Comparative evaluation of reverse transcriptase-quantitative polymerase chain reaction assays for the detection of Japanese encephalitis virus in swine oral fluids

by

Amy Christina Lyons

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Major Professor

Dr. Dana Vanlandingham

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Abstract

Japanese encephalitis virus (JEV) is a mosquito-borne flavivirus maintained among swine and avian species. In infected pigs, replication of JEV leads to the onset of viremia and the development of neurological and reproductive disease in young and naïve pregnant animals. The high-titer viremia levels associated with JEV infection in pigs, whilst important to the enzootic transmission cycle responsible for viral maintenance, also have human health implications within the zoonotic cycle. Sensitive and specific veterinary diagnostic methods capable of readily detecting JEV infection are critical components of JEV surveillance programs in the Asian Pacific region. In this study, reverse transcriptase-quantitative polymerase chain reaction (RT-qPCR) assays were evaluated for use in veterinary diagnosis of JEV. Our hypotheses for this research project were that RT-qPCR assays with fewer oligonucleotide mismatches between the primers and probes of the assays and JEV genomes will be more sensitive for the diagnosis of JEV infection and that oral shedding of JEV in swine would allow for detection of viral RNA using oral fluids. The sensitivity and specificity of three RT-qPCR assays for the detection of JEV were determined using tissue culture fluids of five representative JEV strains belonging to four endemic genotypes. The first assay (assay #1), targeting the highly conserved NS5 gene and 3′UTR regions, provided optimum detection for the current predominant genotype, GI-b. All three assays were highly specific for JEV when tested against other selected flaviviruses in the JEV serocomplex. A rope-based collection method allowed for the simplified collection of oral fluids from three-week-old piglets challenged with endemic JEV strain JE-91. These fluids were then evaluated using RT-qPCR assays for the presence of viral RNA. The results suggest that the shedding of JEV in oral fluids can be readily detected and that non-invasive oral fluid collection can serve as a novel sampling method for the diagnosis and surveillance of JEV in swine.

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Chapter 1 - Introduction to Japanese Encephalitis Virus and Associated Veterinary Diagnostics

Brief introduction

Japanese encephalitis virus (JEV) is a single-stranded positive sense RNA virus approximately 11 kilobases in length. JEV and other related flaviviruses form the JEV serocomplex within the *Flavivirus* genus, the *Flaviviridae* family.

JEV has been known to be perpetuated in a cycle between *Culex* species mosquitoes and avian and swine amplification hosts. Infection of the amplification hosts rarely leads to high mortality. Although reproductive failure has been reported in pregnant sows infected with JEV, most classes of swine rarely show significant clinical symptoms. Infected humans and equines occasionally develop neurotropic disease as incidental hosts, which do not reach viremic levels sufficient to sustain mosquito-borne transmission cycles. Japanese encephalitis (JE) is the leading cause of pediatric encephalitis in the Asian Pacific region and it is estimated that approximately 68,000 human cases of JE occur each year within the endemic regions (Campbell et al., 2011; Cappelle et al., 2016).

The development of diagnostic and surveillance protocols capable of detecting JEV infection in pigs, a main amplification host for the virus, is critical for the monitoring and prediction of JEV outbreaks. In this study, three reverse transcriptase-quantitative polymerase chain reaction (RT-qPCR) assays were assessed for their feasibility in application to veterinary diagnosis of JEV in swine. The tested hypotheses were that the assays will vary in suitability based on differences in oligonucleotide matching between primer and probes and JEV genomes

and that the assays would be able to detect JEV viral RNA in serum as well as oral fluid samples collected from JEV infected pigs.

Genomic structure

The genome of JEV (Figure 1.1) encodes a single open reading frame (ORF) flanked by the untranslated region (UTR) at 5′ and 3′ ends of the genome. Ribosomal translation of the ORF leads to the synthesis of one polyprotein. Individual viral proteins are produced by the proteocleavage of host and viral proteases and lead to three structural proteins and seven non-structural proteins (Mukhopadhyay et al., 2005). The 5′ end is modified by the cap structure through the catalysis of the methyltransferase domain of the nonstructural protein 5 (NS5). Molecular interactions among the 5′ cap structure, 5′ UTR and 3′ UTR regulates the initiation of RNA translation and replication in infected cells (Bollati et al., 2010). The three structural genes encoded in the 5′ quarter of the genome give rise to three structural proteins, capsid (C) proteins, membrane precursor (prM) and envelope (E) (Chambers et al., 1990; Mukhopadhyay et al., 2005). C protein is responsible for the encapsidation of viral genome in progency virions (Unni et al., 2011; Mukhopadyay et al., 2005). prM, a glycoprotein, assists with the proper folding and assembly of E protein in immature virions and prevents the premature viral membrane fusion in the acidic intracellular compartments during the exocytosis process as a molecular chaperone. Virion maturation through the cleavage cellular furin-like protease remove the pr peptide and grants the infectivity of progeny virions prior to the secretion into the extracellular space (Unni et al., 2011). E protein is the most abundant proteinaceous component on the surface of virions. Individual E proteins consist of three distinct domains, domain I (EDI), II (EDII) and III (EDIII), and are involved in the receptor binding, viral entry, membrane fusion of infectious viruses. E

protein is also is a major target of neutralizing antibodies, which can be used as an immunogen for vaccine development (Unni et al., 2011).

Seven nonstructural proteins (NS): NS1, NS2a, NS2b, NS3, NS4a, NS4b, and NS5, are essential for viral replication (Bollati et al., 2010; Mukhopadhyay et al., 2005). NS3 and NS5 are essential enzymes for the synthesis, replication and modification of viral RNA. NS3 contains both N-terminal domain and a C-terminal domain which contains RNA triphosphatase and RNA helicase, enzymes required for capping and viral RNA synthesis (Wengler et al., 1991; Bollati et al., 2010). NS5 contains both an N-terminal methyltransferase (MTase) domain and C-terminal RNA-dependent RNA polymerase (RdRp) domain (Bartholomeusz et al., 1993; Egloff et al., 2002). RdRp uses the positive-sense viral genome as a template to synthesize negative-sense replication intermediates, which becomes the template for the synthesis of progeny genomes. The complex consisting of NS2b and NS3 proteins confers the serine protease activity, which is responsible for the processing of polyprotein (Chambers et al., 1993). In addition to the known functions as replicases, multiple reports have indicated multiple flavivirus nonstructural proteins also promote the establishment of viral infection as immune modulators against host immune signaling pathways.

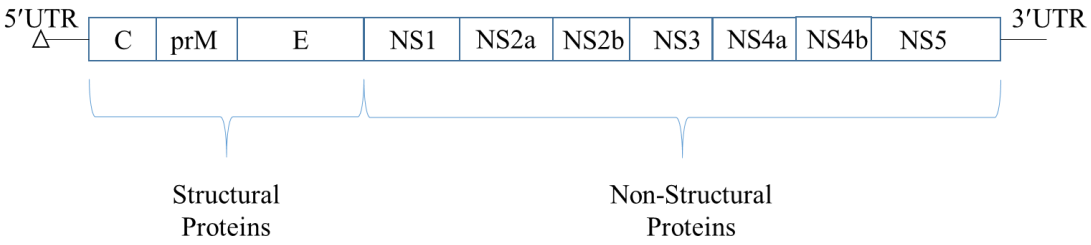

Figure 1.1 Genome structure of Japanese encephalitis virus.

Clinical signs

The majority of JE infections in humans are mild (Figure 1.2), with ratios of asymptomatic to symptomatic infection ranging from 1 in 50 to 1 in 400 depending on geographic location (Weaver & Barrett, 2004). When clinical symptoms present, non-specific febrile illness is usually the first symptom. Patients may then develop other symptoms including headache, vomiting and reduced levels of consciousness. Convulsions and seizures occur more frequently in children than in adults. Changes of the respiratory pattern and abnormalities in posturing and reflexes are signs of poor prognosis (Soloman et al., 2000). Approximately 30 percent of clinical patients die and 30-50% of survivors suffer permanent neurologic, psychiatric and cognitive deficits (Solomon et al., 2000; WHO, 2015).

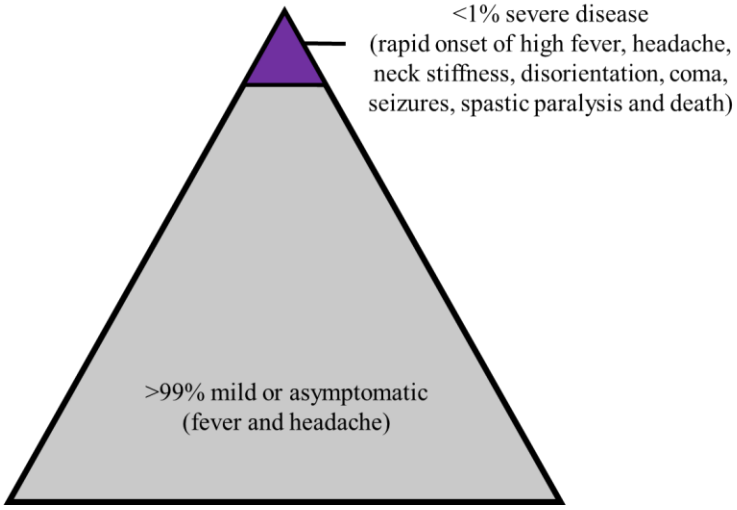

Figure 1.2 Clinical manifestations of Japanese encephalitis in humans (WHO, 2015).

Clinical signs in equids (horses and donkeys) vary with symptoms ranging from moderate fevers and inappetence to more severe symptoms such as high fevers (41°C/106°F), paralysis, aggressive behavior, coma and death (Fernández, 2010; Kheng et al., 1968; Gould et al., 1964).

Cases typically present in one of three described manifestations: transitory type syndrome, lethargic type syndrome or hyperexcitable type syndrome (Fernández, 2010).

Swine are largely impacted by JEV infection through reproductive losses. Sows suffer abortions, usually at term, and sperm quality in boars is compromised (Burns et al., 1950; Van den Hurk et al, 2009; Hsu et al., 1972; Imoto et al., 2010). Piglets that are born with JE suffer neurologic symptoms including tremors, convulsions and death (Fernández, 2010; Yamada et al., 2004). Adult pigs are mostly unaffected by JEV infection.

Transmission

Japanese encephalitis virus is transmitted within an enzootic cycle involving avian and swine amplification hosts and competent mosquito vectors. Traditionally, JE has been associated with rural environments in Asia where the presence of rice paddy fields and pigs are common. A recent study in Cambodia used sentinel pigs to demonstrate that JEV is circulating in periurban areas outside of their capital city (Capelle et al., 2016). JEV has the ability to make use of a range of hosts and vectors, thus providing opportunities for its spread in a wide range of environments.

Humans and equines suffer as incidental or "dead-end" hosts, meaning their viremia does not reach levels adequate to contribute to the maintenance cycle of the virus. Historically, *Culex* species mosquitoes, and more specifically *Culex tritaeniorhynchus*, have been considered the most significant vector for the disease (Rosen et al., 1986). While *C. tritaeniorhynchus* may be involved in the majority of JE outbreaks, there are several mosquito species proven to be competent vectors for JEV including: *Culex annulirostris*, *Culex vishnui* (*C. annulus*), *Culex gelidus*, *Culex juscocephala*, *Culex pipiens*, and *Culex quinquefasciatus* (Rosen et al., 1986;

Ritchie et al., 1997; Wispelaere et al., 2017; Huang et al., 2015). Less clear is the role of *Aedes* species mosquitoes in the JEV cycle; a handful have been proven competent JEV vectors either through experimental challenge or by virus isolation from field samples (Rosen et al., 1986; de Wispelaere et al., 2017). Pigs and ardeid birds, such as herons and egrets, have been proven as the major maintenance reservoirs for JEV and their presence is vital to the transmission cycle (Scherer et al., 1959; Boyle et al.,1983; van den Hurk et al., 2009).

Whilst seroprevalence of JEV is common in several mammalian species (cows, goats, dogs, raccoons, rodents), pigs are the only mammal known to serve as an amplification host (Ohno et al., 2009; Tuno et al., 2017). Pigs are able to fill this role because of their high natural infection rates, duration of high viremia (4 days), host preference of competent mosquito vectors for pigs, and because the high birth rate of pigs allows for quick population turnover, providing susceptible naive hosts (Scherer et al., 1959; van den Hurk et al., 2009; Pitzer et al., 2016; Ricklin et al., 2016; Tuno et al., 2017). Although traditionally we think of domestic pigs as the leading JEV mammalian amplification host, feral swine species can also be implicated in the JEV transmission cycle. According to Ruiz-Fonz et al., JEV is a viral pathogen of high risk for transmission to humans from wild swine (Ruiz-Fonz et al., 2017; Hamano et al., 2007). Infection of JEV in wild boars in Japan has been demonstrated by the presence of neutralizing antibodies and is speculated to have led to human cases of JE (Ohno et al., 2009). JE outbreaks in humans can be predicted by the detection of JEV in nearby swine through the use of sentinel pigs or by diagnosed cases in domestic pig herds (Okuno et al., 1973).

Wading ardeid birds are also known to experience high viremia of adequate duration to infect competent mosquito vectors (Boyle et al., 1983; Nemeth et al., 2012). Their strong presence, alongside competent mosquito species, in the flooded rice paddy fields of Asia has

enabled JEV to thrive there. Although seroconversion in several avian species, including domestic ducks, has been documented, fewer species have demonstrated viremia levels sufficient to contribute to the JEV cycle (Kalaiyarasu et al., 2016; Nemeth et al., 2012). Red-winged blackbirds, rock pigeons, European starlings, house finches, ring-billed gulls have all demonstrated moderate to high levels of viremia experimentally (Nemeth et al., 2012). Whilst the current thought is that wading birds are the most significant avian host, the transmission cycle of JEV is complex and capable of adapting to different environments. As the techniques for the detection of viral infections continue to improve, discoveries of additional species supporting the transmission cycles of JEV remains likely.

Vector-free transmission

A major advancement of our knowledge in the transmission and disease pathogenesis of JEV is the recent discovery showing the transmission of JEV can take place without competent arthropod vectors. Recent studies showed the ability of swine to shed JEV in oronasal secretions as well as their susceptibility to oronasal infection (Ricklin et al., 2016). The spread of JEV from needle-infected pigs through direct contact was demonstrated by co-housing of infected and immunologically naïve pigs. Infectious viruses were isolated from oronasal swabs of both needle-infected and subsequently contact-infected pigs. Under laboratory conditions, domestic pigs were highly susceptible to JEV through direct contact as the minimal infectious doses required for the establishment of infection were as low as 10 median tissue culture infectious dose ($TCID_{50}$)(Ricklin et al., 2016). This process allows immunologically naïve pigs to acquire infection of JEV from infected pigs through direct contact in the absence of competent vectors. The ability of pigs to maintain JEV transmission in the absence of a mosquito vector may explain the outbreaks of JEV-related abortion and stillbirth reported in pig populations, in temperate

regions, prior to the significant proliferation of mosquito populations in nature, as reported in Hokkaido, Japan (Ricklin et al., 2016; Takashima et al., 1988).

Geographic spread

Epidemics of summer encephalitis caused by JEV have been described in Japan since the late 19th century, but the first isolate of the etiological agent was not available until 1935 (Webster et al., 1937). It was first isolated in mice from the brain of a man who succumbed to summer encephalitis in Tokyo, Japan. Since then, JEV has circulated throughout most of Asia and the Pacific region (Figure 1.3). Prior to the development of vaccines, outbreaks leading to thousands of fatal cases were reported throughout East Asia (Rosen et al., 1986). In the late 20th century, JEV spread its geographical presence to countries including Saipan (Paul et al., 1993), Pakistan (Igarashi et al., 1994) and northern Australia (Hanna et al., 1999). In the first two decades of the 21st century, detection of JEV has also begun to be reported outside the Asian Pacific region. A partial genomic sequence of JEV was detected in a pool of *Culex pipiens* mosquitoes collected in Italy suggesting possible circulation in Europe (Ravanini et al., 2012). More recently, infection of JEV was reported in a Sudanese individual dually infected with yellow fever virus (Simon-Loriere et al., 2017). However, neither of the cases of positive molecular detection led to the conclusive evidence based on virus isolation to support the autochthonous transmission.

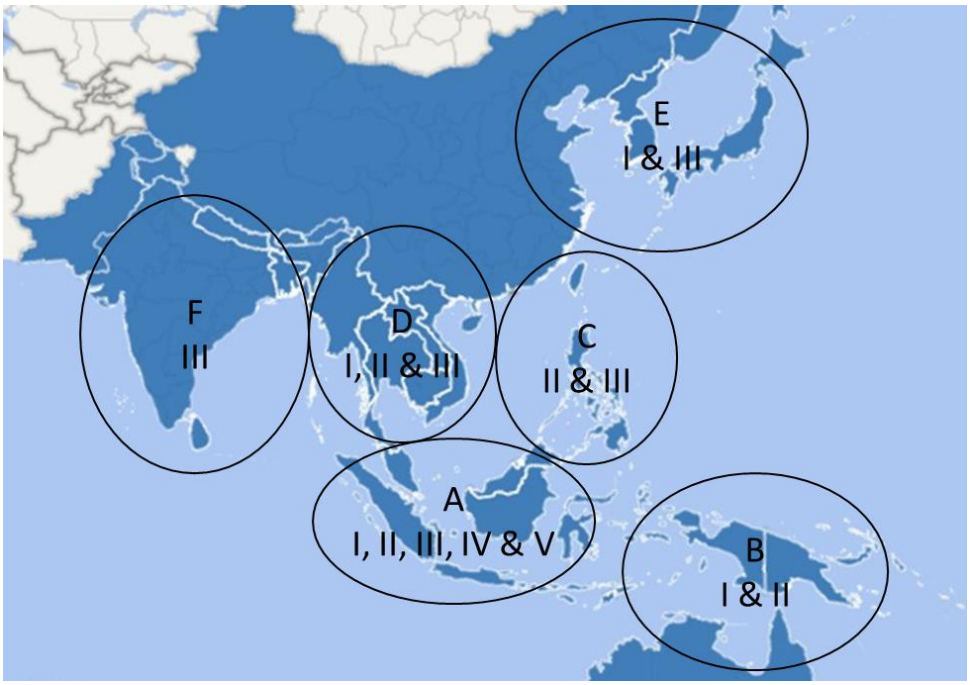

Figure 1.3 Countries reporting JEV cases and geographical distribution of JEV genotypes. Modified from "Origin and evolution of japanese encephalitis virus in southeast asia" by T. Solomon et al., 2003, *Journal of Virology*, 77(5), 3091-3098.

Genotypes

Phylogenetic studies indicate the presence of five genotypes of Japanese encephalitis virus, GI through GV, based on the nucleotide sequences of the prM and envelope (E) genes (Pan et al., 2011). Genotype I of JEV can be separated into two clades, GI-a and GI-b (Schuh et al., 2013). Whilst GIII was the dominant genotype circulating in Asia for several decades, the displacement of GIII by GI-b began in the 1980s and led to the co-circulation of GI-b and GIII in multiple locations (Han et al., 2015). Genotype I, the youngest genotype, is thought to have been a minor strain until the 1970's when it began to spread rapidly (Pan et al., 2011). JEV is unique in that there is little phylogenetic variation between genotypes; there is less than 1% variation between GI and GIII (Han et al, 2015). Genotype V, first seen in Malaysia in the 1940's, went undetected for six decades; in 2009 and 2010 respectively, GV was again detected in isolates

from *Culex* species mosquitoes in the Tibetan Province of China and in South Korea (Li et al., 2011; Takhampunya et al., 2011).

There appears to be an association between climatic conditions and endemic genotypes. Genotype V is found in temperate and tropical regions, GIV is found in only tropical regions, GI-b and GIII are found primarily in temperate regions, and GI-a and GII are found primarily in tropical regions (Schuh et al., 2013).

Diagnostics

Laboratory diagnosis is essential to differentiate JEV from pathogens with similar disease symptoms to JEV. In the endemic region, multiple infectious agents exist that can present with the development of neurotropic disease in humans and animals. There are two approaches to identify JEV; through identification of the agent or by use of serological assays.

Agent identification is typically achieved via virus isolation either in laboratory animals, such as mice, or in cell culture. Chicken embryo, African green monkey kidney (Vero), baby hamster kidney (BHK) and *Aedes albopictus* mosquito C6/36 cells are all acceptable cultures for virus isolation (Hiatt et al., 1951). Indirect fluorescent antibody tests use monoclonal antibodies specific to flavivirus and JEV to identify the virus. The combination of reference antibodies and standard serological assays, such as complement fixation method and hemagglutination inhibition assays, were used as methods to specifically confirm the infection of JEV (Casals and Palacios, 1941; Webster, 1937). The development of contemporary molecular biology methods brought a revolutionary impact to the diagnosis of flaviviruses including JEV. It allows for the direct and specific detection of viral genomic fragments through the use of primers and probes with specific sequences. Molecular diagnostic methods such as reverse transcription-polymerase

chain reaction (RT-PCR) use JEV specific primers to identify JEV in clinical samples or cell culture fluid. Sequences of amplicons derived from RT-PCR reactions were initially confirmed using restriction fragment length polymorphism-based methods (Eldadah et al., 1991). The techniques for nucleotide sequencing further enhanced the throughput of RT-PCR by directly determining the genetic sequences of virus isolates (Sumiyoshi et al., 1986). Their use is limited by the short duration typical of JEV infection and consideration must be made when choosing primers so as to match with currently circulating genotypes. In order to confidently identify an agent, proper samples should be provided, including: a complete set of formalin fixed tissues and fresh brain, spinal cord and/or cerebrospinal fluid (Fernández, 2010; Mansfield et al., 2017).

Serological assays such as enzyme-linked immunosorbent assays, virus neutralization tests, haemagglutination inhibition tests, and complement fixation tests are useful in diagnosing human and animal infections of JE based on antibody levels. A latex agglutination test has also been described for the detection of JEV antibodies in swine (Xinglin et al., 2002). Making a diagnosis in an endemic region based off serological results can be challenging though, due to the prevalence of JEV antibodies in the population from prior exposure or vaccination and the existence of cross-reactive antibodies. For this reason, it is critical to obtain both acute and convalescent samples, collected four to seven days apart, to look for significant rises in antibody titre indicative of recent exposure. In addition, IgM based assays can be used to look for recent infection. Cross reactivity with other serologically related flaviviruses must also be considered and alternative methods of diagnosis may be required for differential diagnosis. Currently serum, heparinized blood and cerebrospinal fluid are the only approved samples for serological testing. Plaque reduction neutralization test is another useful serological assay and is considered the

"gold-standard" for JEV diagnosis due to its high specificity (Fernández, 2010; Mansfield et al., 2017).

Development of Polymerase Chain Reaction and its importance in the diagnosis of JEV

The polymerase chain reaction (PCR) was developed in the mid 1980s by Kary Mullis (Mullis et al., 1985). The technique used DNA polymerase to amplify segments of DNA flanked on each side with a nucleotide primer molecule. He improved upon his original technique with the replacement of DNA polymerase with heat-stable *Thermus aquaticus* (Taq) DNA polymerase to amplify the DNA segments. The ability of Taq polymerase to withstand higher temperatures meant that its use removed the necessity for the addition of polymerase with each cycle, greatly simplifying the procedure. As PCR has gained in popularity, there have been several advancements to the technique. The withholding of either primers or DNA polymerase until the first denaturation step reached a temperature approaching 80°C was labeled Hot Start PCR and reduced mispriming thus increasing the specificity of the assay (Nuovo et al., 1991). Hot Start PCR was then improved upon using tools such as waxes and monoclonal antibodies targeting the DNA polymerase to inhibit the DNA polymerase activity until the first denaturation step (Chou et al., 1992; Dahiya et al., 1995). The "hot start" technique was then applied to reverse transcription PCR allowing the two-step process to be reduced to one-step by combining the reverse transcription step with the PCR process (Seah et al., 1995). The development of quantitative PCR, also known as real time PCR, allows for the quantification of DNA template through use of fluorescent reporter dyes. It has been proven to be more sensitive than conventional PCR (Gadkar et al., 2015; Dahnze et al., 2015).

The development of PCR assays for the detection of JEV genome have followed a similar pathway. Nested PCR assays proved to be highly specific and sensitive for JEV, more so than conventional PCR methods (Eldadah et al., 1991; Jeong et al., 2011). The development of RT-qPCR assays for JEV allowed for the quantification of viral genome. Currently there are several RT-qPCR assays described for the detection of JEV in human, swine and mosquito samples.

Oral fluids as a potential tool for the veterinary diagnosis of JEV in swine

As an alternative sample for detection of viral infection in pigs, oral fluids provide several cost-saving benefits over traditional veterinary diagnostic samples including blood, CSF and swabs. Oral fluid consists of saliva and mucosal transudate and is collected by the placement of an absorbent material in the mouth (Atkinson et al., 1993; Prickett et al., 2008; Prickett et al., 2010). In swine, oral fluids are typically collected by allowing the pigs to chew on a cotton rope attached to their pen. Pigs have three major salivary glands consisting of the parotid, mandibular and sublingual glands (Prickett et al., 2008; Prickett et al., 2010). Serum, the tonsils, the upper and lower respiratory tracts and environmental contamination are all potential pathways for viruses to enter the oral cavity (Jones et al., 2014). Several swine pathogens have been detected in oral fluid samples including vesicular stomatitis virus, foot and mouth disease virus, porcine respiratory and reproductive virus (PRRSV), porcine circovirus 2 (PCV2), swine influenza virus, classical swine fever virus (CSFV) and African swine fever virus (ASFV) and the value of oral fluids as a useful diagnostic sample has been proven (Prickett et al., 2010; Ramirez et al., 2012; Milicevic et al., 2015).

Salivary ribonucleases present within oral fluids can complicate molecular diagnostic results by compromising the viral RNA within samples (Eichel et al., 1964). This is particularly important in field conditions when samples are subject to harsher storage conditions. Stability of

viral RNA in oral fluids when exposed to varying storage and handling conditions has been evaluated for swine pathogens. With PRRSV no significant difference was seen after seven days of storage at 4°C, but a 10-fold reduction in detectable genome was reported at twenty-four hours and a 100-fold reduction at one week when stored at room temperature (Decorte et al., 2013). Hepatitis E virus saw no significant reduction in detectable genome after thirty days of storage at -20°C (Jones et al., 2014). The addition of select saliva RNA stabilizers can inhibit degradation for at least one week at room temperature, but appear to be unnecessary for samples stored at 4°C or frozen at -20°C. In addition, the drying of ropes prior to fluid collection should be avoided as the retrieval of a fluid sample post desiccation will require dilution of the sample.

The potential for diagnosis of JEV and other flaviviruses using oral sampling techniques has been described recently, changing our understanding of flavivirus pathogenesis. Dengue virus (DENV) RNA was found in the saliva of patients infected with DENV (Poloni et al., 2010; Korhonen et al., 2014) and Zika virus RNA has been detected in the saliva of experimentally infected monkeys (Osuna et al., 2016; Newman et al., 2017). In swine, both viral RNA and infectious virus was found in oronasal swabs taken from JEV infected pigs (Ricklin et al., 2016). With this knowledge, the goal of the research described in this thesis was to assess the capabilities of currently described molecular diagnostic techniques at detecting JEV genome in oral fluids of pigs experimentally challenged with JEV.

Chapter 2 - Materials and Methods

Introduction

Three RT-qPCR assays were evaluated for sensitivity using multiple strains of JEV grown in tissue culture fluid, as well as serum and oral fluid samples collected from pigs experimentally challenged with JE-91 strain JEV. Specificity of the assays was addressed through use of synthetic RNA genomes of related flaviviruses.

Cell lines

Aedes albopictus C6/36 cells (CRL-1660) were maintained in Leibovitz-15 (L-15) media (Life Technologies, Carlsbad, CA) supplemented with heat-inactivated 10% fetal bovine serum (FBS), tryptose phosphate broth (TPB), penicillin, streptomycin and L-glutamine. The cells were incubated at 28°C and used for the propagation of all stock viruses.

African green monkey Vero76 cells (CRL-1587) were also maintained in L-15 media supplemented with 10% FBS, TPB, penicillin, streptomycin and L-glutamine. The cells were cultured at 37°C and used for the titration of viral stocks using the $TCID_{50}$ method.

Virus

To evaluate the feasibility of the RT-qPCR assays used in this study for the detection of different genotypes of JEV, five JEV strains were selected to represent four different genotypes. Information of each strain is summarized in Table 2.1. Infectious viruses were passaged twice in *Aedes albopictus* C6/36 cells and used to determine the sensitivity of each assay.

Table 2.1 Information of representative JEV strains for individual genotypes.

Strain	Genotype - clade	Genbank access number
KE-93-83	I-a	KF192510.1
JE-91	I-b	GQ415355.1
JKT1724	II	JQ429304.1; U70404.1
Taira	III	FJ872384.1; FJ515933.1
JKT6468	IV	AY184212.1; U70407.1

Determination of infectious titers

To establish the standard curves of each RT-qPCR assay, titers of all viral stocks were determined using $TCID_{50}$ (Table 2.2). Each stock virus was titrated in duplicate by inoculating 100µL of undiluted virus followed by 10-fold serial dilutions up to 10^{10}-fold dilution. Each dilution was performed by transferring 10µL inoculum into 90µL of L-15 media. Negative control wells received fresh L-15 media. Each well of a 96-well plate received 100µL of Vero76 cells. Plates were sealed with parafilm and incubated at 37°C for 7 days. Cytopathic effects in individual wells were visualized by staining with 200µL of amido black stain containing 1% amido black B10 suspended in 10% glacial acetic acid and 35% isopropanol aqueous solution at room temperature for at least 30 minutes. Stain was washed with regular tap water and read under inverted microscope. Titers were calculated using the Reed-Muench method (Reed & Muench, 1938).

Table 2.2 Median tissue culture infectious dose viral titers for JEV stock viruses harvested after two passages in C6/36 cells.

Genotype	GI-a	GI-b	GII	GIII	GIV
Strain	KE-93-83	JE-91	JKT1724	Taira	JKT6468
Viral Titer ($TCID_{50}$/mL)	$3.3x10^7$	$3.3x10^7$	$1.6x10^8$	$8.9x10^7$	$3.3x10^6$

Extraction of viral nucleic acid

All RNA extractions in this study were performed using the QIAmp viral RNA mini extraction kit (Qiagen Inc., Valencia, CA) according to manufacturer's recommendations. Viral RNA were extracted from tissue culture supernatant of representative strains to compare the sensitivity and specificity of each assay for the detection of each genotype. The standard curve of the JE-91 strain was also used for the quantification of viral loads in infectious samples. Standard curves and limit of detection of each assay were obtained using serially diluted viral RNA extracts from stocks with known infectious titers.

To evaluate the feasibility of utilizing each RT-qPCR assay for laboratory diagnosis of JEV infections, viral RNA from serum and oral fluid collected from experimentally infected pigs were expected using the same procedures as candidate diagnostic samples.

For each sample extraction, 140µL of sample was suspended and pulse-vortexed for 15 seconds in 560µL AVL lysis buffer supplemented with carrier RNA at 1g/L in an RNAse-free microcentrifuge tube. Lysis of virions were performed at room temperature for at least 10 minutes. Precipitation of viral RNA were performed by mixing the lysates with 560µL of absolute ethanol followed by pulse-vortexing for 15 seconds. Microcentrifuge tubes were briefly centrifuged between procedures to avoid contamination caused by the residual fluid present in the lid of individual tubes. To capture viral RNA, 630µL of precipitated lysate was loaded onto the QIAamp RNA mini spin column and centrifuged at 8,000 radius per minute (rpm) for one minute. The procedure was repeated to capture viral RNA from the remaining samples. The spin columns were then washed with 500µL AW1 wash buffer containing 56% of ethanol by centrifugation at 8,000rmp for one minute. The process was repeated using 500µL of AW2 wash buffer containing 70% of ethanol. Residual wash buffers were removed by centrifugation

13,000rpm (max speed) for one minute. Viral RNA was dissolved with 60µL of molecular grade water, carefully added to the silica matrix of the spin column, followed by incubation at room temperature for one minute. Elution of viral RNA was achieved by centrifugation at 8,000rpm for one minute. The eluate was collected and placed in RNAse free microcentrifuge tubes for storage at -80°C. JE-91 virus stock was included as a positive control for each round of viral RNA extraction.

Synthetic nucleic acids

Two types of synthetic nucleic acids with known nucleotide sequences were used as standard samples to determine the specificity and analytical chemistry sensitivity of each RT-qPCR assay. Specificity of each RT-qPCR assay was determined based on the cross-reactivity with RNA genomes of related flaviviruses in the JEV serocomplex. Synthetic RNA genomes of St. Louis encephalitis virus (SLEV) (ATCC® VR-3236SD™) and West Nile virus (WNV) (ATCC® VR-3198SD™) (American Type Culture Collection, Manassas, VA) originally developed as standard samples for laboratory diagnostic assays of SLEV and WNV were tested with each RT-qPCR assay at concentrations up to 10^8 RNA copies/mL to identify any potential cross-reactivity due to the homologous sequences in viral genomes.

As summarized in Table 2.3, cDNA fragments were synthesized based on partial sequences of the NS5 gene and 3′UTR targeted by the three sets of primers and probe. Synthetic cDNA molecules were used as templates to establish the analytical chemical standard curves and determine the limit of detection of each RT-qPCR assay. Stock solutions consisting of lyophilized cDNA fragments suspended with nuclease-free H_2O at the concentration of 10^{10} molecules/mL were stored at -20°C. Synthetic cDNA stocks were further diluted to the

concentration of 10^7 cDNA copies/mL for working stocks to generate templates for each RT-qPCR assay. Standard curves were established using serially diluted cDNA templates at concentrations between $1.0x10^1$ to $1.0x10^7$ cDNA copies/mL. The limit of detection was defined as a Cq value greater than 34.

Table 2.3 Oligonucleotide sequences created for synthetic positive controls.

	Sequence
Assay #1 Synthetic	GACAGACGTTCCGTATGTGGGAAAGCGCGAGGACATCTGGTGTGGC AGCCTCATCGGAACGCGATCCAGAGCAACCTGGGCTGAGAACATCT ATGCGGCGATAAACCAGGTTAGAGCTGTCATTGGGAAAGA
Assay #2 Synthetic	GCAATAGACGAGGTGTAAGGACTAGAGGTTAGAGGAGACCCCGTGG AAACAACAACATGCGGCCCAAGCCCCCTCGAAGCTGTAGAGGAGGT GGAAGGACTAGAGGTTAGAGGAGACCCCGCATTTGCATCAAACAGC ATATTGACACCTGGGAATAGACTGGGAGATCTTCTGCTCTATCTCAA CATCAGCTACTAGGCACAGAGCGCCGAAGTATGTAGCTGGTGGTGA GGAAGAACAC
Assay #3 Synthetic	GTATCTACAACATGATGGGAAAAAGAGAGAAGAAGCCTGGAGAGTT TGGAAAAGCTAAAGGAAGCAGGGCCATTTGGTTCATGTGGCTTGGA GCACGGTATCTAGAGTTTGAAGCTTTGGGGTTCCTGAATGAAGACCA TTGGCTGAGCCGAGAGAATTCAGGAGGTGGAGTGGAAGGCTCAGGC GTCCAAAAGCTGGGATACATCCTCCGTGACATAGCAGGAAAGCAAG GAGGGAAAATGTACGCTGATGATACCGCCGGGTGGGACA

Reverse transcriptase-quantitative polymerase chain reaction

Three RT-qPCR assays targeting the conserved nucleotide sequences in the NS5 gene and 3′UTR were evaluated for use as diagnostic methods to detect JEV infection (Figure 2.1). The first assay (assay #1) detects the presence of JEV genome through the synthesis of amplicons between nucleotide positions of 10,224-10,286 between the 3′ end of NS5 gene and 5′ end of the 3′UTR of the JEV genome (Pyke et al., 2003). The second assay (assay #2) is

designed to target the 3′UTR at nucleotide positions 10,740-10,884 of the JEV genome (Yang et al., 2004). The third assay (assay #3) targets the NS5 gene through the specific recognition of nucleotide positions 9,036-9,301 of the JEV genome (Chao et al., 2006). Sequences for the oligonucleotide primers and TaqMan probe (Integrated DNA technologies, Coralville, IA) used in each RT-qPCR assay are listed in Table 2.4. All TaqMan probes were modified with a 6-carboxyfluorescein fluorophore on the 5′end and a proprietary double fluorescent quencher system including an internal ZEN quencher and an IowaBlack fluorescent quencher on the 3′end.

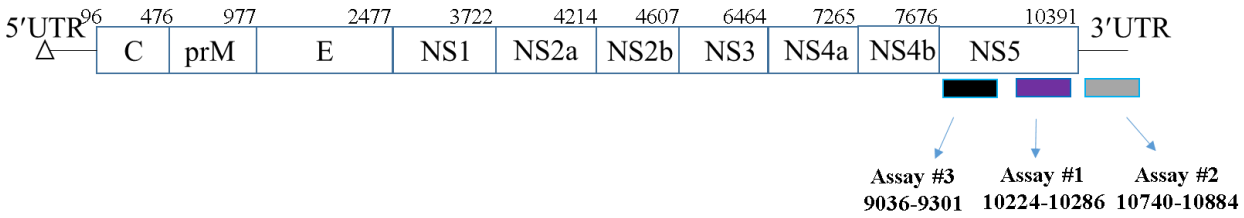

Figure 2.1 Locations of genomic sequences on the JEV genome.

Table 2.4 Oligonucleotide sequences generated through Integrated DNA Technologies and corresponding location in the JEV genome based off the SA14 strain.

		Genomic Sequence	Genomic Position
Assay #1	Forward Primer	ATCTGGTGYGGYAGTCTCA	10224-10242
	Reverse Primer	CGCGTAGATGTTCTCAGCCC	10267-10286
	Probe	CGGAACGCGATCCAGGGCAA	10244-10263
Assay #2	Forward Primer	GGTGTAAGGACTAGAGGTTAGAGG	10740-10763
	Reverse Primer	ATTCCCAGGGTGTCAATATGCTGTT	10861-10884
	Probe	CCCGTGGAAACAACATCATGCGGC	10768-10791
Assay #3	Forward Primer	TACAACATGATGGGAAAGCGAGAGAAAAA	9036-9064
	Reverse Primer	GTGTCCCAGCCGGCGGTGTCATCAGC	9276-9301
	Probe	TCCGTGACATAGCAGGAAAGCAAG	9238-9261

All RT-qPCR reactions were performed using the iTaq Universal Probes One-step Kit (BioRad, Hercules, CA). Fluorescent signal was detected and analyzed with a BioRad CFX96

real-time PCR thermocycler (BioRad, Hercules, CA). In each reaction, 4µL of RNA extracts were loaded to a mixture of 10µL iTaq universal probes reaction mix supplemented with 0.5µL iScript advanced reverse transcriptase, 1µL forward primer, 1µL reverse primer, 0.4µL probe and 3.1µL molecular grade water. Cycling parameters for assay #1 were as follows: reverse transcription, 30 minutes at 50°C; initial denaturation, 10 minutes at 95°C; denaturation, 15 seconds at 95°C; annealing and extension, 1 minute at 60°C for 40 cycles. Cycling parameters for assay #2 were as follows: reverse transcription, 30 minutes at 50°C; initial denaturation, 5 minutes at 95°C; denaturation, 10 seconds at 95°C; annealing and extension, 3 minutes at 55°C for 45 cycles. Cycling parameters for assay #3 were as follows: reverse transcription, 30 minutes at 50°C; initial denaturation, 3 minutes at 95°C; denaturation, 15 seconds at 95°C; annealing and extension, 3 minutes at 48°C for 45 cycles. As an internal control, to provide consistent results among different runs of RT-qPCR assays, a standard curve of JE-91 strain was generated in each experiment. All reactions were carried out with an additional non-template negative control sample consisting of nuclease-free water to exclude the false positive results due to the nonspecific detection of background fluorescence.

Standard curves used to calculate viral loads corresponding to infectious titers of JE-91 strain used for animal challenge studies or cDNA concentrations were created by analyzing RNA or cDNA templates generated by six repeats of ten-fold serial dilution of viral RNA extract from viral stocks with known infectious titers or cDNA stocks with known cDNA concentrations. Reactions leading to quantification cycle (Cq) value less than 34 were considered positive for JEV detection.

An additional set of standard curves was established using viral RNA extracts from five representative strains to compare the sensitivity of three RT-qPCR assays based on the limit of

detection. Negative control samples, as well as samples representing related flaviviruses, were used to assess specificity.

Animals

Two experimental challenges of JEV were conducted using pigs purchased from sources approved by the Comparative Medicine Group, Kansas State University and used to compare of models of infection in pigs intraveneously and intradermally inoculated with JEV. All animal work was performed under BSL-3Ag conditions in the Biosecurity Research Institute on the Kansas State University (Manhattan, KS) campus and under the approval of the Institutional Animal Care and Use Committee of Kansas State University. In addition to characterizing the tissue tropism and persistent infection of JEV infection in pigs, serum and oral fluid samples collected were used for potential diagnostic sample evaluation. Due to limited availability of samples for testing, serum was tested from pigs experimentally infected via the intravenous route and oral fluids were tested from pigs challenged intradermally. Figure 2.2 illustrates the animal challenge and necropsy schedule.

In the first experiment, fourteen 3-week-old white-line crossbred pigs were randomly assigned to control and experimental groups. Four control pigs received 1mL of sterile saline solution and ten experimental pigs received 1mL of 10^7 $TCID_{50}$/mL JE-91 strain JEV through intravenous inoculation. On day three post-infection (d.p.i.), blood was collected from all pigs. The two groups were housed separately to prevent potential transmission through direct contact as described by Ricklin et al. (2016).

In the second experiment, intradermal challenge of JEV was performed with two lineages of pigs. To develop a model of JEV infection in intradermally challenged domestic pigs, fourteen

3-week-old white-line crossbred pigs were used. As a model for JEV infection in feral swine, fourteen 3-week-old HsdSrc:Sinclair miniature pigs (Sinclair Biosciences, Columbia, MO) were chosen to represent the feral swine population in the United States. Upon arrival, pigs from the domestic pig group and the feral pig group were randomly assigned into control and experimental groups with four animals in each control group and ten animals in each experimental group. Each group was housed in separate pens within the same room to avoid direct contact. Control pigs were injected intradermally with 100µL of sterile saline solution. Experimental pigs were injected intradermally with 100µL of diluted viral stock containing 10^7 $TCID_{50}$ of JE-91 strain. Pen-based oral fluid samples were collected on days 0 through 14 post-infection, as well as day 21 and day 28.

In each experiment, two control animals and five challenged animals were euthanized at 3 d.p.i. to characterize the tissue tropism of JEV at the acute phase of infection. The remaining animals were euthanized at 28 d.p.i. to characterize the persistent infection of JEV in challenged animals.

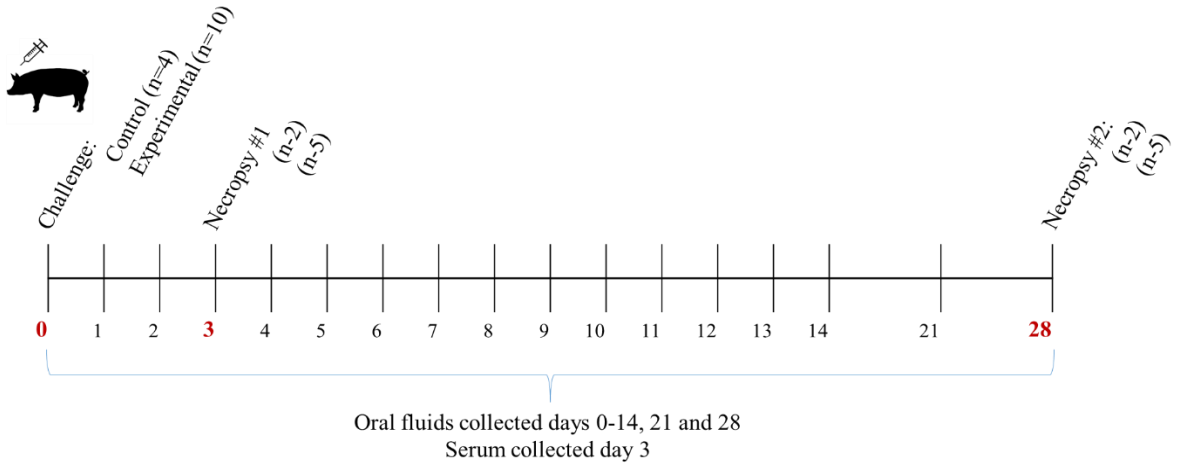

Figure 2.2 Animal study schedule. Same schedule is used for all three animal studies (IV domestic pig, ID domestic pig, and ID feral pig).

Serum collection

Whole blood was collected from pigs via the right external jugular vein into red-top vacutainer tubes at 3 d.p.i. Tubes were centrifuged at 2,000 x g for 10 minutes at 4°C and serum was removed from the blood clot and aliquoted into 2mL microcentrifuge tubes and stored at -80°C.

Oral fluid collection

Oral fluids were collected from each pen of animals using pre-packaged oral fluid collection kits. Cotton rope was tied to a metal bar on the side of the pen at pig shoulder height and left in the pen for the pigs to chew for approximately twenty minutes or until the rope was adequately saturated. Ropes were placed in plastic bags attached to 15mL conical tubes and oral fluid was extracted by manually squeezing fluid into the tubes. Oral fluids were transferred from the conical tubes into 1.5mL microcentrifuge tubes and stored at -80°C.

Statistical analysis

Based on the standard curves constructed with serially diluted RNA extracts, the limit of detection of each RT-qPCR assay was estimated using the simple linear regression model. Repeated measures t test was performed to compare the RNA viral load in oral fluids determined by the RT-qPCR assays. A one way analysis of variance was used to compare the assays on the average quantity of viral RNA detected in serum collected at 3 d.p.i.

Chapter 3 - Sensitivity and Specificity of Three RT-qPCR Assays Against Different Genotypes of JEV

Introduction

The development of RT-PCR-based diagnostic methods has led to exceptional sensitivity and throughput for laboratory diagnosis of flavivirus infections. In contrast to virus isolation using continuous cell lines and laboratory animals, RT-PCR assays provide a significantly shortened turnaround timeframe and eliminate the dependence on the presence of infectious viruses in diagnostic samples. This technique has been applied to the detection of viral RNA in various types of diagnostic samples including serum, central nervous system fluid, and infected tissues (Eldadah et al., 1991). By detecting fragmented viral genomes, the use of RT-PCR in laboratory diagnosis allows the identification of etiological agents after the acute phase of infection (Oliveira et al., 2003). Modifications of amplification strategies provide additional mechanisms to increase the specificity and sensitivity of existing assays. For instance, the gradual reduction of annealing temperature in touchdown PCR methods improves the specificity of diagnosis methods (Johansen et al., 2002). The inclusion of a second round of amplification by nested PCR is a common strategy to increase the sensitivity of assays or achieve the specific detection of different viruses (Eldadah et al., 1991). Although modifications of RT-PCR-based diagnostic methods provides mechanisms for the optimization of existing diagnostic assays, a major limitation to existing RT-PCR-based diagnostic methods is the lack of ability to quantify viral genomes present in diagnostic samples. As conventional RT-PCR methods often take measurements at the plateau phase through the visualization of cDNA amplicons on agarose gels, quantification of viral RNA is highly challenging. The development of RT-qPCR assays by

including fluorogenic oligonucleotide probes in RT-PCR reactions and monitoring the emission of fluorescence in each cycle allows the quantification of cDNA, which is converted from viral RNA at equal mole ratios (Houng et al., 2000). The method not only provides the quantitative results of diagnosis but also further improves the sensitivity of diagnostic assays by detecting the fluorescent signals from cDNA amplicons at low concentrations. By determining the lowest quantity of viral RNA template required for positive detection, comparison of sensitivity can be achieved by determining the limit of detection (LOD) for individual RT-qPCR assays. Similarly, specificity of different RT-qPCR assays can also be demonstrated through the specific detection of fluorescent signals from the amplifications of targeted sequences. Determination of sensitivity and specificity for individual assays is critical for the establishment of protocols for laboratory diagnosis of flaviviruses. The lack of the proofreading capability in viral RNA-dependent RNA polymerase leads to the error-prone nature of viral replication and the accumulation of genetic mutations during viral replication. Whilst the NS5 gene and 3′UTR of flaviviruses are known to contain highly conserved sequences ideal for molecular assays, minor mismatches between the sequences of oligonucleotide primers and probes and viral genomes in this region have been reported to be associated with failures of detection and false negative results in laboratory diagnosis for specific genotypes of DENV (Koo et al., 2016). Therefore, ideal RT-qPCR assays for JEV should demonstrate the high sensitivity against multiple genotypes of JEV present in the endemic region without demonstrable cross-reactivity with other related flaviviruses in the JEV serocomplex.

In this study, sensitivity and specificity of three RT-qPCR assays targeting the highly conserved NS5 gene and 3′UTR were determined using viral RNA or cDNA of different genotypes of JEV and related flaviviruses (Chao et al., 2007; Pyke et al., 2004; Yang et al.,

2004). The objective of the study was to identify a highly sensitive RT-qPCR method for the specific detection of different endemic genotypes of JEV.

Results

Specific detection of four distinct genotypes of JEV

To demonstrate the capability of detecting multiple genotypes of JEV endemic in the Asian Pacific region, viral RNA was extracted from tissue culture fluids of five represented strains and used as template for RT-qPCR assays. Ten-fold serial dilutions of viral RNA were performed to establish the biological standard curve of each RT-qPCR assay and determine the limit of detection and linear responses between the quantity of input viral genomes and quantification cycles (Cq) required for the emission of detectable fluorescent signal. LOD of each assay was calculated based on the corresponding infectious titers at Cq value of 34.

The amplification of JEV KE-93-83 strain, representing isolates from GI-a, by three RT-qPCR assays is summarized in Figure 3.1. The linear relationship between Cq values and input viral RNA templates was observed between $3.3x10^6$ $TCID_{50}$/mL and $3.3x10^1$ $TCID_{50}$/mL. Interestingly, detection of viral RNA in undiluted samples at $3.3x10^7$ $TCID_{50}$/mL was only achieved by assay #1 targeting the 3′UTR of viral genome. The results indicated that assays #2 and #3 are sensitive to the inhibition caused by the overloading of cDNA templates for the PCR reaction. The estimated LOD for the individual assays indicated that assay #1 has the highest sensitivity of less than 1.00 $TCID_{50}$/mL followed by assay #2 (3.98 $TCID_{50}$/mL) and assay #3 (5.89 $TCID_{50}$/mL).

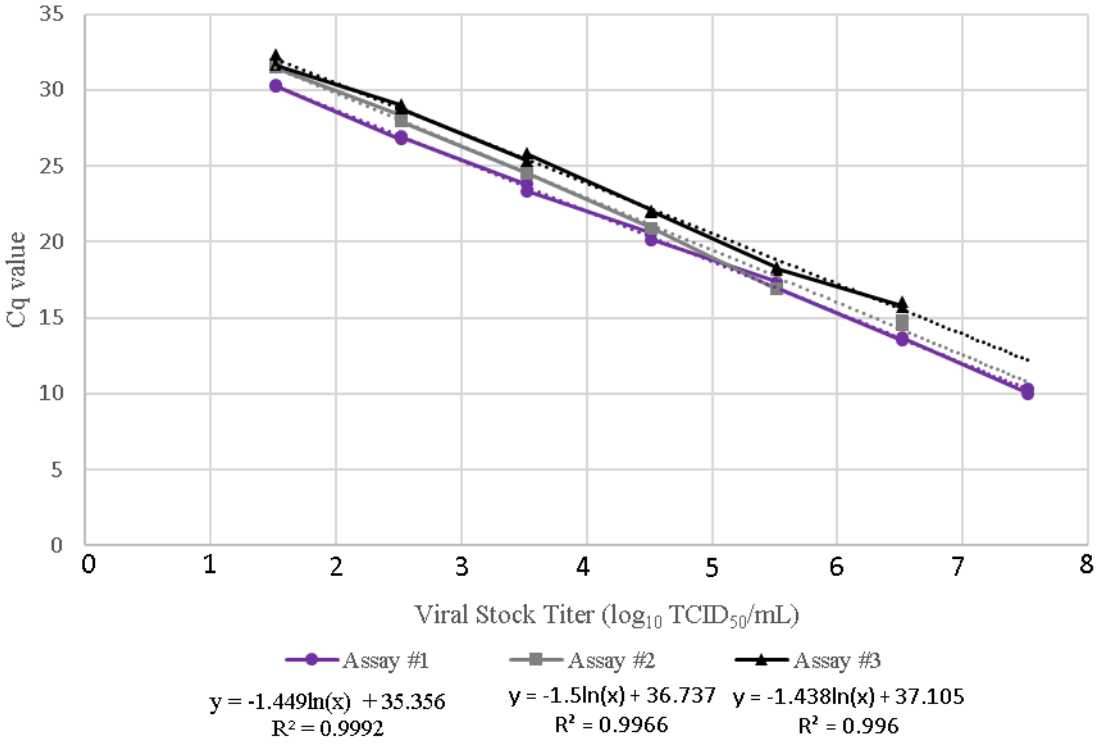

Figure 3.1 Detection of JEV genome in KE_93-83 strain tissue culture fluid. A Cq value <34.0 is considered positive for detection.

Detection of JE-91 strain, a representative for GI-b, consistently yielded positive detections from serially diluted viral RNA extracts representing the infectious titers between $3.3x10^7$ $TCID_{50}$/mL and $3.3x10^2$ $TCID_{50}$/mL as shown in Figure 3.2. Assay #1 demonstrated the highest sensitivity by detecting the presence of viral RNA at $3.3x10^1$ $TCID_{50}$/mL; whereas, the amplification by the other two assays did not lead to any positive fluorescent signals. The estimated LOD of the three assays are in agreement with the amplification of serially diluted viral RNA as assay #1 showed the lowest LOD at 10.27 $TCID_{50}$/mL followed by assay #2 at 19.05 $TCID_{50}$/mL and assay #3 at 21.38 $TCID_{50}$/mL

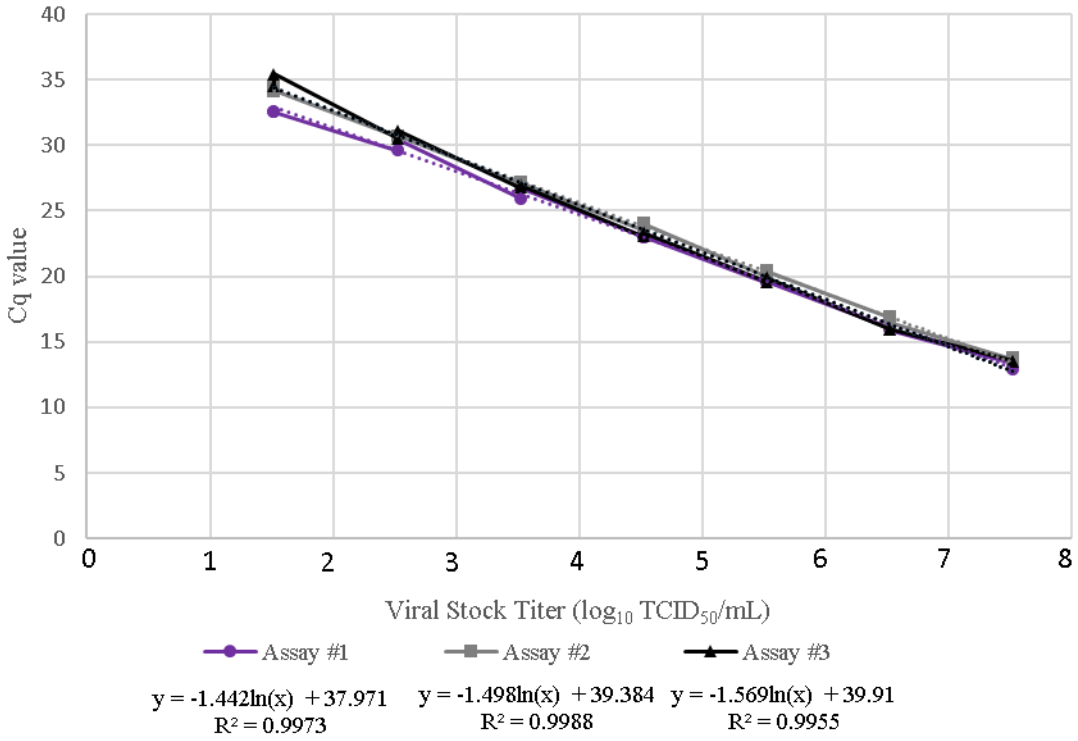

Figure 3.2 Detection of JEV genome in JE-91 strain tissue culture fluid. A Cq value <34.0 is considered positive for detection.

The sensitivity of each assay for the detection of GII of JEV was evaluated using the representative JKT1724 strain (Figure 3.3). Similarly, the linear responses between the input viral RNA and Cq values were observed between $1.6x10^8$ $TCID_{50}$/mL and $1.6x10^2$ $TCID_{50}$/mL. Assay #1 shows the highest sensitivity with LOD at 23.92 $TCID_{50}$/mL. The LODs of assay #2 and #3 were 33.75 $TCID_{50}$/mL and 60.49 $TCID_{50}$/mL, respectively.

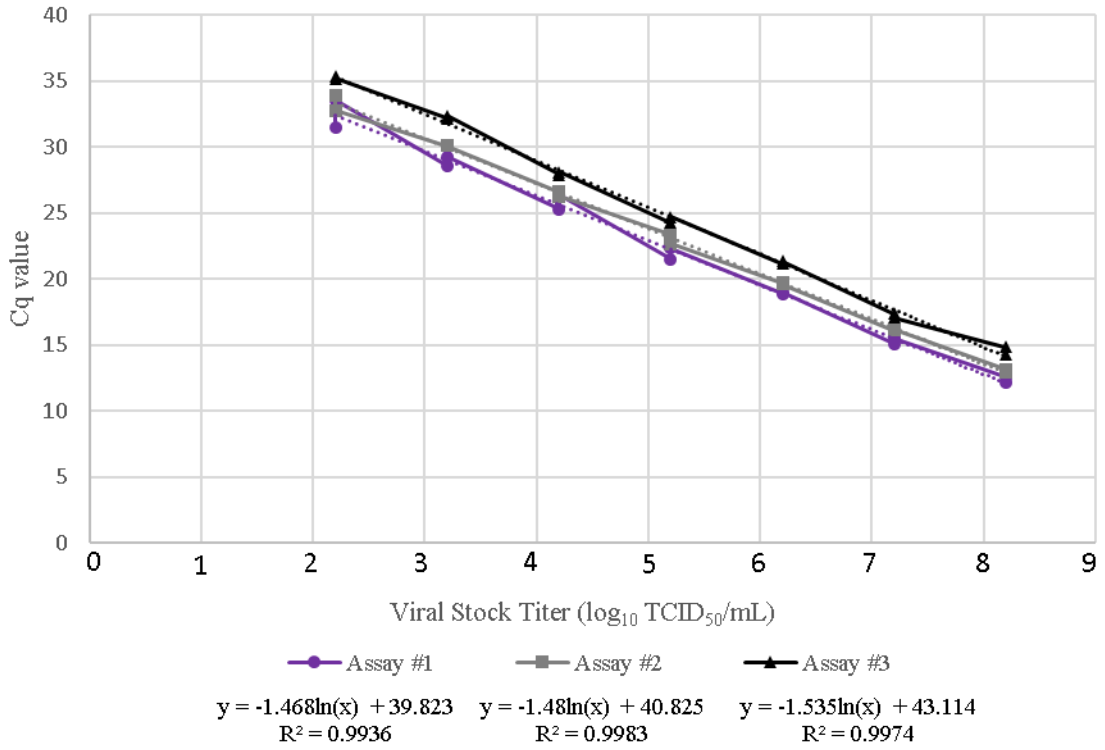

Figure 3.3 Detection of JEV genome in JKT1724 strain tissue culture fluid. A Cq value <34.0 is considered positive for detection

Whilst assay #1 consistently showed higher sensitivity in detecting the presence of viral RNA derived from the representative strains of GI-b and GII, this assay showed the lowest sensitivity for the detection of GIII with an estimated LOD at 90.04 $TCID_{50}$/mL. Assay #2 and #3 were demonstrated to be more sensitive based on the lower LODs at 3.44 and 4.99 $TCID_{50}$/mL, respectively, and the ability to provide positive detections in viral RNA samples at corresponding infectious titers of $8.9x10^{1}$ $TCID_{50}$/mL (Figure 3.4).

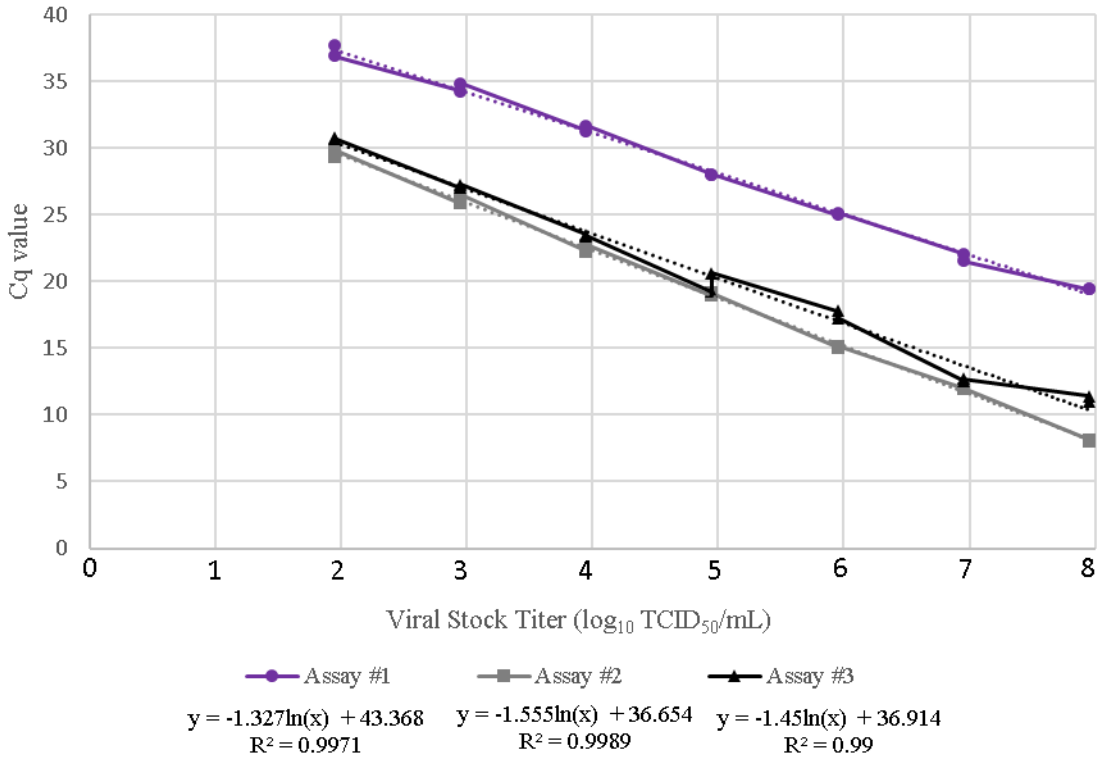

Figure 3.4 Detection of JEV genome in Taira strain tissue culture fluid. A Cq value <34.0 is considered positive for detection.

As summarized in Figure 3.5, the JKT6468 strain, a representative strain for the ancestral GIV, could only be achieved using assays #2 and #3. The two assays showed the linear results in the amplification of viral RNA at corresponding infectious titers from $3.3x10^6$ $TCID_{50}$/mL through 3.3 $TCID_{50}$/mL. Both methods showed remarkable sensitivity with estimated LODs at corresponding viral titers lower than 1.0 $TCID_{50}$/mL.

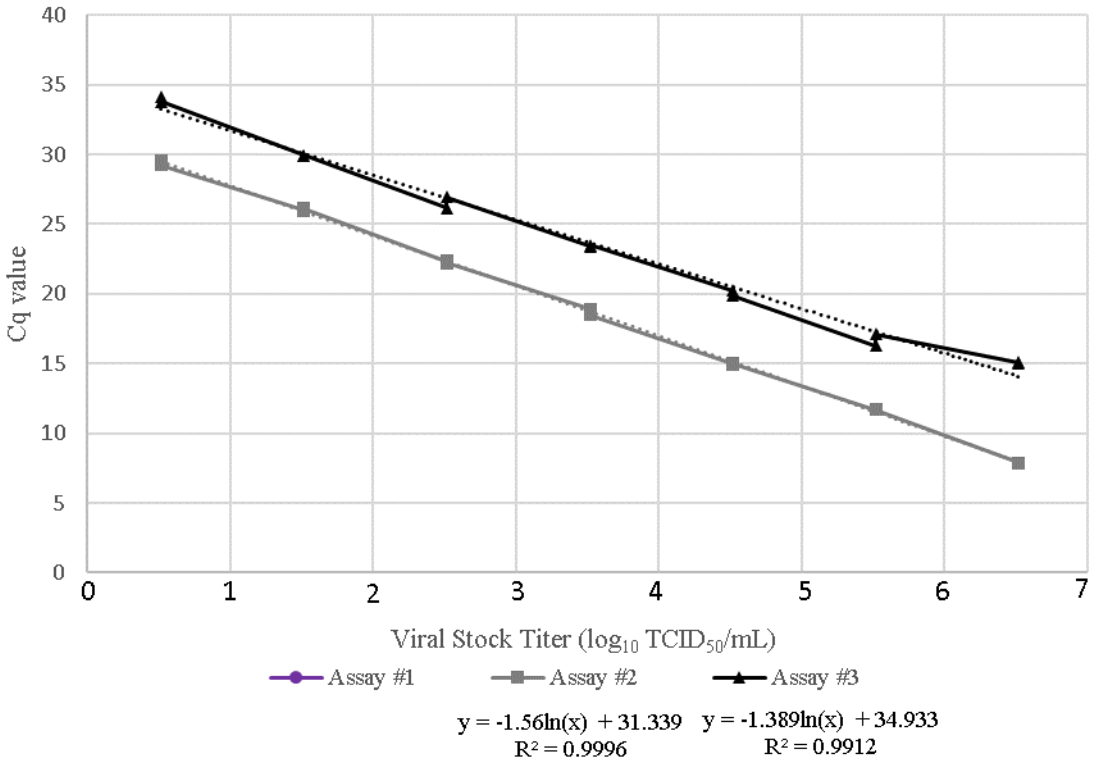

Figure 3.5 Detection of JEV genome in JKT6468 strain tissue culture fluid. A Cq value <34.0 is considered positive for detection.

All three RT-qPCR assays demonstrated high specificity for the detection of JEV as the use of synthetic RNA genome of SLEV and WNV did not lead to positive fluorescent signals between concentrations of $1.0x10^2$ RNA copies/mL and $1.0x10^8$ RNA copies/mL.

Detection of synthetic JEV DNA

To determine and compare the analytical chemistry sensitivity of each assay, cDNA fragments containing the target region of each assay were synthesized based on the genomic sequence of SA14 strain (Figure 3.6). Assay #2 showed the highest analytical chemistry sensitivity with the lowest LOD at 28.29 genome copies/mL. The LODs of assay #1 and #3 were 51.56 and 64.31 genome copies/mL, respectively.

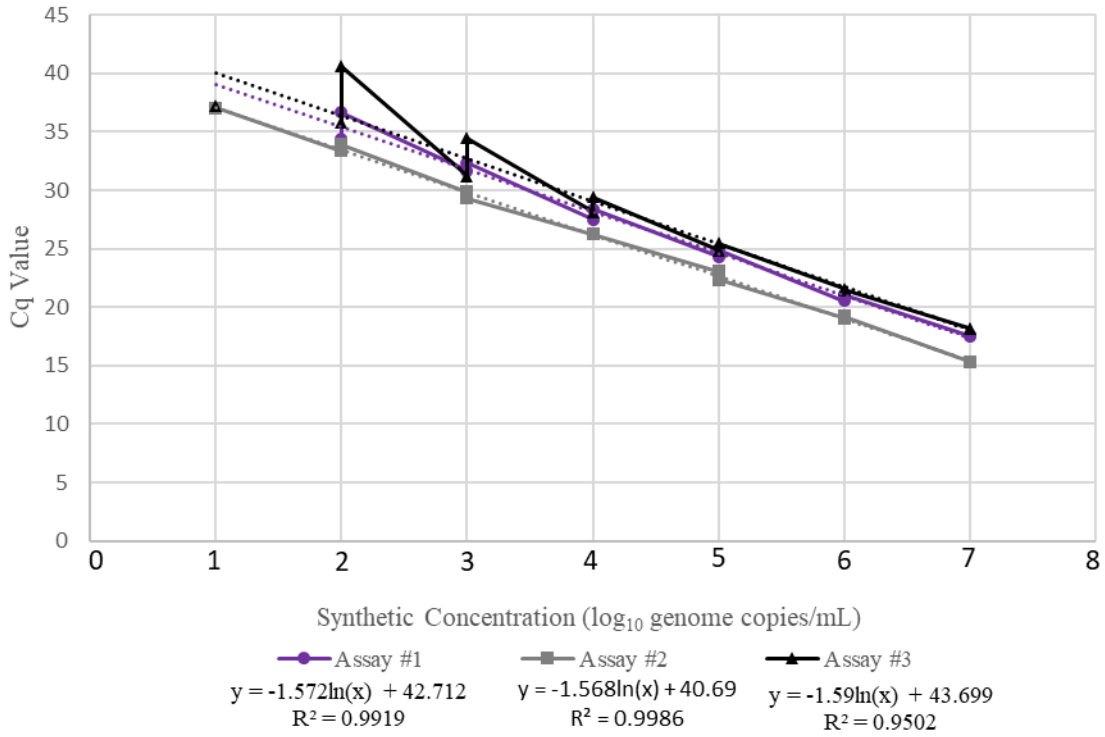

Figure 3.6 Detection of JEV genome in synthetic cDNA fragments based off the SA14 strain. A Cq value <34.0 is considered positive for detection.

Conclusions and discussion

With varying degrees of sensitivity, all three assays were demonstrated to be highly specific for detection of JEV with no observed cross-reactivity with other flaviviruses in the JEV serocomplex. As all three assays successfully quantified viral RNA extracts derived from the three endemic genotypes of JEV, GI-III, our findings support the feasibility of applying the assays evaluated in this study to the laboratory diagnosis of JEV. Although the detection of the representative strain for GIV could only be achieved by assay #2 and assay #3, the confined geographic distribution of GIV in the Indonesia-Malaysia region and the lack of recent isolates

associated with human and animal disease suggest its limited epidemic potential and undermines the need for the development of specific diagnostic protocols.

Interestingly, no single assay consistently demonstrated the highest sensitivity of detection against all representative strains. For example, the lowest LOD against the representative JE-91 strain, belonging to GI-b, demonstrated that assay #1 had the highest sensitivity in detecting the emerging strains under GI-b. However, assay #1 showed the lowest sensitivity and the highest LOD for the diagnosis of GIII, which also co-circulates with GI-b in several geographic regions. Similarly, although assay #2 was a highly sensitive molecular diagnosis method against GIII, the assay showed a lower sensitivity and higher LOD in detecting GI-b than assay #1. The differences in the sensitivity of each RT-qPCR is likely due to the number of mismatches between the sequences of oligonucleotide primers and probes and viral genomes. Oligonucleotid sequences for assays #1-3, as well as sequences representing four genotypes of JEV, are presented in Tables 3.1-3.3. As the primers and probe for assay #1 were developed based on the GII FU strain isolated in Australia, there was only one mismatch nucleotide sequence between the reverse primer and viral genome of strains belonging to GI-b (Pyke et al., 2004). The lower sensitivity against GIII is likely to be associated with the additional mismatch present between the oligonucleotide probe and viral genome. The highly divergent nucleotide sequences between GII and GIV limited the capability of assay #1 in detecting the presence of viral RNA extracted from the representative strain of GIV; a total of five mismatches were identified between viral genome and the oligonucleotide primers and probe used in the RT-qPCR assay. Whilst the NS5 gene and 3′UTR are considered highly conserved regions and ideal targets for the development of molecular diagnostic assays, results

of molecular detection assays targeting this region remain vulnerable to the inference created by variations in nucleotide sequences of different genotypes.

Table 3.1 Oligonucleotide sequences generated through Integrated DNA Technologies for Assay #1. Sequences for strains K94P05, FU, SA14, and JKT6468 were sourced from GenBank.

	Forward Primer	Reverse Primer	Probe
Assay #1	**ATCTGGTGYGGYAGTCTCA**	**GGGCTGAGAACATCTACGCG**	**CGGAACGCGATCCAGGGCAA**
K94P05 Genotype I-b	ATCTGGTGTGGCAGTCTCA	GGGCTGAGAACATTTACGCG	CGGAACGCGATCCAGGGCAA
FU Genotype II	ATCTGGTGCGGCAGTCTCA	GGGCTGAGAACATCTACGCG	CGGAACGCGATCCAGGGCAA
SA14 Genotype III	ATCTGGTGTGGCAGTCTCA	GGGCTGAGAACATCTATGCG	CGGAACGCGATCCAGAGCAA
JKT6468 Genotype IV	ATTTGGTGCGGCAGCCTCA	GGGCTGAGAACATCTATGCA	CGGAACGCGCGCTAGAGCAA

Table 3.2 Oligonucleotide sequences generated through Integrated DNA Technologies for Assay #2. Sequences for strains K94P05, FU, SA14, and JKT6468 were sourced from GenBank.

	Forward Primer	Reverse Primer	Probe
Assay #2	**GGTGTAAGGACTAGAGGTTAGAGG**	**AACAGCATATTGACACCTGGGAAT**	**CCCGTGGAAACAACATCATGCGGC**
K94P05 Genotype I-b	GGTGTAAGGACTAGAGGTTAGAGG	AACAGCATATTGACACCTGGGAAT	CCCGTGGAAACAAAATTATGCGGC
FU Genotype II	GGTGTAAGGACTAGAGGTTAGAGG	AACAGCATATTGACACCTGGGAAT	CCCGTGGAAACAACAATATGCGGC
SA14 Genotype III	GGTGTAAGGACTAGAGGTTAGAGG	AACAGCATATTGACACCTGGGAAT	CCCGTGGAAACAACAACATGCGGC
JKT6468 Genotype IV	GATGTAAGGACTAGAGGTTAGAGG	AACAGCATATTGACACCTGGGAAT	CCCGTGGAAACAACAACATGCGGC

Table 3.3 Oligonucleotide sequences generated through Integrated DNA Technologies for Assay #3. Sequences for strains K94P05, FU, SA14, and JKT6468 were sourced from GenBank.

	Forward Primer	Reverse Primer	Probe
Assay #3	**TACAACATGATGGGAAAGCGAG AGAAAAA**	**GCTGATGACACCGCCGGCTGG GACAC**	**TCCGTGACATAGCAGGAAAG CAAG**
K94P05	TACAACATGATGGGAAAAAGAG AGAAGAA	GCCGATGACACCGCCGGGTGG GACAC	TCCGTGACATAGCAGGGAAG CAAG
Genotype I-b			
FU	TACAACATGATGGGAAAAAGGG AGAAAAA	GCCGATGACACCGCCGGATGG GATAC	TCCGTGACATAGCTGGAAAG CAAG
Genotype II			
SA14	TACAACATGATGGGAAAAAGAG AGAAGAA	GCTGATGATACCGCCGGGTGG GACAC	TCCGTGACATAGCAGGAAAG CAAG
Genotype III			
JKT6468	TACCACATGATGGGGAAAAGAG AGAAGAA	GCCGATGACACTGCTGGATGG GACAC	TCCGTGACATAGCAAGGAAA GAAG
Genotype IV			

Chapter 4 - RT-qPCR Assays for Veterinary Diagnosis of Japanese Encephalitis Virus

Introduction

As an emerging mosquito-borne virus with multiple incidences of dispersal in the past two decades, the demand for highly sensitive and specific diagnostic assays for JEV has become apparent. JEV-specific RT-qPCR assays have been implemented as routine molecular diagnostic procedures among laboratories and surveillance programs in the endemic regions. Existing diagnostic methods were mainly developed for the identification of JEV infection in humans and the detection of JEV in mosquitoes, but more recently, they have been adapted for veterinary use. The first application of RT-PCR based diagnostic assays to the veterinary diagnosis of JEV was not reported until 2002 (Lian et al., 2002). Multiple RT-qPCR assays are now available as veterinary diagnostic methods, especially in infected swine species (Ogawa et al., 2009). These assays often serve as tools to identify the presence of JEV in serum, CNS fluid or organs of infected pigs (Pyke et al., 2001; Cao et al., 2011; Liu et al., 2012) . Although the assays are highly sensitive and specific for the molecular diagnosis of JEV, the collection of diagnostic samples often involves invasive procedures that require highly skilled veterinary professionals and significantly limits the throughput of sample collections.

As the development of systemic infection has been frequently observed in pigs naturally infected or experimentally challenged with JEV, the application of molecular detection methods has led to the significant advancement of our knowledge in the tissue tropism and transmission dynamicss of JEV in infected animals. The detection of viral genomes and infectious viruses from nasal tissues and oronasal secretions has led to the paradigm shifting observation that

oronasal shedding of infectious viruses is a pathological outcome that supports the transmission of JEV in the absence of competent vectors. As isolation of infectious viruses in oronasal secretions and tissues of infected pigs has been reported in two independent studies, detection of viral RNA in oronasal secretions becomes a potential mechanism for the veterinary diagnosis of JEV and introduces the potential for diagnosis through the use of non-invasive oral fluids (Ricklin et al., 2016; Park et al., 2018).

The objective of this study was to evaluate a non-invasive sample collection procedure for veterinary diagnosis of JEV. Our central hypothesis is that viral load in the oral fluid of pigs experimentally challenged with JEV can be used for the monitoring of infection process *in vivo*. The hypothesis was tested by quantifying the viral load of serum and oral fluid samples collected from experimentally infected pigs using three RT-qPCR assays. The results demonstrate the feasibility of utilizing the non-invasive rope-based collection method to obtain oral fluids as diagnostic samples for JEV infection (Lyons et al., 2018).

Results

Viremic profiles of intravenously challenged pigs

To demonstrate the feasibility of utilizing the three assays evaluated in this study for the laboratory diagnosis of viremic animals, viremic profile of domestic pigs intravenously challenged with JEV JE-91 strain at 3 d.p.i. was determined using blood serum (Chao et al., 2007; Pyke et al., 2004; Yang et al., 2004).

Consistent with the relatively higher sensitivity observed using serially diluted viral RNA extract as biological standards, assay #1 showed the highest sensitivity amongst the three assays ($p<0.05$). As illustrated in Figure 4.1, the average viremic titer determined by assay #1 was

993.49± 3360.61 genome equivalent (g.e.q) to $TCID_{50}$/mL, significantly higher than the average viremic titers based on the quantification results of assay #2 (83.29±225.06 g.e.q. to $TCID_{50}$/mL) and assay#3 (101.68±366.82 g.e.q. to $TCID_{50}$/mL). Assay#3 showed the lowest sensitivity, as the assay failed to detect the presence of viral genome in the viremic serum sample collected from animal #13.

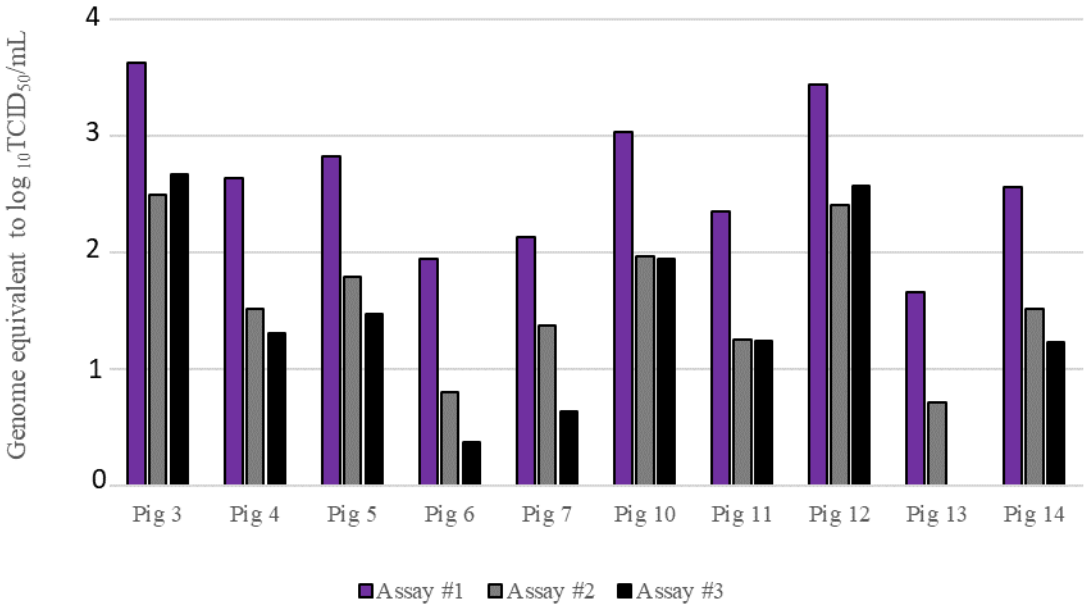

Figure 4.1 RT-qPCR results from serum collected at 3 d.p.i. from pigs IV inoculated with 10^7 $TCID_{50}$ JEV strain JE-91. Pigs 1, 2, 8, and 9 were control pigs and JEV genome was not detected in their serum.

Detection of JEV genome in oral fluid of experimentally infected pigs

Oral fluid samples from three-week-old white-line crossbred domestic pigs and Sinclair™ miniature swine with feral genetic background, intradermally challenged with JE-91 strain, were collected at 0-14, 21, and 28 d.p.i. Concentrations of viral genomes were quantified

using the three RT-qPCR assays evaluated in this study (Chao et al., 2007; Pyke et al., 2004; Yang et al., 2004). The detection of viral RNA in the oral fluids of both the domestic and Sinclair^{TM} miniature pigs demonstrated the feasibility of using oral fluid as diagnostic samples for the detection of JEV infection *in vivo*.

Consistent with the quantification of viral RNA extracted from tissue culture fluid of JE-91 strain, and serum samples collected from the experimental challenge of JE-91 strain, assay #1 showed the highest sensitivity for the detection of viral RNA in oral fluid. In both challenge experiments, assay #3 had the lowest sensitivity and failed to detect viral RNA in oral fluid samples that were demonstrated to be positive by the other two assays.

As depicted in Figure 4.2, similar shedding profiles in experimentally challenged domestic pigs were observed based on the quantification of viral genome by assays #1 and #2. The earliest detection of oral shedding of JEV occurred at 3 d.p.i. Persistence of the viral genome was observed up to 14 d.p.i. after the clearance of viremia at approximately 5-6 d.p.i. (Park et al., unpublished data). The peak of viral load was identified by both assays at 5 d.p.i., exceeding 10^3 g.e.q. to $TCID_{50}$/mL. Assay#1 proved to be consistently more sensitive than assay #2 based on a higher viral load corresponding to the lower Cq values ($p<0.05$). Viral load detected by both assays fell below the cut-off values for positive detection at 12 d.p.i. as Cq values of both assays exceed 34. However, the actual clearance of viral RNA in oral fluid did not occur until 14 d.p.i.

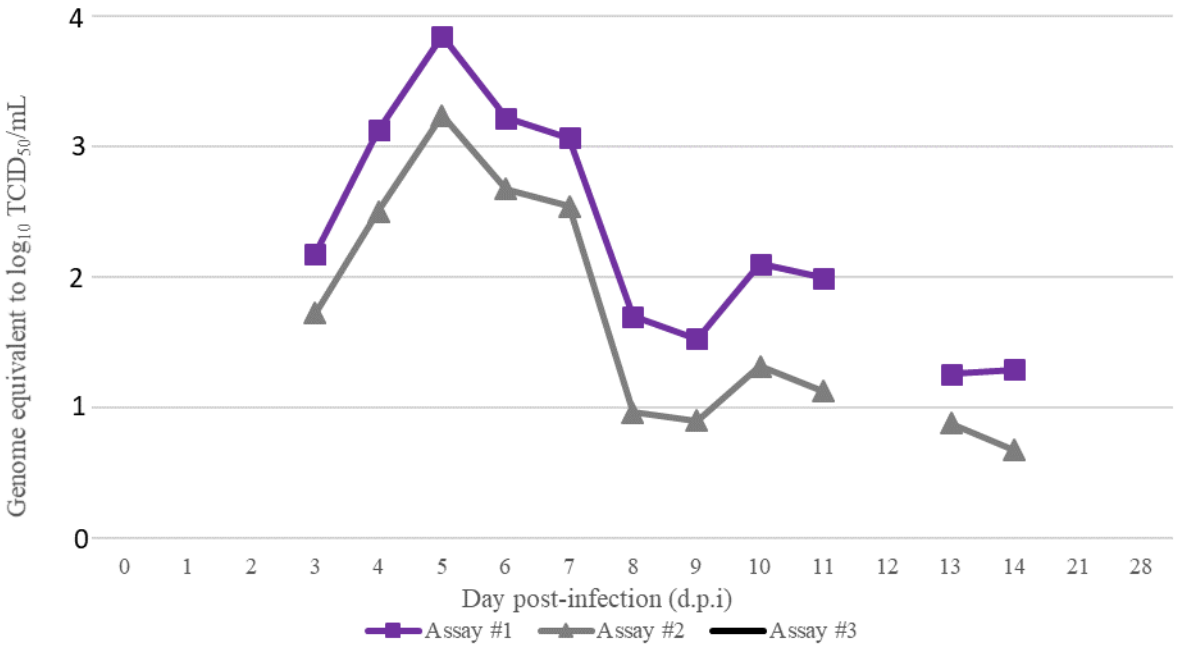

Figure 4.2 RT-qPCR results from oral fluids collected at 0-12, 21, and 28 d.p.i. from 3-week-old white-line cross bred pigs ID inoculated with 10^7 $TCID_{50}$ JE-91. Values below the threshold cutoff Cq value of 34 are not pictured.

Again, as seen in the domestic pigs, assays #1 and #2 demonstrated the capability of detecting JEV viral genome in oral fluids from the Sinclair™ miniature feral swine model. The shedding profiles determined by both assays are shown in Figure 4.3. In contrast to the prolonged period of oral shedding observed in domestic pigs infected with JEV, a shorter period for viral shedding was observed in the oral fluid of Sinclair™ miniature feral swine. Positive detection of viral RNA by assay #1 was observed between 2 and 7 d.p.i.; whereas, assay #2 detected the presence of viral RNA in oral fluid between 4 and 6 d.p.i. This finding was consistent with the higher sensitivity of assay #1 than the other two assays. Both assays showed a peak in JEV viral shedding at 5 d.p.i., with the quantification results of assay #1 exceeding 10^2 g.e.q. to $TCID_{50}$/mL and assay#2 exceeding 10^1 g.e.q. to $TCID_{50}$/mL.

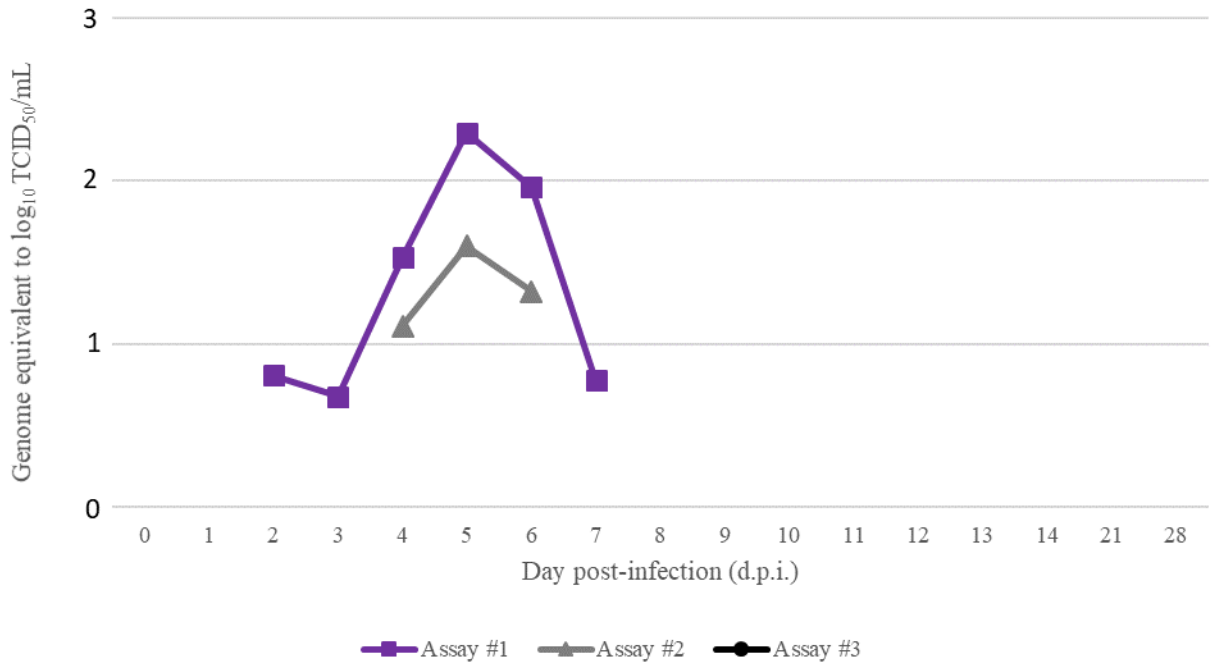

Figure 4.3 RT-qPCR results from oral fluids collected at 0-12, 21, and 28 d.p.i. from 3-week-old Sinclair™ miniature pigs with feral genetic backgroud ID inoculated with 10^7 $TCID_{50}$ JE-91. Values below the threshold cutoff Cq value of 34 are not pictured.

Conclusions and discussion

The findings of this study demonstrated the feasibility of utilizing viremic serum and oral fluid collected from infected pigs for the veterinary diagnosis of JEV. Variation in the sensitivity of each RT-qPCR assay was observed but it was immediately apparent that optimized RT-qPCR assays with the lowest number of nucleotide mismatches provide the best sensitivity for the detection of the viral genome.

Importantly, the detection of viral RNA in oral fluids by two RT-qPCR assays provides a novel strategy to detect infection of JEV in pigs. As the detectable level of viral RNA in oral fluids persisted after the clearance of viremia, the identification of infected animals after the

acute phase of infection is possible through molecular detection of viral RNA in oral fluids. The detection of JEV viral RNA in oral fluid by RT-qPCR assays has a significant advantage over existing diagnostic methods developed to identify infection of JEV at the convalescent phase of diseases. Historically, serological assays such as enzyme-linked immunosorbent assay and neutralization tests are frequently used to demonstrate recent infection. Although the detection of IgM antibodies or an increase in neutralizing antibody titers are reliable markers for recent infection, cross-reactivity of humoral immune responses with other related flaviviruses can potentially complicate diagnostic results. Detection of viral RNA in oral fluids provides definitive evidence required for the veterinary diagnosis of JEV. The non-invasive nature of sample collection will also allow rapid screening of large numbers of animals and increase the throughput of surveillance programs using pigs as sentinel animals.

Chapter 5 - Major Findings, Conclusions, and Discussion

The molecular diagnostic methodologies presented in this thesis provide significant advancements in our capability to diagnose foreign animal and zoonotic disease, specifically JEV, in swine species. This chapter summarizes the results of this study by providing the major findings and conclusions (Table 5.1) based on experimental results and identifies future directions for research in optimizing molecular diagnosis of JEV in animal reservoirs.

Evaluation of RT-qPCR assays for optimum detection of JEV in the presence of related flaviviruses

The three RT-qPCR assays evaluated in this study were able to detect the presence of viral RNA extracted from stocks of two major endemic genotypes. However, not surprisingly, variations in sensitivity were observed due to differences in the genomic sequences of the JEV genotypes. The evolution of JEV has led to the emergence of five distinct genotypes in the endemic region; up to 20% of sequence divergence can be expected between each of the genotypes. The identification of highly conserved regions in viral genomes and the inclusion of degenerate primers may provide potential pathways for the development of highly sensitive methods for the detection of all JEV genotypes (Pyke et al., 2003).

The lack of cross-reactivity with WNV and SLEV demonstrated in this study supports the feasibility of deploying all three RT-qPCR assays for veterinary diagnosis of JEV in areas where JEV is present with other related members within the JEV serocomplex. It is also highly unlikely for the RT-qPCR assays to be cross-reactive with other flaviviruses. Previously published work was able to demonstrate that assay #1 was able to avoid cross-reactivity with other flaviviruses

by targeting the JEV-specific region in the NS5 and the 3′UTR regions of the genome (Pyke et al., 2003). Although assay #3 was developed based on the consensus sequence in the NS5 gene present within multiple medically important flaviviruses, the use of oligonucleotide probes containing virus-specific sequences has been proven to be successful for the differentiation of various flaviviruses including JEV (Chao et al., 2007). As the dispersal of JEV and WNV continues and the geographic distribution of both viruses expands, it becomes increasingly important to maintain the capability of differentiating infection of the two viruses. In addition to the potential application for these assays to detect the presence of viral RNA in swine, these assays could be used with other amplification hosts, especially avian species that can develop viremia for JEV, WNV, and other related flaviviruses.

Detection of viral RNA in serum samples of infected pigs

Since pigs serve the role of an amplification host species, and develop high titer viremia, swine serum is a commonly used diagnostic sample for JEV. Multiple existing surveillance programs utilize serum samples of sentinel domestic pigs for isolation or detection of viruses with the rationale that the early detection of enzootic transmission among pigs may be helpful in preventing epizootic outbreaks. As clearance of viremia often takes place within a few days among experimentally challenged pigs, the relatively short duration of the viremic phase may limit the likelihood for successful isolation of infectious viruses from serum samples of sentinel pigs using conventional cell-based methods. Because of the high sensitivity and specificity, the detection of viral genomes using molecular methods, especially RT-qPCR-based diagnostic assays, has rapidly replaced the virus isolation approach. However, diagnosis of JEV in endemic regions lacks consistency because individual laboratories often utilize different in-house RT-qPCR assays. The lack of a systemic analysis in the sensitivity and specificity of different assays

precludes comparison of diagnostic results and our understanding of disease incidence in different regions.

The results of our study highlight such a technical challenge. Sensitivity of individual assays targeting neighboring regions in the viral genome can still differ significantly, presumably due to the differences in the oligonucleotide primers and probe of each assay. The two assays (assays #1 and #2) utilizing JEV-specific primers and probes showed higher sensitivity in detecting the viremia caused by the infection of GI-b JE-91 strain than the other assay (assay #3) using the combination of the pan-flavivirus mFU1 and CFD2 primers and a JEV-specific oligonucleotide probe (Kuno et al.,1998). As GI-b has displaced GIII as the predominant genotype throughout the Asian Pacific region, the highly sensitive assay #1 targeting the 3′ end of NS5 gene and 3′UTR proves to be an ideal diagnostic method for the optimum detection of JEV strains belonging to the newly dominant GI-b (Schuh et al., 2014).

Diagnosis of JEV using oral fluids

The use of oral fluids for veterinary diagnosis has significantly increased over the last decade with numerous assays and reagents being developed for the detection of swine pathogens. The collection of oral fluid can be achieved using non-invasive rope-based procedures that do not require highly skilled veterinary professionals. Unlike the collection of whole blood or oronasal swabs, which must be collected from individually restrained animals, the rope-based method used for the collection of oral fluid allows for the simultaneous sampling of multiple animals and provides a remarkable improvement in the throughput of sample collection. These advantages can be reflected by decreased costs in veterinary diagnosis and the increased feasibility of testing large numbers of animals for the presence of pathogens.

For the first time, the results of our study demonstrated the feasibility of utilizing oral fluids as diagnostic samples for the detection of JEV in infected pigs. The ability to use oral fluids to detect new infections of JEV could allow for the expansion of surveillance programs using sentinel pigs as well as provide a tool for pig producers to monitor their pig herds for the introduction of JEV. Interestingly, the use of oral fluid as a veterinary diagnostic sample for JEV may not only allow the increased throughput of sample collection but also allow for improved capability in detecting infected hosts. As demonstrated in experimentally challenged animals, the duration of viremia in pigs infected with JEV is relatively short. Detectable levels of infectious viruses or viral RNA normally only persists up to five days followed by the development of neutralizing antibody responses. The detection of viral RNA in the oral fluid of domestic pigs at 14 d.p.i. and feral pigs at 6 d.p.i. indicates that our approach is capable of identifying recent infections of JEV outside the acute phase of infection. Although newly acquired viral infections can be demonstrated by the presence of IgM antibodies, detection of viral RNA by RT-qPCR assays provides direct evidence of infection through the specific identification of viral genome and does not require the delay in diagnosis created by comparing paired serum samples.

Our findings are consistent with previous observations that infection of JEV in pigs leads to detectable levels of infectious viruses shed in oronasal secretions (Ricklin et al., 2016; Park et al., 2018). Our results further define the mechanisms responsible for vector-free transmission of JEV by demonstrating the presence of JEV in the oral fluid of infected animals. Coincidentally, a recent publication also demonstrated that viral RNA of JEV can be detected in the throat samples of infected humans (Bharucha et al., 2018). These findings have challenged our existing knowledge in the tissue tropism of flaviviruses, which are mainly known to cause viremia and neurotropic infections.

Table 5.1 Conclusions regarding RT-qPCR assays and their potential application.

	Pros	Cons	Overall
Assay #1	Most sensitive for GI-b; most sensitive at detecting GI-b infection in swine oral fluids and serum	Unable to detect GIV; Less sensitive than Assay #2 and Assay #3 at detection of GIII	Best for surveillance in regions with dominant GI-b circulation
Assay #2	Capable of detecting all JEV genotypes	Less sensitive than Assay #1 at detection of GI-b	Best for surveillance in areas with multiple JEV genotypes co-circulating
Assay #3	Pan-flavivirus assay	Less sensitive than Assay #1 and Assay #2; unable to detect GI-b infection in swine oral fluids and serum	Best at differentiating JEV infection from co-circulating flaviviruses

Future directions

The results of our study successfully demonstrate the feasibility of using the three RT-qPCR assays for the detection of JEV infection using serum and oral fluid samples collected from experimentally infected animals. The lack of cross-reactivity indicates the three assays are capable of differentiating infections of related flaviviruses. Whilst the use of oral fluids for diagnostic sampling provides a novel approach to improve the existing veterinary diagnosis and surveillance of JEV, it is also important to address the limitations in this study. The newly developed diagnostic assays must be validated using samples collected from the field (i.e. naturally infected animals). Because of the difference in sensitivity of the three assays in detecting the multiple endemic genotypes, it will be helpful to include validation of the oral fluid-based diagnostic method in detecting other endemic genotypes that co-circulate with GI-b

tested in this study. As the experimental set-up of this study provides multiple infected animals with unlimited access to the rope used for sample collection, it remains unclear if the sensitivity of the assays will be sufficient for detection when few infected animals are present within individual pen populations.

With current pig production practices, co-infections of swine pathogens are becoming increasingly more common. Differentiation of these pathogens can be challenging as clinical symptoms oftentimes overlap. Several major swine pathogens can present with reproductive failure similar to JEV, including: PRRSV, CSF, PCV2, porcine pseudorabies virus and porcine parvovirus. Multiplex PCR assays have been developed for the detection of JEV, alongside these pathogens, in tissue samples and while less sensitive than RT-qPCR, can provide time and cost savings by allowing the simultaneous detection and differentiation of multiple infections (Zeng et al., 2014; Zhang et al., 2015). The feasibility of detection using multiplex assays and oral fluids has been demonstrated previously, but not with assays targeting JEV (Grau et al., 2015).

Whilst PCR-based assays are excellent diagnostic tools for JEV infection during the acute phase, they are limited in use for surveillance due to the short window of detection provided. Serological assays remain a critical component of veterinary diagnosis of JEV for serum samples obtained during the convalescent phase of infection. Antibodies to swine pathogens such as PRRSV, influenza A virus, swine vesicular disease virus, and ASFV have been found at detectable levels in the oral fluids (Prickett, 2008; Senthikumaran, 2017; Gimenez-Lirola, 2016; Panyasing, 2014). The evaluation of using oral fluids for serological diagnosis of JEV will provide a potential pathway to further improve JEV diagnostic and surveillance capabilities.

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