	P: FAM-TGA CAG TTC CTG CGG TTT-MGB	400		

ACDP: Australian Centre for Disease Preparedness.

reaction volumes using the QIAcuity One-Step Viral RT-PCR Kit (Catalog no. 1123145, Qiagen) and 26000 24-well Nanoplates (catalog no. 250001, Qiagen). The RT-dPCR reaction mixture consisted of 10 µL of master mix, 800 nM of each forward and reverse primers, 800 nM probe, 0.4 µL of 100 × Multiplex Reverse Transcription Mix, 19.2 µL of DNase and RNase free water, and 5 µL of template nucleic acid. The thermal cycling protocol consisted of reverse transcription at 50 °C for 40 min, followed by an additional 10 min at 50°C, 10 min at 95 °C, and 45 cycles of 30 s at 95°C, and 45 s at 56°C. Two no template controls (NTCs) were included in RT-dPCR run. Data were analyzed using QIAcuity Suite Software version 1.1.3 193 (Qiagen), with quantities expressed as GC/µL of reaction mixture, and RT-dPCR assays performed using automatic threshold and baseline settings. To determine RT-qPCR process limit of detection (PLOD), a total of 80,000 GC of gamma-irradiated JEV were seeded into each of two 10 mL piggery wastewater samples. The PLOD is defined as the lowest concentration of JEV that can be reliably detected after undergoing all sample processing steps, including concentration, extraction, and amplification. A 10-fold serial dilution was performed on each of the seeded wastewater sample, spanning concentrations of approximately 80,000 8000, 800, 80, and 8 GC. Exogenous JEV was concentrated from each wastewater sample using an AE method (Ahmed et al., 2020; Akter et al., 2024). In the AE workflow, 10 mL of wastewater sample was immediately filtered through a MF-Millipore™ 0.45 µm MCE membrane (47 mm; Cat no. HAWP04700) via a magnetic filter funnel (Pall Corporation, Port Washington, New York, USA) and filter flask (Merck Millipore Ltd.) (Ahmed et al., 2020). After filtration, the membrane was aseptically rolled and transferred into a 7-mL bead-beating tube (Axygen, CA, USA) with 1 g of 0.5 mm diameter Zirconium Oxide beads (Next Advance, Inc, NY, USA) for nucleic acid extraction.

Nucleic acid extraction from the membrane was performed separately using the RNeasy PowerWater Kit (Cat. No. 14700–50-NF, Qiagen). For each bead-beating tube containing the membrane, 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 9000 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. To determine the PLOD values, JEV seeded wastewater sample and its dilutions were analyzed using the three JEV RT-qPCR assays. At each dilution, five RT-qPCR replicates were analyzed. The ALOD was determined by probit analysis, which allow the calculation of an ALOD at a desired level of certainty (e.g., 95 %) (Stokdyk et al., 2016). For the estimation of PLOD, results obtained for two wastewater samples (A and B) were pooled.

2.6. Comparison of assay performance using field-derived piggery wastewater nucleic acid samples

The piggery wastewater nucleic acid samples used in this study for RT-qPCR assay comparison were obtained from a recent study (Ahmed et al., 2025). In total, 30 effluent samples were selected that had been collected between 12/02/2025 and 03/03/205 from a piggery. Grab wastewater samples (500 mL to 1 L) were collected from shed and effluent pond. An 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 4750 *g* 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 and colleagues (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.

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 9000 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.

2.7. RT-PCR inhibition

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). SARS-CoV-2 was selected as the inhibition control because it is not expected to be present in piggery effluents, ensuring that any detected amplification reflects the seeded control and not environmental contamination, thus allowing accurate assessment of PCR inhibition. 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 two Cq values of the reference Cq value (Staley et al., 2012).

2.8. Quality assurance/controls and data interpretation

Nucleic acid extraction and RT-PCR setup were performed in separate laboratories to minimize potential contamination. Wastewater

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. Samples were considered indeterminate when the Cq value was between 40 and 45. A sample was considered not-detected (ND) or negative when none of the three replicates showed amplification. A McNemar's test was conducted to compare the sensitivity of the Universal JEV and ACDP JEV G4 RT-qPCR assays for detecting JEV nucleic acid in piggery wastewater samples. A significant result ($p < 0.05$) indicates that the ACDP JEV G4 assay detects significantly more positive samples than the Universal JEV assay, confirming higher sensitivity.

3. Results

3.1. JEV RT-qPCR assay performance characteristics

The performance characteristics of three RT-qPCR assays for detecting JEV were evaluated and are summarized in Table 2. The Universal JEV assay exhibited an efficiency of 92.3 %, with an R^2 value of 0.994, a slope of −3.59, and a y-intercept of 37.9. The ACDP JEV G4 assay demonstrated the highest efficiency at 99.7 %, with an R^2 of 0.994, a slope of −3.33, and a y-intercept of 37.5. The VIDRL2 JEV G4 assay showed an efficiency of 98.2 %, with an R^2 of 0.978, a slope of −3.37, and a y-intercept of 38.9. All assays displayed high linearity ($R^2 \geq 0.978$) and efficiencies within the acceptable range of 90–110 %, consistent with the Minimum Information for Publication of Quantitative Real-Time PCR Experiments (MIQE) guidelines. These results confirm the robust performance of all assays, meeting MIQE standards for reliable RT-qPCR experiments (Bustin et al., 2009). All wastewater samples were free of PCR inhibitors.

3.2. JEV RT-qPCR assays ALOD comparison

The ALOD for three RT-qPCR assays targeting JEV was determined using RT-PCR detection rates (Supplementary Table ST3) and is presented in Table 3. The Universal JEV assay demonstrated ALOD values of 2.10, 3.80, and 5.50 copies/reaction at 50 %, 80 %, and 95 % detection probabilities ($ALOD_{50}$, $ALOD_{80}$, and $ALOD_{95}$, respectively), respectively. The ACDP G4 JEV G4 assay showed comparable ALOD values of 2.20, 3.90, and 5.70 copies/reaction at the same detection thresholds. In contrast, the VIDRL2 JEV G4 assay exhibited higher ALOD values of 5.60, 12.6, and 27.4 copies/reaction for 50 %, 80 %, and 95 % detection probabilities, respectively. All assays achieved reliable detection at low viral loads, with the Universal JEV and ACDP JEV G4 assays demonstrating superior sensitivity compared to the VIDRL2 JEV G4 assay.

3.3. JEV RT-qPCR assays PLOD comparison

The PLOD for the JEV RT-qPCR assays was evaluated at 50 %, 80 %, and 95 % detection probabilities ($PLOD_{50}$, $PLOD_{80}$, and $PLOD_{95}$, respectively) (Table 4) using RT-PCR data (Supplementary Table ST4). The Universal JEV assay demonstrated an $PLOD_{50}$ of ~80.8 copies, an $PLOD_{80}$ of ~300 copies, and an $PLOD_{95}$ of ~800 copies. The ACDP JEV G4 assay exhibited higher sensitivity, with an $PLOD_{50}$ of ~72.0 copies, an $PLOD_{80}$ of ~146 copies, and an $PLOD_{95}$ of ~282 copies. In contrast, the VIDRL2 JEV G4 assay showed a lower sensitivity, with an $PLOD_{50}$ of ~1000 copies, an $PLOD_{80}$ of ~3000 copies, and an $PLOD_{95}$ of ~8000 copies. These findings indicate that the ACDP JEV G4 assay has the highest detection sensitivity among the tested assays, followed by the Universal JEV assay, while the VIDRL2 JEV G4 assay requires much higher viral copy numbers for detection.

Table 3
Japanese encephalitis virus (JEV) RT-qPCR assay limit of detection (ALOD).

RT-qPCR assays	Assay limit of detection (ALOD)		
	$ALOD_{50}$	$ALOD_{80}$	$ALOD_{95}$
Universal JEV	2.10	3.80	5.50
ACDP JEV G4	2.20	3.90	5.70
VIDRL2 JEV G4	5.60	12.6	27.4

3.4. Recovery efficiency of JEV from wastewater samples

The recovery efficiency of JEV from two piggery wastewater samples (A and B) was calculated using Universal JEV, ACDP JEV G4, and VIDRL2 JEV G4 assays. Recovery efficiency was calculated as the percentage of seeded viral copies detected, with results presented as mean recovery efficiency ± standard deviation (SD) in Table 5 for two wastewater samples. For the Universal JEV assay, mean recovery efficiencies in wastewater Sample A were 34.3 ± 5.24 % at 80,000 copies, 45.9 ± 2.18 % at 8000 copies, and 27.8 ± 12.0 % at 800 copies. In wastewater Sample B, recovery efficiencies were lower, at 9.35 ± 16.9 % for 80,000 copies, 8.16 ± 3.31 % for 8000 copies, and not detected (ND) at 800 copies, as the viral concentration was below the PLOD. The ACDP JEV G4 assay showed mean recovery efficiencies in wastewater Sample A of 26.6 ± 2.76 % at 80,000 copies, 25.0 ± 1.70 % at 8000 copies, and 21.0 ± 10.2 % at 800 copies. In wastewater Sample B, recovery efficiencies were 20.3 ± 3.38 % at 80,000 copies, 21.3 ± 5.20 % at 8000 copies, and 14.9 ± 6.15 % at 800 copies, demonstrating consistent detection across all tested concentrations.

The VIDRL2 JEV G4 assay exhibited the highest recovery efficiency in wastewater Sample 1 at 80,000 copies (70.2 ± 9.35 %), followed by 56.2 ± 22.1 % at 8000 copies; however, JEV was not detected at 800 copies. In wastewater Sample B, recovery efficiencies were notably lower, at 7.13 ± 26.1 % for 80,000 copies and 1.80 ± 1.10 % for 8000 copies, with no detection at 800 copies due to the viral concentration being below the PLOD.

3.5. Detection of JEV I piggery wastewater samples

In total, 30 piggery wastewater samples (PWW 1–30) using three RT-qPCR assays targeting Universal JEV, ACDP JEV G4, and VIDRL2 JEV G4 RT-qPCR assays (Table 6). Each assay was run in triplicate per sample, and positive detection was recorded based on Cq values. Universal JEV assay detected JEV nucleic acid in 17/30 samples (≥1/3 replicates positive). ACDP JEV G4 assay detected JEV nucleic acid in 23/30 samples (≥1/3 replicates positive). The VIDRL2 JEV G4 assay showed no positive detections in any sample. These results suggest that the ACDP JEV G4 assay is more sensitive than the Universal JEV assay for detecting JEV in piggery wastewater. The VIDRL2 JEV G4 assay failed to detect JEV in any sample.

McNemar's test was used to compare paired proportions to determine whether the ACDP JEV G4 assay detected significantly more samples than the Universal JEV assay. Only discordant pairs were considered for the analysis. Among the tested samples, seven were

Table 2
Japanese encephalitis virus (JEV) RT-qPCR assay characteristics.

RT-qPCR assays	Assay performance characteristics			
	Efficiency (%)	R^2	Slope	Y-intercept
Universal JEV	92.3	0.994	−3.59	37.9
ACDP JEV G4	99.7	0.994	−3.33	37.5
VIDRL2 JEV G4	98.2	0.978	−3.37	38.9

Table 4
Japanese encephalitis virus (JEV) RT-qPCR process limit of detection (PLOD).

RT-qPCR assays	Process limit of detection (PLOD)		
	$PLOD_{50}$	$PLOD_{80}$	$PLOD_{95}$
Universal JEV	~80.8	~300	~800
ACDP JEV G4	~72.0	~146	~282
VIDRL2 JEV G4	~1000	~3000	~8000

Table 5
Recovery efficiency (%) of Japanese encephalitis virus (JEV) from piggery wastewater samples.

RT-qPCR assays	Number of copies seeded	Mean recovery efficiency ± SD (%)	
		Wastewater Sample A	Wastewater Sample B
Universal JEV	80,000	34.3 ± 5.24	9.35 ± 16.9
	8000	45.9 ± 2.18	8.16 ± 3.31
	800	27.8 ± 12.0	ND
ACDP JEV G4	80,000	26.6 ± 2.76	20.3 ± 3.38
	8000	25.0 ± 1.70	21.3 ± 5.20
	800	21.0 ± 10.2	14.9 ± 6.15
VIDRL2 JEV G4	80,000	70.2 ± 9.35	7.13 ± 26.1
	8000	56.2 ± 22.1	1.80 ± 1.10
	800	ND	ND

SD: standard deviation; ND: Not detected; JEV was below the process limit of detection at this concentration, preventing calculation of recovery efficiency.

positive by the ACDP JEV G4 assay but negative by the Universal JEV assay, while none were positive by the Universal assay alone. The results showed that the ACDP JEV G4 assay detected significantly more positive samples than the Universal JEV assay ($p < 0.05$), suggesting higher sensitivity. Overall, the ACDP JEV G4 RT-qPCR assay demonstrated the highest sensitivity for detecting JEV nucleic acid in piggery wastewater. Statistical analysis confirmed it outperformed the Universal JEV assay.

4. Discussion

The objective of this study was to identify the most sensitive RT-qPCR assay for detecting JEV in piggery wastewater by comparing three assays through experiments determining the ALOD and PLOD, as well as evaluating their detection sensitivity on real-world piggery wastewater samples. The study's results highlight differences in the sensitivity of the three RT-qPCR assays. The Universal JEV and ACDP JEV G4 assays demonstrated equal sensitivity based on ALOD. In contrast, VIDRL2 JEV G4 assay exhibited notably lower sensitivity under the conditions of our laboratory experiments. The VIDRL2 JEV G4 assay's reduced sensitivity, despite further optimization, likely stems from its degenerate primers, which may reduce binding efficiency and increase non-specific amplification risks. In this study, we employed a one-step RT-qPCR for all three assays in contrast to the two-step RT-qPCR used by Martin and colleagues (Martin et al., 2023), which may have reduced the sensitivity of the VIDRL2 JEV G4 assay. One-step RT-qPCR was chosen to minimize variability, contamination risk, and hands-on time due to its single-tube format, making it more suitable for high-throughput applications (Trindade et al., 2025). A recent study has reported one-step RT-qPCR may be more sensitive than two-step for detecting SARS-CoV-2 in wastewater (Raya et al., 2024).

While ALOD, commonly used in clinical diagnostics to determine the lowest detectable viral nucleic acid concentration in a sample, it does not account for the complexities of environmental samples (Hospodsky et al., 2010; Qu et al., 2024). PLOD, defined as the lowest concentration of JEV reliably detected after sample processing steps including virus concentration and nucleic acid extraction, is more relevant for wastewater surveillance (Ahmed et al., 2022; Ribeiro et al., 2023; Chen and Bibby, 2025). The ACDP JEV G4 assay's superior PLOD values compared to the Universal JEV and VIDRL2 JEV G4 assays underscores its robustness for wastewater applications, where low viral loads and complex matrices necessitate high process sensitivity for effective surveillance (Ahmed et al., 2022). However, the study's finding that all wastewater samples were free of PCR inhibitors suggests that the VIDRL2 JEV G4 assay's performance was probably due to intrinsic assay limitations rather than sample matrix effects.

The recovery of viral pathogens including JEV from piggery wastewater samples is a critical component of early detection strategies for surveillance, enabling timely interventions to prevent outbreaks in both

Table 6
Detection of Japanese encephalitis virus (JEV) in piggery wastewater samples using three RT-qPCR assays: Comparison of assay sensitivity based on number of positive replicates and Cq values.

Piggery wastewater sample ID (Farm location and sampling date)	Sample matrices	JEV RT-qPCR assays (no. of replicates positive/no. of replicates tested) (Cq values/ reaction)		
		Universal JEV	ACDP JEV G4	VIDRL2 JEV G4
PWW 1 (Shed, 12/02/2025)	Solid	2/3 (35.8, 37.8)	1/3 (36.6)	0/3
PWW 2 (Effluent Pond, 12/02/2025)	Solid	3/3 (36.3, 34.2, 35.0)	3/3 (34.8, 34.9, 34.2)	0/3
PWW 3 (Effluent Pond, 12/02/2025)	Liquid	1/3 (35.9)	3/3 (36.4, 36.5, 35.8)	0/3
PWW 4 (Shed, 21/02/2025)	Solid	2/3 (36.7, 37.0)	2/3 (36.3, 36.6)	0/3
PWW 5 (Shed, 21/02/2025)	Liquid	1/3 (35.3)	3/3 (36.8, 37.5, 36.3)	0/3
PWW 6 (Effluent Pond, 21/2/2025)	Solid	2/3 (35.3, 37.6)	3/3 (35.6, 36.7, 35.7)	0/3
PWW 7 (Effluent Pond, 21/02/2025)	Liquid	0/3	0/3	0/3
PWW 8 (Shed, 24/02/2025)	Solid	1/3 (37.5)	1/3 (36.2)	0/3
PWW 9 (Shed, 24/02/2025)	Liquid	1/3 (37.9)	2/3 (42.2, 37.2)	0/3
PWW 10 (Effluent Pond, 24/02/2025)	Solid	0/3	2/3 (35.1, 37.4)	0/3
PWW 11 (Effluent Pond, 24/02/2025)	Liquid	2/3 (36.5, 35.3)	0/3	0/3
PWW 12 (Shed, 27/02/2025)	Solid	1/3 (38.7)	1/3 (39.6)	0/3
PWW 13 (Effluent Pond, 27/02/2025)	Solid	1/3 (35.6)	2/3 (37.1, 36.3)	0/3
PWW 14 (Effluent Pond, 27/02/2025)	Liquid	0/3	1/3 (37.2)	0/3
PWW 15 (Shed, 03/03/2025)	Solid	3/3 (33.2, 32.9, 33.5)	3/3 (33.8, 33.4, 33.0)	0/3
PWW 16 (Shed, 03/03/2025)	Liquid	2/3 (35.5, 34.5)	3/3 (36.0, 34.4, 34.8)	0/3
PWW 17 (Shed, 06/03/2025)	Solid	0/3	1/3 (37.8)	0/3
PWW 18 (Shed, 06/03/2025)	Liquid	0/3	1/3 (36.5)	0/3
PWW 19 (Effluent Pond, 06/03/2025)	Solid	1/3 (39.9)	1/3 (41.0)	0/3
PWW 20 (Shed, 10/03/2025)	Solid	2/3 (36.7, 35.1)	3/3 (36.2, 35.6, 37.0)	0/3
PWW 21 (Shed, 10/03/2025)	Liquid	0/3	2/3 (37.8, 37.4)	0/3
PWW 22 (Effluent Pond, 10/03/2025)	Liquid	0/3	0/3	0/3
PWW 23 (Shed, 12/03/2025)	Solid	1/3 (36.7)	3/3 (39.2, 38.4, 38.4)	0/3
PWW 24 (Shed, 12/03/2025)	Liquid	0/3	0/3	0/3
PWW 25 (Effluent Pond, 12/03/2025))	Solid	0/3	1/3 (39.3)	0/3
PWW 26 (Effluent Pond, 12/03/2025)	Liquid	0/3	0/3	0/3
PWW 27 (Effluent Pond, 10/03/2025)	Liquid	0/3	0/3	0/3
PWW 28 (Effluent Pond, 10/03/2025)	Solid	0/3	1/3 (36.5)	0/3
PWW 29 (Shed, 27/02/2025)	Liquid	1/3 (37.6)	1/3 (35.5)	0/3
PWW 30 (Effluent Pond, 03/03/2025)	Liquid	0/3	0/3	0/3

animal and human populations (Ahmed et al., 2020; Ikner et al., 2012). However, little is known about the recovery efficiency of JEV from piggery wastewater, which differs from urban wastewater due to its unique composition, including higher organic matter and microbial content (Ahmed et al., 2025). In this study, for JEV recovery from wastewater samples, an AE method was used. This method can concentrate both enveloped and non-enveloped viruses from water/-wastewater (Miura et al., 2021; Ahmed et al., 2025). However, the virus extraction efficiency of the AE method in piggery wastewater has not been explored.

Data from the PLOD assessment, where known quantities of gamma-irradiated JEV (ranging from 80,000 to 8 copies) were seeded into wastewater samples, enabled the calculation of percent recovery for the AE method. The Universal JEV assay showed moderate recovery efficiencies (e.g., 34.3 % at 80,000 copies in wastewater Sample A, but only 9.35 ± 16.9 % in Sample B), while the ACDP G4 JEV assay demonstrated more consistent recoveries across concentrations (e.g., 26.6 % at 80,000 copies and 21.0 % at 800 copies in Sample A). The VIDRL2 JEV G4 assay achieved the highest recovery efficiency at 80,000 copies in Sample A (70.2 %) but failed to detect JEV at lower concentrations (800 copies) and showed poor performance in Sample B (e.g., 7.13 % at 80,000 copies). In this study, recovery efficiencies varied between samples and across RT-qPCR assays. This variability may be attributed to differences in the wastewater matrix composition and inconsistencies in the RT-qPCR standard curve parameters such as slope and Y-intercepts, which influenced recovery efficiency estimates. Using dPCR may improve accuracy in these instances, as it quantifies nucleic acids directly without relying on a standard curve. This could reduce errors caused by wastewater matrix inhibitors or inconsistencies in the qPCR standard curve, potentially leading to more reliable recovery efficiency measurements across diverse wastewater samples.

The ACDP JEV G4 assay's ability to detect JEV in 23 out of 30 real-world piggery wastewater samples, compared to 17 for Universal JEV, underscores its higher sensitivity. This aligns with its lower ALOD and PLOD values, indicating that the assay can reliably detect lower viral concentrations both in controlled conditions and in real world samples. The ACDP JEV G4 assay's ability to detect JEV at low Cq values further supports its sensitivity, making it well-suited for piggery wastewater surveillance where viral loads are typically low. The VIDRL2 JEV G4 assay failed to detect JEV in any of the 30 field samples under our laboratory conditions, consistent with its higher PLOD values, suggesting lower sensitivity for wastewater surveillance. However, these findings may require further verification possibly in other laboratories to fully assess the assay's performance.

The study has several limitations. First, the use of gamma-irradiated JEV may not fully replicate the behavior of infectious virions in wastewater, as irradiation could affect viral nucleic acid integrity or recovery efficiency during the AE method. Future studies should explore using non-infectious surrogates or inactivated JEV that better mimic live virus behavior. Second, the study tested only two wastewater samples for PLOD assessment and recovery efficiency, limiting the generalizability of results across diverse piggery effluents. Variability in wastewater composition (e.g., organic matter, pH) and standard curves likely contributed to differences in recovery between Samples A and B, and further research should include a larger sample set to account for matrix effects. Third, the study did not investigate the impact of different concentration methods beyond AE, such as ultrafiltration or polyethylene glycol (PEG) precipitation, which could improve recovery efficiency and lower PLOD values (Ahmed et al., 2020). Comparing multiple concentration methods could optimize JEV detection in low-viral-load samples.

5. Conclusions

- Based on empirical data, the ACDP JEV G4 assay is recommended for wastewater surveillance in regions with known genotype 4 circulation due to its high sensitivity and consistent performance.
- In areas with multiple genotypes, a dual-assay approach combining the ACDP JEV G4 and Universal JEV assays could balance sensitivity and broad detection capability.
- Implementing wastewater surveillance programs should involve regular monitoring of piggery effluents, integrated with clinical and serological data, to provide a comprehensive picture of JEV circulation.

CRediT authorship contribution statement

Angela Freddi: Writing – review & editing, Methodology, Investigation. **Metasebia Gebrewold:** Writing – review & editing, Methodology, Investigation. **Wendy Smith:** Writing – review & editing, Methodology. **Elena Manini:** Writing – review & editing, Supervision. **Stuart Simpson:** Writing – review & editing, Resources. **Warish Ahmed:** Writing – original draft, Supervision, Methodology, Formal analysis, Conceptualization.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.jviromet.2025.115272.

Data availability

Data will be made available on request.

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