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FINAL RESEARCH GRANT REPORT FORMAT

Project Title and Project Identification Number
Establish and validate RT-rtPCR for detecting Japanese Encephalitis Virus in porcine samples, Project #23-045 SHIC

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Industry Summary:
Japanese Encephalitis Virus (JEV) is a severe disease affecting pigs and humans. It is spread mainly by mosquitoes and is endemic in many parts of Asia and the western Pacific regions. However, its recent outbreak in Australia posed a renewed threat to the US pig industry and public health. Therefore, it is crucial to have a reliable way to detect JEV in pigs before it causes an outbreak. The current project aims to detect JEV rapidly using viral RNA and Reverse Transcription real-time polymerase chain reaction (RT-rtPCR) assays. This project established RT-rtPCR assays for JEV and its five genotypes (G-1, -2, -3, -4, -5) and compared the newly established test with a previously published assay. The findings from this project suggest that the newly developed RT-rtPCR assay is more specific and can detect all five genotypes accurately and efficiently. It also had a low detection limit, meaning it could detect very small amounts of the virus. Validation of genotype-specific RT-rt PCR assay and evaluation of the assay performance on known status clinical samples in collaboration with Australia is ongoing. Our project developed a novel and reliable test for JEV that can help the pig industry and public health authorities monitor and prevent JEV outbreaks.

Keywords: Japanese Encephalitis Virus (JEV), RT-rtPCR, real-time PCR, genotypes, diagnostic

Scientific Abstract:
Japanese Encephalitis Virus (JEV) is a mosquito-borne virus that causes viral encephalitis. Pigs become infected through a bite from infected mosquitoes, primarily Culex species. In general, transmission is through the movement of infected mosquitoes, often over long distances, due to wind dispersal and the movement of migratory water birds. Water birds can carry the virus but do not show illness. JEV is endemic in many parts of Asia, but with the re-emergence of JEV in February 2022 in Southern Australia, a potential outbreak in the pig population in the USA can have detrimental economic and zoonotic implications globally. Therefore, it is crucial to be vigilant and have a screening method for early detection of this virus in the pig population when clinical signs suggest JEV as a differential.

The current study aims to establish an RT-rtPCR for JEV (species level) and its five genotypes (G-1, -2, -3, -4, -5). The TaqMan-based primer/probe (p/p) sequences used in this study were either designed or previously published to target viral genes NS and M. All reactions included controls with the same thermal cycling conditions. Standard curves generated with known quantities of synthetic RNA oligos were used to determine the limit of detection (LOD) of RT-rtPCR assays. The current study developed a species-level RT-rtPCR assay for JEV and compared its specificity to the previously published assay. The screening RT-rtPCR assays were negative for St. Louis encephalitis, Zika, Dengue, Yellow fever, and other arthropod-borne viruses (Tahyna orthobunyavirus, Getah Virus, and Sindbis Virus). In addition, the RT-rtPCR assay did not cross-react with PRRSV, PRV, PHEV, PPV1, PTV, PEDV, PDCoV, TGEV, PSV, porcine Astro3/Astro4, and PPV2. However, p/p from the previously published assay cross-reacted with West Nile virus RNA. In the case of genotype-specific RT-rtPCR assays, p/p of G-1 and G-3 were specific for different regions of viral gene NS2 (non-structural protein 2) and can detect as low as 125 copies/µL of synthetic RNA. The genotype-specific RT-rtPCR amplification efficiency for JEV G-1 and -3 was 100.1% and 106.1%, respectively. Five virus strains belonging to G-1 and G-3 were tested and had a Ct < 20 with both screening and genotype-specific RT-rtPCR assays. This study developed a species-level RT-rtPCR for JEV and evaluated the analytical sensitivity and specificity of JEV RT-rtPCR for G-1 and G-3. Currently, more studies

are in progress to validate the RT-rtPCR assays for G-2, G-4, and G-5, and the assay is further evaluated for diagnostic sensitivity and specificity in Australia.

Introduction:

Japanese Encephalitis Virus (JEV) is a mosquito-borne virus that causes viral encephalitis. Pigs become infected when infected mosquitoes bite them, primarily Culex species. Infected pigs do not transmit the infection to other animals. However, mosquitoes may transmit the virus while the animals have it in their blood [4]. Hence, it acts as a reservoir for JEV and is crucial in transmitting the virus to humans. Humans and other animals such as horses, cats, dogs, cattle, sheep, and goats are dead-end carriers. They cannot usually transmit the disease back to mosquitoes [1,6]. In general, the spread is through the movement of infected mosquitoes, often over long distances, due to wind dispersal and the movement of migratory water birds [5]. Water birds can carry the virus but do not show illness [3]. JEV is endemic in many parts of Asia, including China, Japan, Korea, and Southeast Asia. The virus has also been reported in Australia, the Pacific Islands, and South Asia. There are five genotypes (Genotypes I, II, III, IV, and V) of JEV, which are classified based on the nucleotide sequence of the envelope gene. Genotypes I and III are the most prevalent in Asia, while Genotype II is primarily found in Indonesia and Australia. Detection of genotype IV in southern Australia was attributed to the dissemination of JEV to local mosquitoes in the Tiwi islands, followed by migratory birds with subsequent amplification by domestic and feral pigs, which was postulated as the possible route [3]. The clinical signs of JEV infection in pigs can vary depending on the severity of the disease. JEV can cause respiratory signs, reproductive losses (abortions, mummified or still-born fetuses), and neurological signs such as ataxia, tremors, and convulsions leading to encephalitis. In mild cases, infected pigs may show no signs or only exhibit mild fever, lethargy, depression, and anorexia. After the virus enters the body through a mosquito bite, it replicates in the lymph nodes and then spreads to the central nervous system. The virus can infect other organs, such as the liver, spleen, and kidneys.

Statement of the problem:

With the re-emergence of JEV in February 2022 in Southern Australia [2], the North American domestic pig population is at risk of infection based on experimental studies conducted in the United States (US)[7]. In October 2022, SHIC sponsored a symposium on JEV dedicated to gaining deeper insights into the JEV outbreak in Australia [10]. Understandably, a potential outbreak in the pig population in the USA can have detrimental economic and zoonotic implications globally. Therefore, it is crucial to be vigilant and have a screening method to detect this virus very early in the pig population when clinical signs suggest JEV as a differential. Although it is impractical to determine the specific transmission route, it is possible to establish a robust diagnostic assay for a differential diagnosis that includes JEV and its genotypes. The most common diagnostic methods for JEV infection in pigs include serological tests such as the enzyme-linked immunosorbent assay (ELISA) and virus isolation from tissue samples [8]. However, RT-rtPCR can be a quicker, more reliable, and cost-effective approach to detect the presence of JEV and its genotypes in porcine samples. Unfortunately, there is currently no FADDL or NAHLN-approved RT-rtPCR that can detect JEV or its genotypes. Unlike ASF/CSF/FMD, the (National Veterinary Services Laboratories) NVSL provides no proficiency testing panel to VDLs across the US to further validate an RT-rtPCR. Henceforth, there is an immediate need to establish an RT-rtPCR for JEV and its genotypes following NAHLN laboratory guidelines and with cooperation from FADDL.

Objectives:

1. Establish a reverse transcriptase-real-time polymerase chain reaction (RT-rtPCR) assay for detecting Japanese Encephalitis Virus (JEV) and its five genotypes based on the American Association of Veterinary Laboratory Diagnosticians (AAVLD) and the National Animal Health Laboratory Network (NAHLN) guidelines.
2. Validate analytical sensitivity of RT-rtPCR for JEV and its genotypes using synthetic targets and inactivated virus isolates from reference laboratories.
3. Perform analytical specificity RT-rtPCR for JEV and its genotypes using pathogens recommended for differential diagnosis by the World Organization for Animal Health and other viruses associated with encephalitis.

Materials & Methods:

Samples used:

All the samples used in this study followed the Iowa State University Biosafety guidelines and were obtained after signing a Material Transfer Agreement (MTA) with the World Reference Center for Emerging Viruses and Arboviruses (WRCEVA), and Biodefense and Emerging Infections (BEI) Research Resources Repository. In case of Porcine reproductive and respiratory syndrome virus (PRRSV) or Betaarterivirus suid 1, Pseudorabies virus (PRV),

Porcine haemagglutinating encephalomyelitis virus (PHEV), Porcine parvovirus 1 (PPV1), Porcine parvovirus 2 (PPV2), Porcine teschovirus, Porcine epidemic diarrhea virus (PEDV), Transmissible gastroenteritis virus (TGEV), Porcine deltacoronavirus (PDCoV), Porcine sapelovirus (PSV), Porcine astrovirus 3 (Past3), and Porcine astrovirus 4 (Past4), known positive samples in ISU-VDL were used for this study. The samples used in this project were stored in -80°C freezers continuously monitored as AAVLD guidelines and voided repeated freeze-thaw cycles.

Viral RNA extractions:

Total viral RNA from JEV and other arthropod-borne viral samples in TRizol reagent (ThermoFisher Scientific) was isolated using chloroform phase separation followed by column-based RNA extraction protocol according to kit manufacturer's instructions (QIAGEN, Hilden, Germany). The total RNA elutes obtained for each sample was 50 µL. In the case of Zika, Dengue, and Sindbis samples, viral RNA elutes were supplied in 100 µL format. All other viral RNA extraction was performed using the MagMAX Pathogen RNA/DNA Kit isolation kit (Thermo Fisher Scientific) and a Kingfisher flex instrument (Thermo Fisher Scientific) according to the NVSL-SOP-0644.04 with minor modifications. All reagents and consumables were stored per the manufacturer's instructions to maintain integrity.

Data analysis:

Data obtained from RT-rtPCR assays was analyzed using design and analysis software 2.6 (ThermoFisher Scientific), Microsoft Excel, and GraphPad Prism 10. Standard curves were plotted using GraphPad with Ct values on the y-axis and log concentration per milliliter on the x-axis. Ct values above 36 were considered as threshold cut-off.

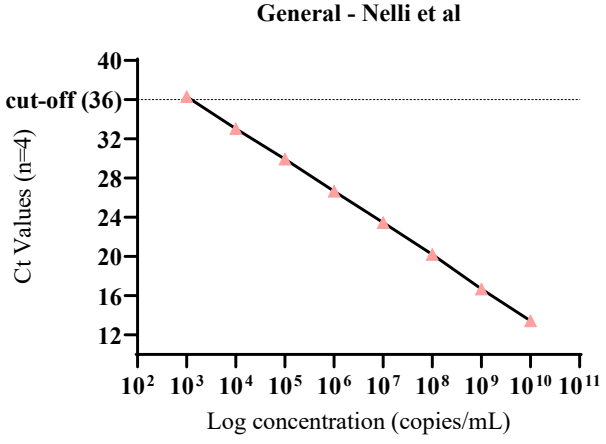
Results:

Objective 1 – Establish RT-rtPCR assay:

For this study, p/p specific for different genotypes of JEV were from previously published sequences (Table 1[9]). Primers and probes that detect all genotypes of JEV, i.e., species-level (JEV All) were designed by Dr. Nelli and compared against the designs of Shao et al., 2018. A primer concentration of 800 nM and a probe concentration of 400 nM was used for all JEV RT-rtPCR assays. For positive extraction control, exogenous internal positive control (XIPC) had a primer concentration of 400 nM and probe concentration of 150 nM. Along with samples, all assays included a positive extraction control, positive amplification control (PAC, synthetic RNA), and negative extraction control (nuclease-free water). The analytical sensitivity of RT-rtPCR assay was performed using synthetic Ultramer RNA Oligos (IDT), synthesized for each of the target regions of JEV. All RT-rtPCR assays were carried out in ABI-7500 Real-Time PCR instruments with 4X TaqMan Fast Virus 1-Step Master Mix using thermal cycling conditions: 50 °C for 5 min holding for reverse transcription, 95°C for 20 s denaturation, 40 cycles of 95°C for 3 s, and 60°C for 30 s for amplification.

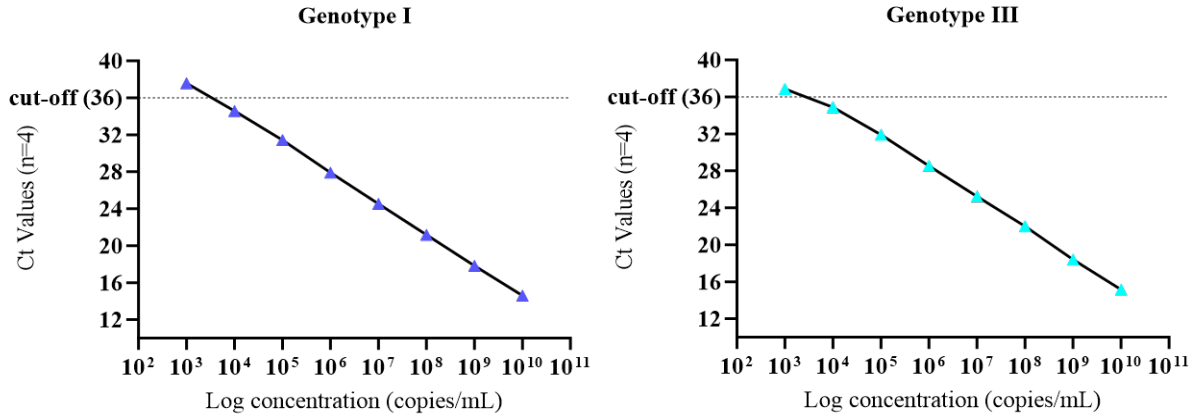
Table 1: *Primer and probe sequences (IDT, Coralville, Iowa)*

Primer/probe	Sequence (5-3)	Genomic region	Amplicon
JEV G5 For Shao	TGCGACAAACAAGCCGTGTA	M	87
JEV G5 Rev Shao	TTGCACTGACACAGATCTTCTACTTCT		
JEV G5 Probe Shao	CGTTGCACGAGGACCAGGCACTC		
JEV G4 For Shao	TTCAATATGGACGGTGCACAA	M	73
JEV G4 Rev Shao	CCATGCGTGTGGACAGACA		
JEV G4 Probe Shao	AACCTCACACTCCCAGACAAGCAGGAGATC		
JEV G2 For Shao	GAAGACACCATCACCTACGAATGTC	M	73
JEV G2 Rev Shao	CACACCAGCAATCCACATCCT		
JEV G2 Probe Shao	CAAGCTCACCACAGGCAATGACCCA		
JEV G1/G3 For Shao	GGTCTGCAACCCAAACAAGAA		
JEV G1/G3 Rev Shao	GCCAGCATGAAGGGTATTGACAT		
JEV G1 Probe Shao	TTGTGGGAGGTCTAGCCGAGTTGG	NS2	132
JEV G3 Probe Shao	TCGTAGGTGGTTTGGCCGAGTTG	NS2	132
JEV All For Shao	GCCACCCAGGAGGTCCTT	NS1	62
JEV All Rev Shao	CCCCAAAACCGCAGGAAT		
JEV All Probe Shao	CAAGAGGTGGACGGCC		
JEV All For Nelli	[redacted]	[redacted]	[redacted]
JEV All Rev Nelli	[redacted]		
JEV All Probe Nelli	[redacted]		



Objective 2- JEV RT-rtPCR analytical sensitivity:

Because of the limited availability of the inactivated virus isolates from the reference laboratories, we could not generate RT-rtPCR assay standard curves. However, to evaluate the analytical sensitivity of the JEV RT-rtPCR assays, synthetic RNA called Ultramers were used to establish the standard curves. The newly developed RT-rtPCR assay specific for species-level JEV genotypes (Nelli) has a standard curve with a slope of -3.2648, giving it an amplification factor of 2.02 and PCR efficiency of 102.5%. The assay can detect as low as 1322 copies of the JEV NS1 gene in a milliliter sample. The JEV RT-rtPCR assay specific for genotypes I and III (Shao et al.), GI and GII have slopes of -3.319 and -3.238, giving them an amplification factor of 2.00; 2.04 and PCR efficiencies of 100.1%, 102.5% with lowest detection of 3561; 3119.5 copies of JEV-GI and GIII NS2 gene in a milliliter of sample, respectively.



***Objective 3*- JEV RT-rtPCR analytical specificity as recommended by WOAH**

The p/p established by Shao et al., 2018 for all five genotypes of JEV and another p/p set (Nelli), designed by Dr. Rahul Nelli, PI of this project, were used for this study. Both these p/p sets were compared for their specificity in strains of St. Louis encephalitis virus, Zika virus, Dengue virus, Yellow fever virus, and other arthropod-borne viruses (Tahyna orthobunyavirus, Getah Virus, and Sindbis Virus). Interestingly, the p/p set developed by Shao cross-reacted with West Nile Virus strains (B956 and TX 40909D1660), while the p/p set developed by Nelli did not cross-react (Table 2).

Table 2: *Comparative JEV All RT-rtPCR assay specificity results*

Specimen	Strain	Nelli et al.	Shao et al.
SLEV Virus	V 07457	>= 40	>= 40
SLEV Virus	TX AR 9-6038	>= 40	>= 40
Zika Virus	Mex 2-81	>= 40	>= 40
Zika Virus	FLR	>= 40	>= 40
Dengue Virus	Type 1 (hawaii)	>= 40	>= 40
Dengue Virus	Type 2 (Denv-2)	>= 40	>= 40
Dengue Virus	Type 3 (Denv-3)	>= 40	>= 40
Dengue Virus	Type 4 (H241)	>= 40	>= 40
Yellow Fever Virus	17D	>= 40	>= 40
Tahyna Virus	92	>= 40	>= 40
Getah Virus	MM 2021	>= 40	>= 40
Sindbis Virus	EgAr 339	>= 40	>= 40
West Nile Virus	B 956	>= 40	27.57
West Nile Virus	TX 4090 9D1660)	>= 40	24.13
Positive amplification control	Synthetic RNA	17.26	17.03

In addition, the analytical specificity was further confirmed by testing negative (>=40) against the following viruses: PRRSV, PRV, PHEV, PPV1, PPV2, PEDV, TGEV, PDCoV, PSV, Past3, Past4, and Porcine teschovirus. All the controls performed as expected. The negative extraction and amplification controls had Ct >=40. Internal positive control XIPC ranged between 29-33 Ct, while positive amplification control for JEV was < 20 Ct.

Discussion:

This project is focused on establishing an RT-rtPCR assay for JEV and its genotypes. The aim is to validate this assay per AAVLD and NAHLN guidelines. However, obtaining virus isolate samples from the WRCEVA and BEI was a significant challenge due to the multiple legal and biosafety approvals required at different stages. In addition, there are limitations on the number of samples that can be requested within a fiscal year. Identifying the JEV genotypes from the WRCEVA biobank list proved challenging because they were poorly cataloged and annotated. JEV genotype V was particularly problematic as there was only one virus isolate, and it was contaminated with *Mycoplasma sps*.

Consequently, WRCEVA did not provide that sample, which means that the RT-rtPCR assay for JEV genotype V cannot be validated in this project. Similarly, the JEV RT-rtPCR assay validation for genotypes II and IV requires further validation as there was a delay in getting virus isolate samples from WRCEVA. Additionally, it appears that the p/p designed by Shao et al. did not detect all JEV genotypes, which further complicated the validation process. Therefore, Dr. Nelli has designed an additional p/p set currently validated for GII and GIV genotypes.

The species-level RT-rtPCR assay Dr. Nelli and Shao designed can detect all JEV genotypes with comparable Ct values. However, when analyzed *in silico,* the p/p set designed by Shao et al. showed a 100% identity with West Nile virus isolate JA 22 and 22C2285. This in-silico analysis was further confirmed by the RT-rtPCR assay when tested with West Nile virus strains B956 and TX 4090 9D1660. In contrast, the p/p set designed by Nelli did not cross-react with the West Nile virus or other arboviruses and common porcine viruses, indicating a robust analytical specificity. The RT-rtPCR assay by Nelli for species-level JEV detection also had a PCR efficiency greater than 100%, making it an ideal assay.

The current research involving JEV detection by Nelli's RT-rtPCR assay has shown promising results. However, before the assay can be used for routine diagnostics, it must be further validated for diagnostic specificity and sensitivity. To accomplish this, a collaborative effort will be undertaken with Dr. Peter Kirkland, utilizing samples from Australia. This collaborative research aims to ensure that the assay is accurate and reliable in detecting JEV in various conditions and clinical samples. By validating the assay using these methods, we can have greater confidence in its ability to help identify and prevent the spread of JEV in pigs. The assay can be further expanded to be used in other animals and possibly humans.

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Research and/or collaboration outcomes:

1. Nelli RK*, Bradner LK, Gauger PC, De Jong A. Development and evaluation of RT-rtPCR assay for detecting Japanese Encephalitis Virus in porcine samples – an update. American Association of Veterinary Laboratory Diagnosticians (AAVLD), 66th Annual Meeting. National Harbor, Maryland. October 12-18, 2023.
2. Nelli RK*, Bradner LK, Gauger PC, De Jong A. Evaluation of RT-rtPCR assay for detecting Japanese Encephalitis Virus in porcine samples. 2023 NAPRRS/NC229: International Conference of Swine Viral Diseases. Chicago, Illinois, USA. Dec 2-4, 2023.
3. Established collaborative opportunities with Dr. Peter Kirkland, Adjunct Professor, James Cook University, Australia.
4. Established close working opportunities with NAHLN assay validation team Dr. Chung, Chungwon, Section Head, Proficiency and Validation Service Section, Foreign Animal Disease Diagnostic Laboratory, Plum Island Animal Disease Center, National Veterinary Services Laboratories.

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