Characterizing the fecal shedding of swine infected with Japanese encephalitis virus

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Abstract

Japanese encephalitis virus (JEV) is an enveloped, single-stranded, positive sense *Flavivirus* with five circulating genotypes (GI to GV). JEV has a well described enzootic cycle in endemic regions between swine and avian populations as amplification hosts and *Culex* species mosquitoes which act as the primary vector. Humans are incidental hosts with no known contributions to sustaining transmission cycles in nature. Vector-free routes of JEV transmission have been described through oronasal shedding of viruses among infected swine. The aim of this study was to characterize the fecal shedding of JEV from intradermally challenged swine. The objective of the study was to advance our understanding of how JEV transmission can be maintained in the absence of arthropod vectors. Our hypothesis is that JEV RNA will be detected in fecal swabs and resemble the shedding profile observed in swine oral fluids, peaking between days three and five. In this study fecal swabs were collected throughout a 28-day JEV challenge experiment in swine and samples were analyzed using reverse transcriptase-quantitative polymerase chain reaction (RT-qPCR). Quantification of viral loads in fecal shedding will provide a more complete understanding of the potential host-host transmission in susceptible swine populations. Our results show that fecal shedding of JEV appears to mimic that of oral shedding, with peak viral loads detected around day five post-infection. The detection of JEV viral genomes in fecal specimens indicate a potential for fecal shedding to contribute to transmission of JEV in the absence of vectors. These findings are significant in developing control strategies to mitigate the agricultural and public health threats created by JEV in endemic regions.

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Chapter 1 - An Overview of Japanese Encephalitis Virus

Introduction

Arthropod-borne viruses contain several emerging pathogens of human and animal public health significance. Specifically, mosquito-borne viruses of family *Flaviviridae* have repeatedly demonstrated the ability to rapidly disperse and establish transmission cycles among immunologically naïve hosts. In the past three decades, emerging flaviviruses under the Japanese encephalitis virus (JEV) serocomplex have been reported in new regions and contribute to human and animal disease burden worldwide. This includes the emergence of Usutu virus in Europe (Ashraf et al. 2015; Lühken et al. 2017) and the dispersal of West Nile virus (WNV) across Europe and into the New World (Asnis et al. 2000; Nash, O'Leary, and Sherman 2001).

JEV is the leading cause of acute encephalitis syndrome (AES) in children throughout the South Pacific region. The conventional knowledge is that JEV is maintained through an enzootic transmission cycle involving swine as amplification hosts, avian species as pathogen reservoirs, and *Culex* species mosquitoes as the primary vector (Lord, Gurley, and Pulliam 2015; Buescher, Scherer, Rosenberg, Gresser, Hardy, et al. 1959). Although JEV remains geographically confined in the Asia Pacific region, numerous studies have demonstrated mosquito, avian and swine species around the world are susceptible to JEV and vertebrate species can develop viremia to support transmission (Lord, Gurley, and Pulliam 2015; Park et al. 2018; Oesterle et al. 2012; de Wispelaere, Després, and Choumet 2017; Huang et al. 2015). Potential dispersal of JEV outside the endemic region might have also occurred without the establishment of enzootic transmission. For instance, fragments of JEV genome have been found in *Culex pipiens* mosquitoes and birds in Italy (Ravanini et al. 2012; Platonov et al. 2012) and a human case in Sudan (Simon-Loriere 2017).

The diversity and dispersion of susceptible vertebrate hosts and ubiquity of JEV competent mosquitoes make JEV a threat for geographic expansion. New evidence suggests that JEV may also be transmitted in the absence of competent vectors through contact with oral secretions of infected swine (Ricklin, García-Nicolás, et al. 2016; Lyons et al. 2018). This report will characterize the fecal shedding profile of JEV infected swine and discuss its potential influence on vector-free transmission of JEV in swine populations. The hypothesis is that JEV RNA can be detected in fecal swabs collected from JEV infected swine. If large quantities of genomic RNA are detected, the excrement may contain viable JEV virions which could in turn provide a vector-free route of transmission to immunologically naïve swine through contact with their highly susceptible oronasal cavity. To test this hypothesis, reverse-transcriptase-quantitative-polymerase-chain-reaction (RT-qPCR) is the molecular diagnostic technique implemented to detect genomic viral fragments in fecal samples during animal challenge studies. Understanding the kinetic shedding profile of infected swine may inform advancements in control and provide alternative samples for use in diagnostics and surveillance.

History

Reports of summer encephalitis, consistent with the manifestation and clinical symptoms of Japanese encephalitis, date back to 1871 in Japan (Lewis et al. 1947). Small outbreaks were reported during rainy seasons over the next 50 years with a large outbreak reported in 1924 consisting of 6,000 cases and a 60% case fatality (Solomon et al. 2000). At this time, rabbits and mice were used to isolate an unknown filterable agent from a fatal human case. This agent was identified in 1935 by Japanese scientists after multiple studies demonstrated encephalopathy in mice and monkeys following infection by intracerebral inoculation of human brain suspension (Miyake 1964; Webster 1938). Vaccines became available in the 1950's in the form of inactivated

mouse-brain derived genotype-III strains (Warren, Smadel, and Rasmussen 1948). Serological diagnostic tests were then developed for JEV based on the presence of specific neutralizing antibody in recovered patients (Erlanger et al. 2009; Calisher et al. 1988).

Astute observations by early investigators attempting to reveal natural reservoirs and transmission pathways prevailed when virus was isolated from *Culex tritaeniorrhynchus* in 1936 (Mitamura et al. 1936) and mosquito-borne transmission of JEV was suggested in 1938 (Rosen 1986a; Erlanger et al. 2009; Mitamura et al. 1938). These findings provided opportunities to interrupt the transmission cycle using vector control strategies. *Culex tritaeniorhynchus* remains to be the most prolific vector of JEV, while *Cx. vishnui, Cx. gelidus, Cx. pseudovishnui, Cx. sitiens,* and *Cx. fuscocephala* species have also demonstrated vector competency in studies that followed (Hurk, Ritchie, and Mackenzie 2009; Pearce et al. 2018). Field studies by Buescher and Scherer in the 1950's demonstrated the role of aquatic birds and pigs in the viral enzootic cycle (Buescher, Scherer, Rosenberg, Gresser, and Hardy 1959).

Ecology

Arthropod-borne viruses (arboviruses) rely on the interaction of competent invertebrate vectors and susceptible, viremic vertebrate host species within particular climatic parameters to establish and maintain enzootic transmission cycles. The climatic conditions and agricultural practices in the South Pacific, namely intense rice farming and swine production, are major factors contributing to the maintenance of JEV. Transmission cycles are attributed to dense populations of *Culex* species mosquitos and amplification hosts such as swine and other susceptible vertebrates, particularly waterfowl, which develop sufficient viremia to support continued transmission.

When discussing the ecological conditions that support JEV transmission it is important to first consider the climatic conditions necessary to support the mosquito lifecycle. Tropical areas provide adequate temperatures for mosquitoes to continuously breed and maintain low levels of JEV transmission year-round. Further from the equator, or at higher elevations, seasonal variation in temperature and precipitation restrict the mosquito lifecycle and result in predictable patterns of JEV outbreaks as they occur during warmer months or rainy seasons (Miller et al. 2012). When factoring in anthropogenic influences, epizootic potential is highest amongst the copious amounts of rice cultivation and pig farms in this region (Hurk, Ritchie, and Mackenzie 2009). Numerous investigations have supported the correlation between increased rice cultivation and higher incidence of JEV (Hurk, Ritchie, and Mackenzie 2009; Miller et al. 2012; Liu et al. 2010; Takagi et al. 1997). This is attributed to the low lying stagnate water bodies associated with paddy fields, providing the preferred conditions for *Culex* species mosquitoes to breed, lay eggs, and complete larval and pupal development. A correlation between pig farming intensity and increased JEV prevalence has also been described since the 1950's (Lord, Gurley, and Pulliam 2015; Buescher, Scherer, Rosenberg, Gresser, and Hardy 1959). This has implicated pigs as the main amplification host for JEV. Although, this should be interpreted with caution because pig farming is not as prevalent in other regions where JEV incidence is also high (Bustamante and Lord 2010).

Whilst transmission of JEV is known to peak during the summer and fall, the underlying mechanisms that support the inter-epidemic outbreaks and viral maintenance remain unclear. Outbreaks of JEV have been observed during inter-epidemic periods in temperate zones absent of infected mosquito populations (Hashimoto et al. 1988). There are multiple proposed explanations for these occurrences. One proposed hypothesis is that JEV is reintroduced each year, either through bird migration or infected mosquito introduction. Another hypothesis is that JEV is

maintained locally in its mosquito vector, possibly through transovarial transmission (Rosen et al. 1978). Yet another theory involves local over-wintering of JEV in vertebrate reservoir hosts, such as pigs and birds (Rosen 1986a). This has instigated investigations aimed at re-evaluating the contribution of JEV hosts in transmission dynamics of JEV.

Epidemiology of Japanese Encephalitis Virus

Geographic Distribution of Japanese Encephalitis Virus

Epidemics of JEV were first reported in Japan during summer outbreaks in 1924, 1927, 1934, and 1935. The Korean peninsula and China began reporting cases in the late 1930's and 1940's. The geographical area in which JEV cases are reported has expanded over the past 50 years (Campbell et al. 2011). By 1960, JEV had spread across Eastern Asia and began appearing in the Pacific islands of Philippines and Indonesia. Westward expansion was reported throughout the late 20th century, appearing first in India and reaching Nepal in 1978 and Pakistan in 1983. JEV continued to expand South to Papua New Guinea and Northern Australia in 1995 (Misra and Kalita 2010; Erlanger et al. 2009; Hanna et al. 1995). More recently evidence of JEV has been reported outside of the Asian Pacific region. The detection of genomic JEV from *Culex pipiens* in Italy suggests Europe may have JEV circulation (Ravanini et al. 2012). Additionally, an isolated human case of JEV was reported in Sudan in 2017 which is believed to be locally acquired (Simon-Loriere 2017).

Similar to other mosquito-borne flaviviruses, geographic distribution is dependent on the presence of vector and vertebrate populations that support viral replication and transmission (Kuno and Chang 2005; Miller et al. 2012). One must appreciate the capacity for JEV and related flaviviruses to adapt quickly to new hosts and ecological conditions. These evolutionary tendencies facilitate a robust range of hosts which may aid in transboundary distribution and subsequent

persistence (Elena and Sanjuán 2005). Migratory birds and bat populations are suspected to play a role in JEV distribution and maintenance, although conclusive data representing the role of bats is currently lacking (Kuno and Chang 2005; Lord, Gurley, and Pulliam 2015; Rosen 1986b). Water birds, such as egrets, herons, and ducks reside in close proximity to habitats one would expect to find ample mosquito breeding. In regions such as India where these bird species are domesticated and in close proximity to homesteads or sold in live market-places, there is potential for spillover to human populations (Lord, Gurley, and Pulliam 2015).

Incidence

Vector-borne diseases account for 17% of global human infectious disease incidence. While many of those may be related to malaria or various tick-borne bacterial pathogens, viral pathogens constitute over 25% of emerging infectious diseases from 1940-2004 (Jones et al. 2008). Additionally, mosquito-borne pathogens such as dengue, Zika, chikungunya, and yellow fever account for millions of infections each year (Daep, Muñoz-Jordán, and Eugenin 2014; Gould and Solomon 2008). There are currently twenty-four countries found primarily throughout Asia and the Western Pacific with endemic transmission of JEV. This area is home to more than one-third of the global population. A 2011 report released by the World Health Organization (WHO) estimates global incidence of JEV to be 67,900 cases per year with 13,000-20,000 deaths; a total incidence of 1.8/100,000 (Campbell et al. 2011). A vast majority of these cases (up to 86%) occur in China and India (Campbell et al. 2011). The 0-14 age group incurs 75% of cases with annual incidence of 5.4 per 100,000. Prolonged impairment of JE infections are estimated to have a global impact of over 700,000 disability-adjusted life years (DALYs) (Mathers, Ezzati, and Lopez 2007). It is noted that these estimates are based on data sets that likely include non-JE cases, but this discrepancy is overshadowed by the assumed under-reporting of actual cases.

Given that most human JEV infections are sub-clinical, large fluctuations are common when assessing the global impact of JEV. A majority of reported cases happen during epidemic periods. Generally, there are two epidemiological patterns for JEV; 1) Tropical and subtropical areas experience relatively consistent, endemic JE transmission while 2) temperate locations may only experience epidemic transmission for five or six months during the warm rainy season (Hurk, Ritchie, and Mackenzie 2009). General expectations of JEV incidence can be estimated when considering air temperature, precipitation, and vaccine coverage of particular regions. Anthropogenic influences are also correlated with JEV incidence and can make it difficult to produce accurate annual predictions of JEV incidence (Pearce et al. 2018). Unprecedented population growth in JEV endemic regions is compounded with increased rice farming and industrial rearing of domesticated animals (Erlanger et al. 2009). These practices increase breeding grounds for *Culex* species mosquitoes and turnover rates of susceptible vertebrate populations for mosquitoes. Much of this activity is associated with rural communities where vaccine coverage may be low and contact with infected animal populations may be high (Lord, Gurley, and Pulliam 2015).

Human and Animal Diseases Caused by Japanese Encephalitis Virus

JEV is a zoonotic pathogen known to cause diseases in mammalian species. Humans are incidental hosts that develop low viremic titers that are not sufficient to support transmission in nature. However, immunologically naïve individuals, particularly children, can succumb to neurotropic diseases. Although most infections in swine are asymptomatic, clinical presentation in young swine can present as mild neurological impairment in limbs (Park et al. 2018). Disease in adult swine is known to be mild but has been reported to cause reproductive loss when infection takes places during pregnancy (Lindahl et al. 2012). In either case, the effects of JEV associated

morbidity and mortality, human or swine, have economic tolls related to public health and agricultural loss.

Greater than 98% of human JEV infections are asymptomatic (Fan et al. 2017; Campbell et al. 2011). Clinical manifestation in symptomatic individuals presents as acute febrile illness with associated symptoms including fever, dizziness, and flu-like illness (Miyake 1964). Advanced JE infections progress to acute encephalitis syndrome (AES) characterized by inflammation of the brain (encephalitis). AES has neurotrophic involvement including headache, vomiting, reduced level of consciousness, and occasional convulsions. Some cases report acute flaccid paralysis in the legs and arms (Solomon et al. 2000). Children are more prone to severe disease outcome and display higher rates of convulsions and seizures than adults. Some patients have rapid spontaneous recovery while others that experience multiple or prolonged seizures, changes in respiratory pattern, and abnormal reflexes are associated with poor outcomes. Human mortality following clinical onset of symptoms is estimated at about 30% and may account for nearly 20,000 deaths each year, dubbing JEV as the leading cause of viral encephalitis associated death (Campbell et al. 2011). About half of the recovered individuals display prolonged motor deficits, cognitive and language impairment, or further convulsions resulting from neurological sequelae (Solomon et al. 2000).

Swine populations are significant amplification hosts for JEV. Contrary to human and equine infections, swine support high levels of viremia for 2-4 days (Ricklin, Garcìa-Nicolàs, et al. 2016; Sasaki et al. 1982; Park et al. 2018). Clinical manifestation of experimentally infected piglets includes transient fever and depression recovery. Adult swine are typically asymptomatic but have been reported to encounter reproductive losses as sows experience stillbirths and abortions (Hashimoto et al. 1988). Prolonged infection of the tonsils has recently been reported

but has not been associated with any clinically significant consequences (Ricklin, García-Nicolás, et al. 2016; Park et al. 2018).

Control

In the absence of antiviral therapy, only supportive therapies and preventive measures can be taken to control JEV. Surveillance and vaccination have proved to be an effective means to control JEV in endemic regions with well-established public health care. China, Japan, Nepal, South Korea, Sri Lanka, Taiwan, and Thailand have successful vaccination programs that have reduced disease burden (Erlanger et al. 2009). However other countries such as Bangladesh, Cambodia, India, and Laos do not have established vaccination programs and continue to see a rise in JEV incidence (Erlanger et al. 2009).

Mouse brain-derived Isolates of JEV from 1935 and 1949 provided prototype Nakayama and Beijing (P1) strains that were widely used to produce inactivated vaccines during the last half of the twentieth century (Yun and Lee 2014; Ku et al. 1994; Tirrell et al. 1999). While this inactivated vaccine provided protection for millions, the production was costly and there were occasional reports of reactogenicity (Hoke et al. 1988). Today, cell culture-derived live-attenuated (SA14-14-2) and killed-inactivated (JEBIK V; ENCEVAC; IC51; IXIARO) vaccines are available in addition to genetically engineered live-attenuated chimeric vaccines (ChimeriVax-JE) which are available in Taiwan and Australia. Although all JEV genotypes are grouped within a single serotype (Beasley et al. 2004), antigenic variations are known to exist among circulating JEV genotypes as shown by neutralization tests that demonstrate the differences in neutralizing antibody titers against different genotypes (Kobayashi et al. 1984; Fan et al. 2012). Currently, all licensed JEV vaccines are derived only from GIII JEV strains. Here lies the potential for vaccine

breakthrough issues to arise as G1-b strains have become the dominant circulating genotype in recent decades (McAuley et al. 2017; Fang et al. 2019).

In the absence of vaccines, personal precautions can be taken to limit an individual's exposure to mosquito bites (Omodior, Luetke, and Nelson 2018). Additionally, modifying pig rearing and agricultural practices could limit mosquito activity and JEV circulation. One approach to reducing outbreaks of JEV is to improve irrigation methods as to reduce standing water and therefore mosquito breeding. Alternatively, the use of insecticide and larvicide treatments can be used but are costly and effective for a relatively brief period of time (IRRI 1989).

Global surveillance networks and advanced molecular diagnostic techniques have provided necessary resources to track the incidence and dissemination of JEV and other arthropod-borne viruses. These tools inform vaccination campaigns and could be applied to determine where biocontrol measures may be most effective at interrupting JEV transmission.

Transmission

Enzootic transmission cycles of JEV in the Asian Pacific region are influenced by seasonal rains, increased rice farming, and industrial production of domestic animals like swine and birds (Erlanger et al. 2009; Huang, Higgs, and Vanlandingham 2019; Pearce et al. 2018). The transmission of JEV is facilitated by the hematophagous nature of mosquito vectors and their interactions with pigs for amplification and susceptible birds for maintenance and dissemination in nature. This mode of transmission is attributed to viral characteristics and aspects of community ecology such as host availability, susceptibility, density, and behavior (Antonovics et al. 2017; Agarwal, Parida, and Dash 2017).

The ecological context is significant when considering the source of blood meals and the type and density of vertebrates available. JEV nucleic acid has been detected in a wide range of

vertebrates in nature (Oliveira et al. 2018; Lord, Gurley, and Pulliam 2015). Mammalian hosts with documented seroprevalence of JEV are not limited to rodents, goats, cows, horses, dogs, raccoons, bats, and pigs (Oliveira et al. 2017). The dilution of competent hosts with dead-end or less permissive hosts is important when considering the rate of pathogen spread (Lord, Gurley, and Pulliam 2015; Schmidt and Ostfeld 2001). Pigs, birds, and rodents may support a period of JEV viremia and have high turnover rates that provide constant populations of susceptible young, enabling continuous transmission between vectors and vertebrates (Endy and Nisalak 2002; Page et al. 2014). In some regions that are predominantly Islamic, such as India and Bangladesh, swine have a lesser presence but JEV cases are still frequently reported. This indicates that enzootic cycles with different key vertebrate host species likely exist but are not currently well defined.

It is also important to consider anthropogenic influences that may support the transmission of JEV. South-east Asia has experienced increases in industrial pig rearing and the expansion of rice cultivation over the last 50 years. The proximity of the paddy fields and pig farms purvey opportunities for female mosquitoes to transmit JEV while ingesting the blood meal necessary for her to produce eggs. Additionally, the zoophilic nature of some *Culex* species mosquitoes, particularly *Cx. tritaeniorhynchus*, are fitting vectors for JEV because of the host preference on avian and mammalian hosts of JEV (Wada et al. 1970; IRRI 1989; Pearce et al. 2018).

Mosquitoes can also contribute to transboundary dissemination of JEV and other flaviviruses. Globalization has provided opportunities for mosquitoes to hitch rides on cargo ships transporting consumer goods and waste. Additionally, mosquitoes may be carried by wind over great distances to regions where they may infect susceptible hosts and establish transmission cycles in naïve populations (Pearce et al. 2018). This is believed to be the route which introduced JEV to Northern Australia in the 1990's (Ritchie and Rochester 2001).

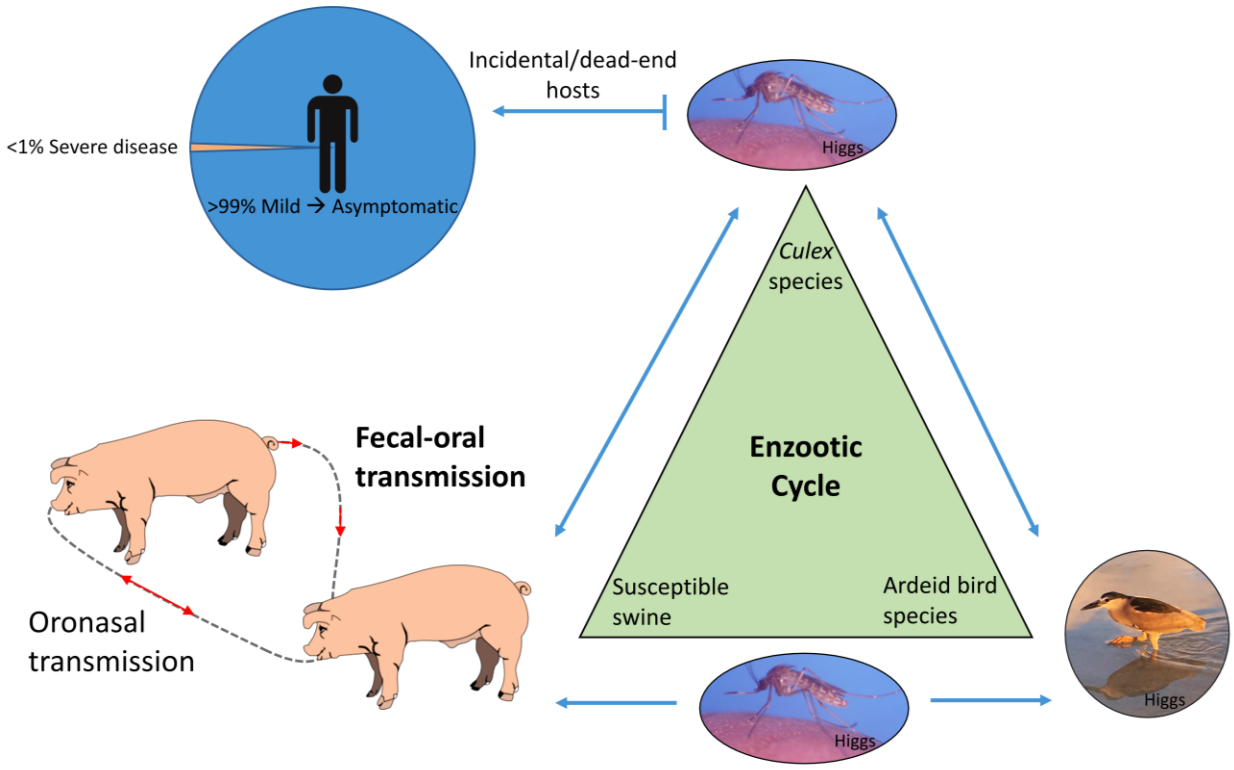


Figure 1.1. Enzootic transmission cycle of JEV is supported by competent mosquito vectors (*Culex* species), swine amplification hosts, ardeid avian species reservoir hosts. Humans are considered incidental, dead-end hosts. Dotted lines represent hypothesized (fecal-oral) and experimentally demonstrated (oronasal) transmission events that occur between swine and are not known to occur in nature.

Host-Pathogen Interactions

There are several host-pathogen interactions and spatiotemporal factors to take into account when contemplating the ecological and evolutionary development that has allowed JEV to emerge and maintain successful enzootic transmission cycles. Assuming the availability of susceptible vertebrate hosts and competent vectors within supportive climatic parameters, JEV has many intra- and inter-host barriers to overcome in order to proliferate and continue transmission. The virus

must replicate in both vertebrate and invertebrate hosts, producing ample progeny, viremia, and/or tissue tropism for viral shedding to occur and support subsequent transmission events.

When vertebrate hosts are exposed to JEV via biological transmission in mosquito saliva, viral characteristics, such as the mutational capacity derived from the high fidelity of RNA-dependent RNA polymerase (RdRp) based replication, provide JEV the opportunity to gain affinity to vertebrate host cell receptors. Further optimization of JEV viral proliferation in vertebrate hosts creates parasitic relationships in which JEV can exploit host resources and establish new hosts, allowing for enzootic transmission cycles to develop between viremic vertebrate hosts and mosquitoes.

Vector – Pathogen Interaction

One obvious governing factor perpetuating the transmission of JEV is the hematophagous activity of *Culex* species mosquitoes. Although systemically and persistently infected there is an apparent absence of pathology within this vector, allowing for continuous feeding and reproduction. Many studies have investigated the infectivity of JEV to a variety of mosquito sub-species with the aim to demonstrate vector competence. Results reveal certain species to be more competent than others (Lord, Gurley, and Pulliam 2015; Turell et al. 2006; Huang et al. 2015). Oliveria et al. recently conducted a meta-analysis of JEV infection, dissemination, and transmission rates in vectors (Oliveira et al. 2018). Results show that *Aedes japonicas* challenge *Culex* species for rank among highest infection and transmission rates. However, the authors point out that these results should be interpreted with caution because literature has disproportional experimental studies conducted on each species and large variability among findings. In conjunction with this, Bustamante and Lord make clear that infection rate alone is not a suitable indicator for virus transmission since infectious mosquitoes are not consistently produced in

variable regions and climatic conditions (Bustamante and Lord 2010). It is significant to note that JEV has been isolated from *Anopheles* and other *Aedes* species in nature, which could be attributed to recent engorgement from a viremic host or taken as further demonstrating the wide range of susceptible vector species.

Factors which influence vector competence are the midgut infection and escape barriers and the salivary gland infection and escape barriers. Some basic intra-host interactions have been outlined. For JEV to sustain replication within its mosquito host it must overcome innate immune responses and intra-host tissue barriers.

After a female mosquito has ingested a viremic bloodmeal from a host, virions must first attach to and enter epithelial cells lining the midgut lumen. If this is successful, ER-membrane associated viral RNA replication takes place. Mature virions then cross the basal lamina of the midgut epithelium either through budding from the midgut epithelial cells or directly pass into the haemocoel through a “leaky” midgut (Vogels et al. 2017). Now the virions must disseminate into mosquito secondary tissues in order to make its way to the salivary glands. Current literary searches reveal that various hemocytes are the most viable path for virions to arrive at the salivary glands, but other secondary tissues can be fat bodies, nerve tissue, or muscle tissue. The permissiveness of secondary tissues appears to be virus-mosquito species specific. In order to infect the salivary glands the virions are thought to be endocytosed at the distal lateral lobes of the salivary glands (Franz et al. 2015). Further intra-cellular RNA replication takes place and mature virions move to acinar cells which deposit proteins and the viral load into the next host that is fed upon (Franz et al. 2015).

Vertebrate Host – Pathogen Interaction

A relatively broad host range normally involves amplification hosts which support viral replication and contribute to transmission and incidental hosts which succumb to severe diseases without apparent roles in transmission cycle. The role of the vertebrate in enzootic cycles is to serve as an amplification host and/or reservoir for infectious agents. In the case of JEV, avian populations act as a reservoir host and swine are implicated as the primary amplification host. In some instances, avian or bat populations are suspected to play a role in transboundary dissemination or re-introduction in temperate areas (Rosen 1987). Recent experimental evidence also demonstrated that swine are capable of transmitting JEV directly between one-another in the absence of a mosquito vector (Ricklin, García-Nicolás, et al. 2016), although this has not been shown to have an epidemiologically significant role in nature. Conversely, some vertebrates such as humans and horses are dead-end hosts such that they are less permissive to infection and do not produce sufficient viremia to infect mosquitoes. However, it is important to note that Higgs et al. demonstrated non-viremic transmission can occur in closely related WNV between co-feeding mosquitoes (Higgs et al. 2005; 2007). These findings could revitalize the role of non-viremic hosts when mosquito population densities and infections rates are high.

The susceptibility of vertebrate hosts is complex and multi-faceted. The outcome of interactions between the virus and the host immune system is dependent on viral load, host age, sex, nutritional status, and previous exposure (Huang, Higgs, and Vanlandingham 2019). The duration of viremia is a significant factor dictating the contribution of the vertebrate to subsequent transmission events. In the case of JEV, biological transmission occurs when an infected mosquito introduces virus directly into the skin and blood while feeding on the host. The virus is taken up by immune cells and transported via the lymph to secondary immune organs. Persistent infections

up to 28 days have been observed in the tonsils of pigs (Ricklin, García-Nicolás, et al. 2016; Park et al. 2018).

The contribution of avian hosts is an area of active research. Water birds, such as egrets, herons, and ducks are known to be JEV reservoir hosts as they support a period of viremia and their habitat overlaps with *Culex* species mosquitoes (Yang et al. 2011; Buescher, Scherer, Rosenberg, Gresser, Hardy, et al. 1959). The contribution of birds may be regionally bias due to *Culex* preference to feed on larger hosts when available (Lord, Gurley, and Pulliam 2015). Regions such as India and Bangladesh have a much higher proportion of domestic bird populations to pig densities, implying birds may be the primary host responsible for human JEV spillover. Furthermore, waterfowl, such as herons and egrets, may contribute to further dissemination as they develop viremia and/or latent infections and provide a JEV laced blood meal to neighboring communities of competent mosquitoes (Rosen 1986a).

Vector-Free Transmission

Evolutionary phylogenetic analysis of *Flaviviridae* provides evidence for the emergence of non-vectored transmission prior to vector borne (Kuno et al. 1998; Moureau et al. 2015). This suggests that vectored transmission was favored in some context and evolved secondary from direct transmission (Kuno et al. 1998). These phylogenetic analyses support hypothesis describing *Flavivirus* genomic alterations in response to pressures associated with host availability (Moureau et al. 2015; Simón et al. 2017). The genomic alterations support host switching though a selection process involving the gain and/or loss of genetic material over time to adapt to hosts and achieve optimal fitness (Simón et al. 2017; Lobo et al. 2009; Parrish et al. 2008; C. H. Calisher and Higgs 2018; Coffey et al. 2013).

Assuming these hypotheses are correct, it is possible that transmission may deviate from traditional routes provided appropriate ecological context and viral characteristics. For instance, non-viremic transmission has been described for various arthropod-borne viruses. When vectors are abundant and infection rates are high, co-feeding can facilitate transmission of virus from one vector to another in the absence of viremia (Higgs et al. 2005; 2007). In addition to transmission vectored by competent mosquitoes among viremic hosts, unconventional routes of transmission without the involvement of arthropod vectors has been demonstrated with a limited number of flaviviruses under laboratory conditions and described as "vector-free transmission." Vertebrates may acquire infectious virus intranasally, orally, venereally, through contact with the cornea, abrasions in the skin, or other mucosal tissues. Sources of virus could stem from food, contaminated water, aerosols, body secretions (urine, fecal matter, saliva, milk, mucus), hair, feathers, or skin. Animal behavior that may support these events include eating, drinking, sniffing, licking, aggressive behavior, and insectivorous tendencies (Kuno and Chang 2005).

The mode of transmission and site of infection may be significant factors dictating pathogenesis, immune system activation, and subsequent transmission events. Depending on the host, the mode of transmission may result in rapid clearance with mild clinical outcomes. A short history of *in vivo* JEV transmission experiments has provided evidence for the oronasal susceptibility of swine, macaques, mice, hamsters, guinea pigs, rats, squirrels, and hamsters to JEV (Innis et al. 1999; García-Nicolás et al. 2018; Ramakrishna et al. 1999; Chai et al. 2019). Most recently, JEV was also demonstrated to shed in the oronasal secretions of swine and successfully infect naïve swine via direct contact (Ricklin, García-Nicolás, et al. 2016; Lyons et al. 2018). Direct transmission of JEV though contact in the nasal epithelium in swine provides a more direct pathway to neuroinvasion via the occipital nerve.

Direct transmission has also been described for other *Flaviviruses* that were thought to be strictly arthropod-borne and this has become a subject of great concern. Sexual transmission of Zika virus (ZIKV), the causative agent linked to increased incidence of microcephaly in South America, was described in 2007 (Foy et al. 2011; Atkinson et al. 2016). Saliva, urine, breast milk, and amniotic fluid of infected individuals have also tested positive for ZIKV (Grischott 2016). Isolation of WNV from birds during mosquito dormancy, as reported during the winter of 1999 in New York, instigated further investigations into WNV transmission (Garmendia et al. 2000). Experimental evidence supports that birds can directly transmit WNV through cloacal shedding, carnivorous activity, or feather plucking behavior (Banet-Noach, Simanov, and Malkinson 2003; Komar et al. 2002; Hayes et al. 2005)

Molecular Biology of Japanese encephalitis virus

JEV is a single-stranded positive-sense enveloped RNA virus. Genomes of JEV are approximately 11kb in length and encode one single open reading frame (ORF) flanked by 3′ and 5′ noncoding regions (NCRs) and modified with a 5′ cap structure. The single ORF is translated into a polyprotein, which is proteolytically processed into three structural proteins (capsid (C), pre-membrane (prM) and envelope (E)) and seven non-structural proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5). The C protein is involved in the encapsulation of progeny virions. The surface of virions consist of 180 copies of the prM-E heterodimer arranged in an anti-parallel fashion (Wang et al. 2017). The E protein plays the crucial role of interacting with host cell receptors for attachment and internalization of the virion.

The non-structural proteins interact with vertebrate host factors to form replication complexes in association with the membrane of the endoplasmic reticulum (Selisko et al. 2014). NS5 encodes the RNA-dependent-RNA-polymerase for primer-independent transcription of the

positive-sense genome into negative sense RNA for use as a template for viral replication. NS3 and co-factor, NS2B, contribute to the catalytic functions of viral serine protease used in processing of the viral polyprotein. In addition to the protease function, NS3 also acts as other important enzymes in viral replication including RNA helicase and nucleoside-triphosphatase (Apte-Sengupta, Sirohi, and Kuhn 2014). NS1 has diverse roles as a component involved in virus replication and modulating the cell antiviral responses (Muller and Young 2013, 1). A programed ribosomal frameshift of NS1 segment produces an NS1' phenotype to serve as an eleventh protein in WNV and JEV (Mason 1989). The NS1' product has been examined extensively and is shown to have roles in immune modulation and neuroinvasiveness (Melian et al. 2010; 2014; Rastogi, Sharma, and Singh 2016; Muller and Young 2013). NS2A, NS4A, and NS4B are hydrophobic proteins that closely associate with the intracellular membranes to support viral RNA replication and immunomodulation (Apte-Sengupta, Sirohi, and Kuhn 2014; Muller and Young 2013; Melian et al. 2014).

The 3′ and 5′ NCRs contain elements folded into secondary structures and participate in multiple functions throughout the viral life cycle. These regions are essential components to support negative sense RNA synthesis and translation initiation (Gritsun and Gould 2006; Mazeaud, Freppel, and Chatel-Chaix 2018; Wy et al. 2017). Direct repeats function as promoters and replication enhancers in the 3′ untranslated region (Gritsun and Gould 2006; Wy et al. 2017). These have evolved variable expression associated with specific needs to increase replication and transmissibility in vertebrate and invertebrate hosts. ZIKV and dengue (DENV) have been observed to use internal ribosomal entry sites at the 5′ UTR, enhancing replication in both vertebrate and invertebrate hosts (Song et al. 2019).

Although current literature is inconclusive regarding host receptors used by most flaviviruses, the diversity associated with *Flavivirus* hosts range and tissue tropism suggests a variety of receptors may be utilized for attachment and entry at a given tissue (Barrows et al. 2018; Laureti et al. 2018; Dai et al. 2016; Zhang et al. 2017). JEV is known to associate with a variety of host receptors for viral attachment and entry. The most well studied are glycosaminoglycans (GAGs), such as heparan sulfate, which are found ubiquitously amongst mammalian cells (Laureti et al. 2018; Yun and Lee 2018; E. Lee and Lobigs 2008). GAGs are also used for viral entry of WNV, Murray valley encephalitis virus (MVEV), ZIKV, dengue virus (DENV), yellow fever virus (YFV), and tick-borne encephalitis virus (TBEV). This process is mediated through a negatively charged sulfate group on the GAG that bind to positively charged residues on the viral E glycoprotein. Alternatively, Ca^{2+} dependent glycan-binding proteins, like C-type lectins, are also attachment factors associated with flavivirus entry (Yun and Lee 2018). This method of entry is reliant on the interaction of N-linked mannose-rich glycans on the viral E protein with dendritic cell-specific or liver/lymph node specific intercellular adhesion molecule (ICAM)-3-grabbing non-integrin (DC-SIGN/L-SIGN) which are highly expressed on dendritic cells and macrophage cell surfaces and endothelial cells of the liver and lymph nodes, respectively.

Viral pathogen history has shown to be an important factor determining infectivity upon initial infection of naïve hosts (Barrows et al. 2018). The factors that determine the composition and structure of the virion surface are host-cell derived (attachment factors) as well as intrinsic to the species of *flavivirus* (E protein). Budding from mosquito-derived cells creates high mannose N-linked glycans at the carbohydrate attachment site of E. This facilitates initial infection of ZIKV and WNV to dermal dendritic cells and macrophages mediated by DC-SIGN lectins present on their cell surface (Rey, Stiasny, and Heinz 2017). Mammal-cell derived virions contain complex

carbohydrates at this site, restricting use of DC-SIGN in exchange for DC-SIGNR, a homologue of DC-SIGN, that likely facilitates additional rounds of virus replication in diverse cell types found throughout mammalian hosts. (Rey, Stiasny, and Heinz 2017).

Advanced imaging of *Flavivirus* envelope composition provided by cryo-electron microcopy reveal a dynamic and heterogeneous population of partially matured virion particles that may facilitate diversity observed in host cell attachment and fusion of viral and host membranes (Wang et al. 2017). Embedded in the host-derived lipid envelope of immature virion are E-prM heterodimers. Exposure to low pH of the trans-golgi-network induces conformational changes and prM is cleaved by host furin-like serine proteases producing a smooth, mature virion surface of E dimers. This process is temperature and pH sensitive and relies on efficient proteolytic cleavage (Wang et al. 2017; Simón et al. 2017). Inconsistency in this process results in incomplete maturation of the virion. This produces heterologous envelopes with variable amounts of furin cleavage, altering the binding affinity and variable (mosaic) exposure of host-binding factors which partially dictate tissue tropism. Additionally, a phenomena described as virion "breathing" exposes C and M (or PrM) proteins that are otherwise buried residues masked by E-protein dimer structures (Dowd and Pierson 2018). The structural heterogeneity and transient state of glycoproteins on the surface of flavivirus envelopes provide alternative cellular attachment factors and entry receptor interactions. These interactions may explain the diversity of *Flavivirus* tissue tropism and the capacity for host switching and viral shedding potential (Rey, Stiasny, and Heinz 2017; Barrows et al. 2018; Moureau et al. 2015).

Arboviruses are required to adapt to both vertebrate and invertebrate hosts while maintaining genetic integrity during genome replication. Investigating the mutational capacity of the *Flavivirus* genome and variable protein expression amongst species is necessary to understand

the mechanisms involved in diverse disease pathogenesis and transmissibility within this family. Mutations accumulate at higher than average rates in single stranded RNA viruses due to low fidelity and lack of proofreading associated with RNA-dependent-RNA-polymerase (RdRp). This creates a population of progeny with slight mutations that may have alternative levels of fitness which can be selected for under changing environmental or host pressures (Sanjuán and Domingo-Calap 2016). This selection process influences infectivity of hosts and disease progression within hosts (Stapleford et al. 2014). Additionally, mechanisms involved with viral replication, such as ribosomal frameshifting, account for differences in coding potential and protein products that may alter pathogenesis between hosts (Melian et al. 2010, 2014).

Advancements in molecular biology coupled with increased global surveillance and networking have provided whole genome sequences which facilitate taxonomical organization based on nucleotide sequence. There are three genera identified within family *Flaviviridae*; *Flavivirus, Pestivirus,* and *Hepacivirus*. (Kuno et al. 1998; Simón et al. 2017; Moureau et al. 2015; Schuh et al. 2013). All viruses of *Flaviviridae* are similar in virion morphology, genome organization, and replication strategies but exhibit diverse biological associations and lack serological-cross reactivity (Poidinger, Hall, and Mackenzie 1996). As such, the more than 70 viruses of genus *Flavivirus* have been categorized into nine serogroups defined by serological cross-reactivity. Furthermore, four clades have been described for the genus based on their associated vector; Mosquito-borne (MBFV), Tick-borne (TBFV), Insect-specific (ISFV), and No-known Vector (NKV). The NKV group is believed to be the ancestral root of *Flaviviridae* and environmental/host pressures have selected for variants that adapt traits optimizing their transmissibility and host range capacity. (Kuno et al. 1998). Evidence of this provides proof of concept that evolutionary dynamics are linked to close associations between vector-vertebrate-and

virus. Viral evolution and fitness of mosquito and tick-borne flaviviruses (MBFV and TBFV) are tied to constant host-switching and necessity to maintain infectivity and replication in vertebrate and invertebrate hosts (Simón et al. 2017). Whereas ISFV and NKV flaviviruses are constrained to their respective insect or vertebrate hosts, respectively. These associations have been examined in depth recently (Kuno et al. 1998; Woolhouse, Haydon, and Antia 2005; Moureau et al. 2015). Interestingly, phylogenetic analysis reveals some members of NKV clade diverged within the MBFV clade, specifically the *Aedes* spp. lineage, but have lost the ability to replicate in insect hosts and are typically isolated from bats and rodents (Cook and Holmes 2006). Tracking these changes and the mechanisms responsible has led to a better understanding of how transmission may evolve from that of traditional mosquito-borne flaviviruses.

Broad categorization based on historical introductions of the MBFV to new regions divides the clade between old world and new world viruses. Old world viruses, such as YFV and DENV, are prevalent in the tropics and primarily associated with *Aedes* species mosquitoes and hemorrhagic clinical manifestation. JEV, WNV, and other members of its serogroup are primarily considered new world viruses which are associated with *Culex* species and clinical presentation of encephalopathy (Huang et al. 2014).

The advancement of our knowledge in molecular virology helped to develop molecular diagnostic tools for JEV, which leads to the discovery and classification of different genotypes. Four JEV genotypes (GI – GIV) are known to circulate in the Asian Pacific region while the fifth genotype has not been detected for some time. These genotypes are believed to have developed from evolution in different mosquitoes and amplification hosts. Environmental factors contribute to selective pressures that account for genotypic variation within a species. This is best

characterized in JEV genotypes that have predominant associations with particular geographic areas, although co-circulation is common (Agarwal, Parida, and Dash 2017; Pan et al. 2011).

Genotype I, further subdivided into G1a and G1b, has become the most predominant genotype isolated since the 1990's and typically found in Japan, Korea, Northern Thailand, and Cambodia. Genotype II is partial to the Southeastern Asian countries like Malaysia and Indonesia and is suspect to be the sentinel genotype introduced to Australia. Genotype III was once the most frequently isolated genotype and is widely distributed across Asia. Genotype IV displays the largest antigenic and phylogenetic differences and believed to be the primordial genotype (Erlanger et al. 2009). Genotype V is presumed to be the archetype of JEV genotypes.

Diagnosis and molecular detection of Japanese encephalitis virus

Japanese encephalitis presents as acute encephalitis syndrome (AES) as do other neurotrophic diseases in JE endemic regions. Since there are no specific signs or symptoms that distinguish JE from other causes of AES, laboratory testing is needed to determine if JE is the cause of illness (Kennedy 2004). Preliminary investigations relied on virus isolation for infected individuals to identify etiological agents of disease. Virus could be isolated from brain tissue, blood, or cerebrospinal fluid (CSF) of humans, large animals, and mice. Later, *in vitro* cell culture techniques were applied in substitution of live specimen. Chicken embryos, African green monkey kidney (Vero), baby hamster kidney, and *Aedes albopictus* mosquito C6/36 cells are still commonly used today to isolate JEV (Shope 1994). To confirm JE, microscopic observation can be implemented through use of JEV specific monoclonal antibodies for detection via immunofluorescence or immunohistochemistry (Kobayashi et al. 1984). Hemagglutination-inhibition and complement fixation tests were useful in diagnosing JE based on the interaction of reference antibodies and the presence of JEV in the sample provided.

Molecular biology has contributed robust diagnostic methods with high specificity and sensitivity. Sanger-sequencing was introduced in 1977, making use of labeled dideoxynucleosides and chain termination to determine the nucleotide sequence of genomes of interest. Complementary to these efforts, exponential amplification of DNA was made available in 1983 with polymerase chain reaction (PCR). Provided these advancements, small amounts of isolates could be amplified and characterized using restriction fragment length polymorphism-based methods creating genomic libraries. This opened the door for new diagnostic methods to be developed using specific primers and probes to detect viral genomic fragments unique to their agents. Reverse-transcription polymerase chain reaction (RT-PCR) can detect JE viral RNA in clinical samples or cell culture fluid using primers based on conserved sequences specific to JEV (Lyons et al. 2018; Pyke et al. 2004; Chao, Davis, and Chang 2007).

The rapid response of immune systems clears viral RNA from the blood within the first few days after infection. This short period of viremia makes viral isolation and antigen detection unlikely methods to identify the causative agent. Advanced serological assays were developed and relied on antibody-based methods to provide high sensitivity detection after viremia has faded (Johnson et al. 2016). However, it is necessary to collect both acute and convalescent samples to demonstrate a fourfold increase in titer over five-ten days, that would be indicative of active infection. This is important in endemic regions where populations are potentially exposed previously to JEV through natural infection or vaccination. There is also the possibility of cross-reactive antibodies which may require alternative methods for differential diagnosis. The WHO manual for laboratory diagnosis of JEV indicates IgM-capture ELISA is the gold standard for detection (World Health Organization 2007).

Chapter 2 - Materials and Methods

Introduction

During the spring of 2017, a 28-day JEV pig challenge study was conducted under BSL-3AG conditions at the Biosecurity Research Institute (BRI) at Kansas State University. Experimental procedures and animal use were approved by the Kansas State University Institutional Animal Care and Use Committee. The aim of this study was to develop a model of JEV infection in intradermally (ID) challenged domestic pigs. Fourteen three-week-old white line crossed commercial piglets were involved in the study. Ten piglets were housed in two separate cages and challenged ID with 10^7 $TCID_{50}$/ml of JEV-91, representative of genotype 1-b. The four remaining piglets were housed separately and used as negative controls. Seven piglets were necropsied on day three and day 28 post challenge to provide samples characterizing the acute and convalescent stage of infection, respectively.

Fecal swabs were collected during daily health checks at 0-14, 21, and 28 days post-infection (dpi). Viral RNA was extracted and then quantified using reverse-transcriptase-quantitative-polymerase-chain-reaction (RT-qPCR) to construct a kinetic profile of fecal shedding throughout a JEV infection in domestic piglets.

Cell lines

Immortalized C6/36 cells (CRL-1660™), derived from *Aedes albopictus* larvae, were incubated at 28°C in Leibovitz-15 (L-15) media (Life Technologies, Carlsbad, CA). The media was supplemented with heat-inactivated 10% fetal bovine serum (FBS), tryptose phosphate broth (TPB), and L-glutamine for nutrients. Penicillin and streptomycin were also added to reduce the chance of biological contaminants. These cells were used for the propagation of all stock viruses prior to my involvement in this study.

Vero76 cells (CRL-1587™), derived from the kidney of African green monkeys, were used to determine the titer of viral stock via the median tissue culture infectious dose ($TCID_{50}$) method. These cells were maintained at 37℃ with L-15 media supplemented with 10% FBS, TPB, L-glutamine, penicillin, and streptomycin.

Virus

JEV strain JE-91, representative of genotype I-b, (Genbank access number: GQ415355.1), originally isolated from mosquitoes collected in Korea in 1991 and acquired from existing virus culture collection in the laboratory of Dr. Alan D. T. Barrett, University of Texas Medical Branch, was used for positive controls during each RNA extraction as well as for standard curve titration on each RT-qPCR assay.

Determination of Infectious Titers

This process was performed by colleagues in the lab prior to my involvement in this experiment. Titration of stock JE-91 was performed on a 96-well plate to determine the $TCID_{50}$ of JE-91 used for the RT-qPCR standard curve. Ten serial dilutions were carried out in duplicate beginning with 100µl of undiluted virus. 10µl of inoculum were transferred from this sentinel well into downstream wells containing 90µl of L-15 media, producing a 10^{10}-fold dilution in the final well. Negative control wells contained only fresh L-15 media. Each well then received 100µl of Vero76 cells.

These plates were covered with parafilm and incubated at 37℃ for seven days. A solution containing 200µl amido black stain, 1% amido black B10, 10% glacial acetic acid, and 35% isopropanol was prepared and applied to the 96-well plate for 30 minutes at room temperature for visualization of cytopathic effects in each well. The plate was washed with tap water over the sink

until the runoff was clear. A light microscope was used to observe the wells and the Reed-Muench method (Reed & Muench, 1938) was used to calculate the titer, 8.95 x 10^8 $TCID_{50}$/ml.

Animals

Fourteen three-week-old U.S. commercial white-line crossed piglets were obtained from sources approved by the Comparative Medicine Group of Kansas State University and transported to the BRI in March of 2017. All animal work was approved by the Institutional Animal Care and Use Committee of Kansas State University. Upon arrival, pigs were housed in BSL-3Ag facilities and randomly assigned as control (n=4) or experimental (n=10) groups and provided five days to acclimate.

Ten pigs were intradermally challenged with 1ml of JE-91 JEV strain at 10^7 $TCID_{50}$/ml and four pigs were given 1ml of sterile saline. Challenged pigs were split into two pins and housed separately from control pigs to avoid direct transmission as described by Ricklen et al. (2016). Seven pigs (two control and five challenged) were euthanized at three days post infection (dpi) to assess acute phase pathology. The other seven pigs were euthanized on 28 dpi to evaluate the convalescent stage of infection. Health checks were conducted daily to observe changes in behavior, temperature, and weight for the entirety of the 28-day study.

Sample Collection

Sample collection was performed by colleagues in the laboratory prior to my involvement in this experiment.

Fecal swabs were collected on days 0-14, 21, and 28 during the study. Sterile cotton swabs were used to collect fecal remnants from the anus of all pigs. Swabs were then placed in appropriately labeled 15ml conical tubes that contained 1ml of supplemented L-15 media. The conical tubes were vortexed and stored at -80℃.

Extraction of RNA

All RNA extractions were performed using Zymo Direct-zol™ RNA MiniPrep Plus kits following manufacturer's recommendations. All BSL-3 laboratory work was carried out by colleagues and provided in inactivated form to work with under BSL-2 conditions.

Conical tubes containing a suspension of L-15 media and fecal matter were thawed in a 37℃ water bath in a BSL-3 laboratory. 250µl of this suspension was added to 750µl of TRIZOL LS Reagent® in a 2ml microcentrifuge tube (Eppendorf Safe-Lock) and allowed to sit for five-minutes. This step inhibits RNase activity and inactivates infectious agents by lysing cellular membranes and viral envelopes. The tubes were properly disinfected and transferred into a BSL-2 laboratory for further processing.

Microcentrifuge tubes containing the lysate were centrifuged for 15 seconds after transport to ensure there was no residual liquid in the lid of the Eppendorf tube. Spin columns, wash buffers, and enzymes provided by Zymo Direct-zol™ RNA MiniPrep Plus Kits were used to isolate viral RNA from the TRI Reagent® /fecal sample suspension. An equal volume of 100% ethanol was first added to the lysate followed by centrifugation at 13,000 x g for 30 seconds. Three separate aliquots (≤666µl) and centrifugations (13,000 x g for 30 seconds) were used to transfer this mixture into a Zymo-Spin™ IIICG column. Flow through was captured in a collection tube and discarded. The column containing viral RNA was transferred into a new collection tube and treated with 400µl RNA wash buffer and centrifuged as previously described. DNase treatment (5µl DNase + 75µl DNA Digestion Buffer per sample) was prepared in a 15ml conical tube and added directly to the column then incubated at 25℃ for 15 minutes. 400µl of RNA PreWash was passed through the column then centrifuged (13,000 x g for 30 seconds) and repeated. Lastly, 700µl of RNA wash buffer was added and centrifuged for 2 minutes, allowed to sit, and then centrifuged again for 30

seconds to ensure complete removal of the buffer. The column was then transferred into a 1.5ml RNase-free microcentrifuge tube (Eppendorf Safe-Lock) and eluted with 50µl of DNase/RNase-free water and centrifuged one last time. The eluted RNA was aliquot evenly into two labeled 1.5ml microcentrifuge tubes and stored frozen at -80℃.

RT-qPCR

Absolute quantification of JE-91 is carried out using an iTaq Universal Probes One-step Kit from BioRad (BioRad, Hercules, CA). Previous experiments carried out by Lyons et al. (2018) validated the most sensitive and specific primer sets to detect JE-91 strain of JEV in oral fluids from this same study. The primer and probe were designed to target nucleotide positions of 10,224-10,286 between the 3′ end of the NS5 gene and 5′ end of the 3′ UTR of the JEV genome (Pyke et al., 2003). Probes were designed with a 6-carboxyfluorescein fluorophore on the 5′ end, a double fluorescent quencher with an internal ZEN quencher to minimize background signal, and an IowaBlack fluorescent quencher on the 3′ end. Fluorescent signal was detected with a BioRad CFX96 real-time PCR thermocycler (BioRad, Hercules, CA) provided by the Integrated Genomics Facility in the Department of Agronomy at Kansas State. Upon each thermocycle, Taq polymerase extends the genome where the primers have attached. The synthesis of specific amplicons is detected by fluorescent signal as Taq degrades the probe, disrupting interactions between the reporter and quencher. The fluorescence produced is proportional to the amount of product and recorded by the BioRad CFX96 real-time PCR thermocycler.

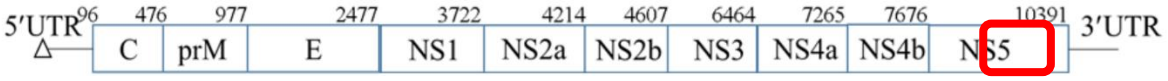


Figure 2.1. JEV genome with structural (C, prM, E) and non-structural (NS1, NS2, NS3, NS4, NS5) genes and associated nucleotide positions. The targeted nucleotide positions at 10224 – 10286 for the primer and probe are highlighted in red. Modified from Lyons 2018.

Each sample was run in duplicate and carried out in 96-well plates. Reactions consisted of 4µl RNA extract, 10µl iTaq universal probes reaction mix, 0.5µl iScript advanced reverse transcriptase, 1µl forward primer, 1µl reverse primer, 0.4µl probe, and 3.1µl of molecular grade water. The CFX96 thermocycler was programed to cycle 40 times as follows: 30 minutes of reverse transcription at 50℃; 10 minutes of initial denaturation at 95℃; 15 seconds of further denaturation at 95℃; 1 minute of annealing and extension at 60℃.

Six repeats of ten-fold serial dilutions from a known concentration (8.95×10^8 $TCID_{50}$) of stock JE-91 were run in duplicate on each 96-well plate to generate a standard curve of JE-91, shown in Figure 3. The standard curve provided a consistent means to extrapolate viral load of unknown samples between RT-qPCR runs based on liner regression. The limit of detection (LOD) was 14.865 genome equivalent (g.e.q.) to $TCID_{50}$/mL based on corresponding Cq value of 34 as described in previous experiments carried out by Lyons et al. (2018). Additionally, negative control wells, containing nuclease-free water, were present on each plate to validate the absence of false positive results that could result from cross-contamination or background fluorescence. Positive controls of known concentration from each extraction were also run to ensure extractions worked properly.

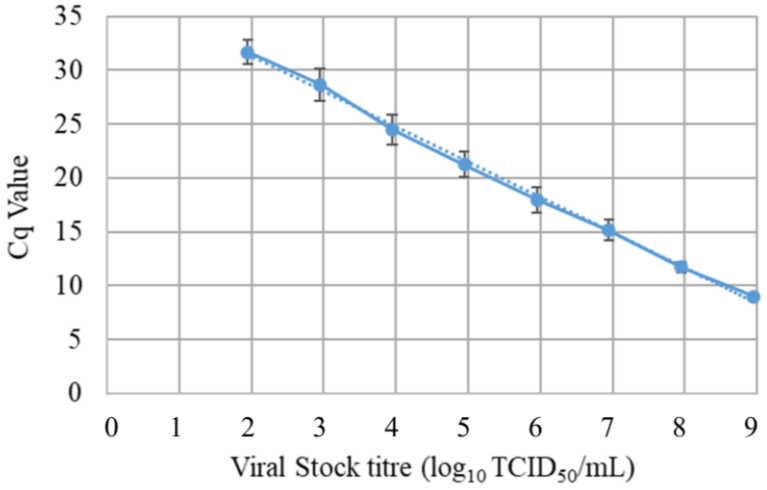


Figure 2.2. Standard curve generated from JE-91 stock of 8.95 x 10^8 $TCID_{50}$/mL. Y= -1.422ln(x)+37.838, R^2=0.9979, LOD at Cq value of 34 = 14.865 g.e.q. to $TCID_{50}$/mL.

Chapter 3 - Profile of Fecal Shedding Throughout JEV Infection in Domestic Pigs

Introduction

Domestic pigs are the primary amplifying hosts in the enzootic cycles of JEV in endemic regions (Buescher, Scherer, Rosenberg, Gresser, Hardy, et al. 1959; Rosen 1986b). Industrial pig rearing has increased dramatically in recent decades to meet demands of the pork industry and increasing population (Erlanger et al. 2009). Recent investigations have demonstrated that vector-free transmission is possible via direct contact through oronasal secretions of JEV infected pigs to naïve pigs (Ricklin, García-Nicolás, et al. 2016; Lyons et al. 2018). The nasal epithelium has been shown as highly susceptible to JEV infection (Ricklin, García-Nicolás, et al. 2016; García-Nicolás et al. 2018). To better understand the contribution direct transmission may have in transmission of JEV in pigs it is necessary to further characterize the shedding profile of JEV infected swine. Fecal-oral transmission has been described for WNV, a closely related *Flavivirus* (Banet-Noach, Simanov, and Malkinson 2003). To our knowledge, this has not yet been explored in the context of JEV transmission in swine. The presence of infectious virus in fecal excrement could contribute to the persistence of JEV in regions with vigorous vector control programs and the maintenance of JEV in temperate zones or during inter-epidemic periods, particularly in crowded industrial settings. As millions of susceptible pigs are birthed each year in JEV endemic regions it is necessary to investigate this as a potential route of transmission and reassess control measures to keep JEV from hindering production or spilling over into human populations. Additionally, fecal swabs may provide a cheap, fast, and less invasive sampling method to increase JEV surveillance in rural areas with intensive pig farming; similar to oral swabs as described by Lyons et al. (Lyons et al. 2018).

The objective of this study was to determine if JEV genome could be detected in fecal swabs collected from 3-week-old intradermally challenged white-line crossbred domestic pigs. Our hypothesis is that fecal shedding profiles may mimic that of oral shedding, with peak viral loads detected around 5 days post infection. This hypothesis was tested by collecting rectal swabs from infected pigs and quantifying the viral load using RT-qPCR assay specific for JEV-91. The results of this study show the presence of JEV genome in fecal shedding. Further investigations are necessary to determine the infectivity of shed JEV to determine if its contribution in nature or industrial settings.

Results

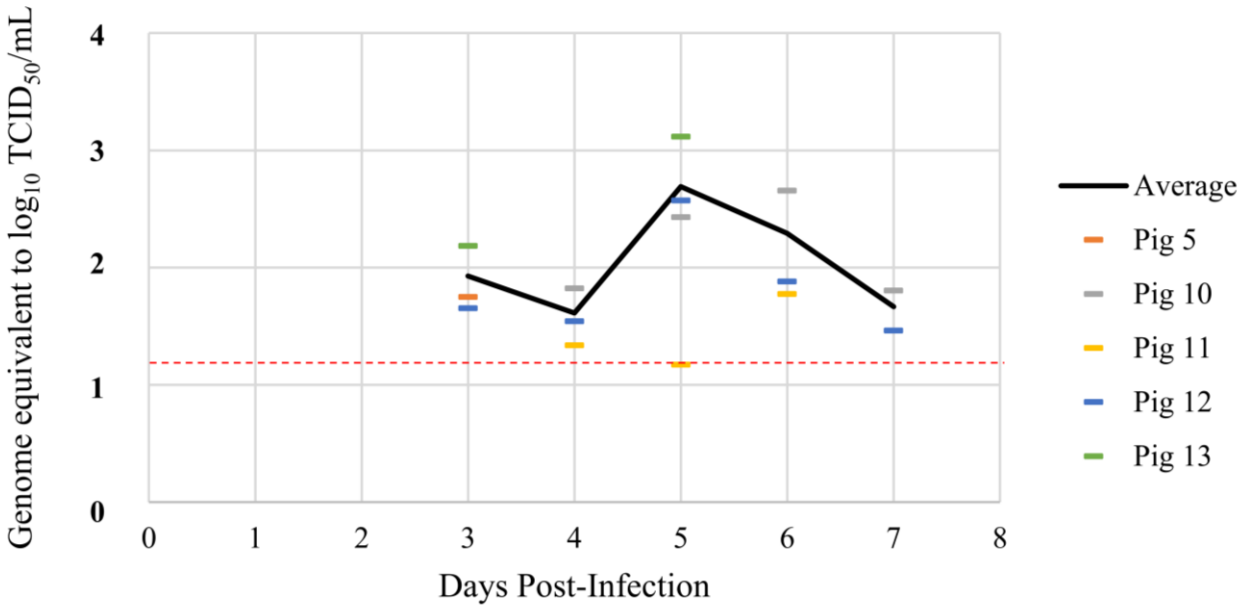
Kinetic Profile of Intradermally Challenged Domestic Pigs

Fecal samples were collected on days 0-14, 21, and 28 dpi. from three-week-old white-line crossbred domestic pigs ID challenged with 10^7 $TCID_{50}$ JE-91. RT-qPCR with JEV-91 specific primers and probes were used to detect and quantify viral loads. The specificity of these primer sets and threshold cutoff Cq value of 34 was previously validated and determined by Lyons et al. (2018).

A scatter plot representing viral RNA detected each day in the fecal shedding of pigs is displayed in Figure 4. Provided this graphic it is possible to track onset of shedding and daily fluctuations in shedding of individual pigs. Although pigs 1-7 were euthanized at 3 dpi., which is the earliest onset of detectable shedding, the shedding profile of pigs 8-14 are shown through 7 dpi. Fecal shedding surpassed peak viremia which occurred at approximately 5-6 dpi (Park et al. 2018). In total, five out of the ten pigs had detectable amounts of JEV genome in fecal swabs by 7 dpi. Pig 12 shed JEV for the longest duration from 3 dpi. through 7 dpi. Pig 13 is an anomaly with highly transient shedding and the highest recorded shedding (three times that of the next highest

recorded) at 5 dpi. The earliest onset of shedding was observed in pigs 5, 12, and 13 with detectable JEV at 3 dpi. Pigs 10 and 11 did not begin shedding until 4 dpi. (Figure 4. b-c).

a)



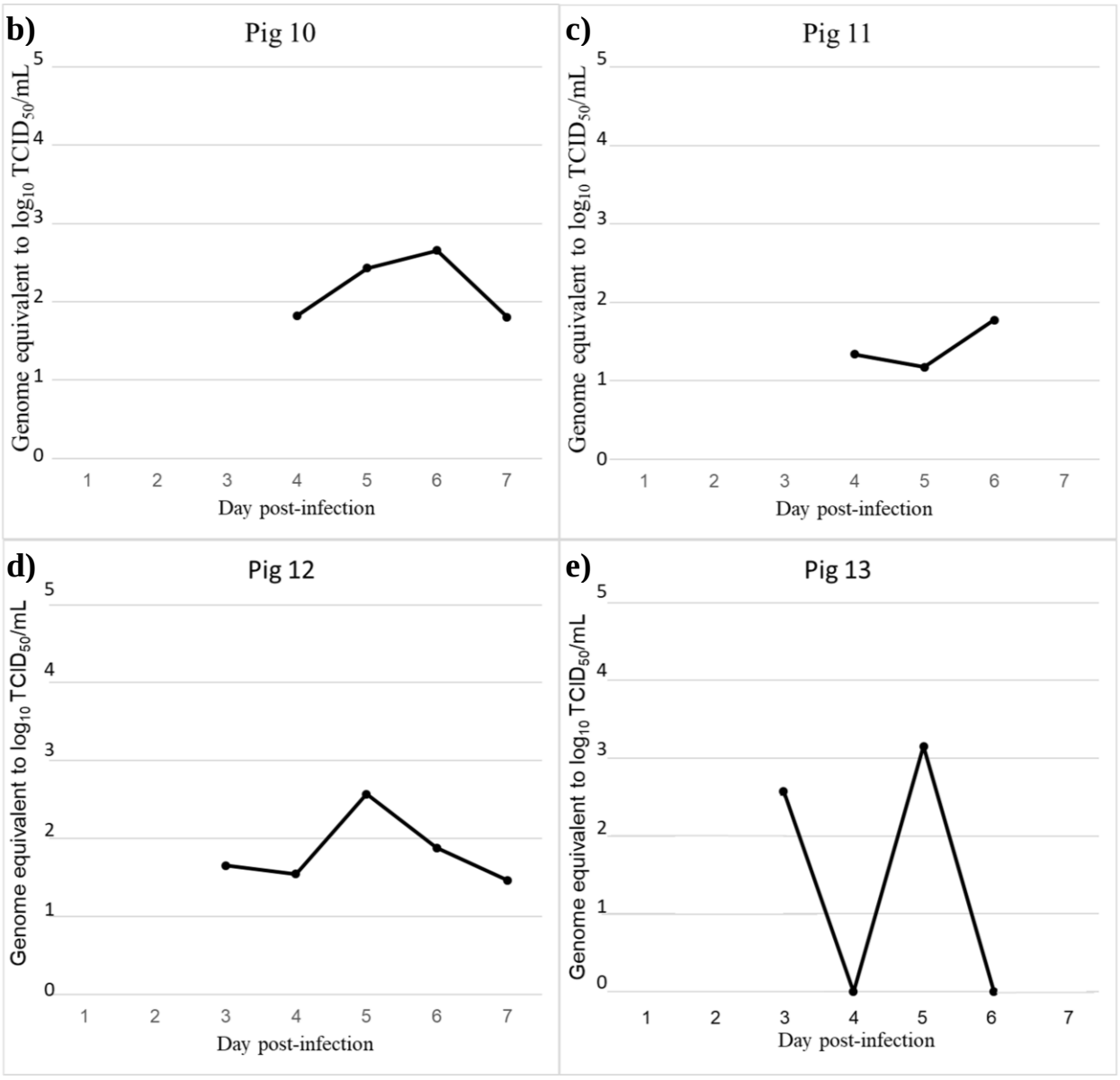


Figure 3.1. Average of daily RT-qPCR results from fecal swabs collected on days 0-7 dpi. from 3-week-old white-line crossbred pigs ID inoculated with 10^7 $TCID_{50}$ JE-91. b-e) Daily profile of individual pigs with detectable viral RNA. Values below the threshold cutoff Cq value of 34 (red line) are not pictured.

A box-and-whisker plot is displayed in figure 5 to represent the range, mean, and median amounts of virus shed from individual pigs throughout the course of the first seven days. Upper

and lower-quartile range could only be assessed for pigs 10 (407.45 – 64.43 g.e.q. to $TCID_{50}$/mL) and 12 (224.92 – 32.05 g.e.q. to $TCID_{50}$/mL) as detectable amounts of virus were shed for four and five days, providing the necessary data points. Pig 13 recorded the highest average (730.915 g.e.q. to $TCID_{50}$/mL) although only two days of shedding were detected. Average viral shedding in pigs 10, 11, and 12 were 213.285 g.e.q. to $TCID_{50}$/mL, 32.115 g.e.q. to $TCID_{50}$/mL, and 111.81 g.e.q. to $TCID_{50}$/mL, respectfully.

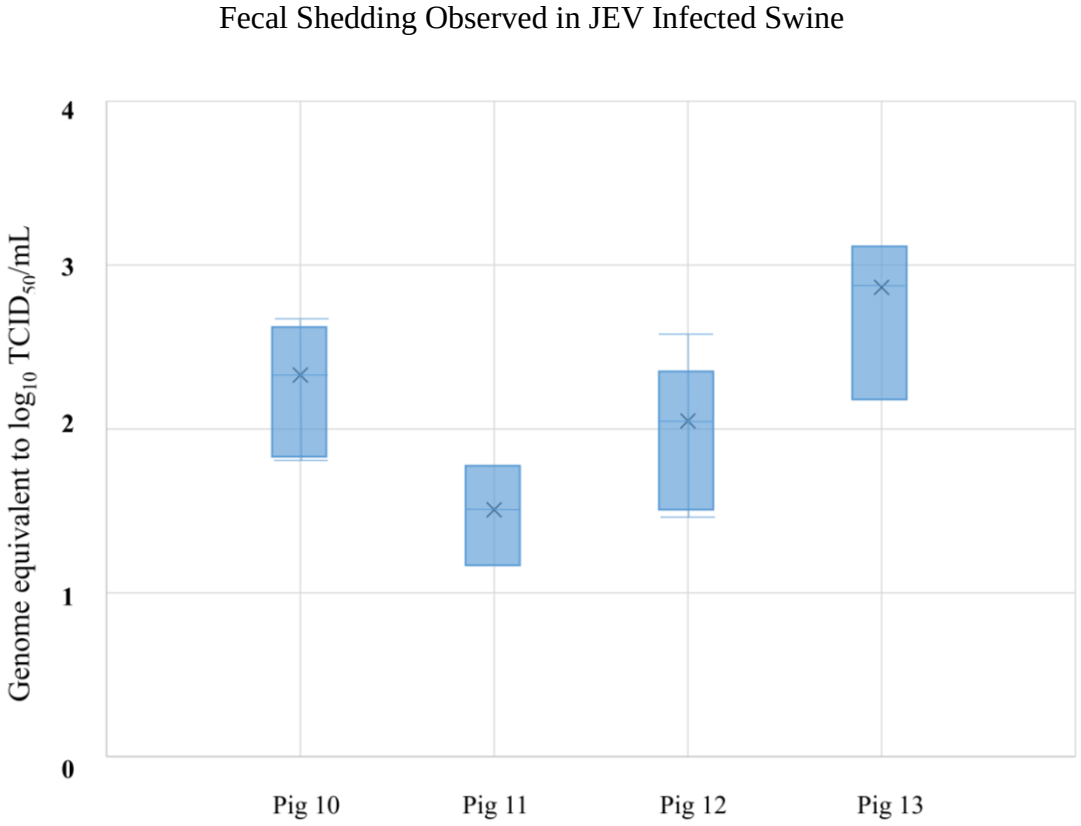


Figure 3.2. Box-and-whisker plot representing the mean (X), median, range, and interquartile range through 7 days of fecal shedding in domestic pigs inoculated ID with JEV 10^7 $TCID_{50}$ JE-91. Values below the threshold cutoff Cq value of 34 are not pictured.

Conclusion

The data collected in this study has provided evidence that genomic fragments of JEV are present in the fecal shedding of pigs infected ID with 10^7 $TCID_{50}$ JE-91. Of the ten pigs inoculated, five of them shed detectable amounts of JEV in their excrement. Fecal shedding was present as soon as 3 dpi and persisted for up to 5 days in at least one pig. Three logs of RNA were detected in one pig for one day, however it was more common to observe only one to two logs of RNA that would persist for three or four days.

Chapter 4 - Conclusion and Future Directions

The goal of this study was to characterize the fecal shedding of domestic swine ID inoculated with JEV. The purpose of doing this was to provide preliminary data for use in determining whether fecal-oral transmission may be a contributing factor to the vector-free transmission of JEV and to evaluate the feasibility of using fecal swabs for diagnostic samples. Through the process of collecting rectal swabs from JEV infected pigs, extracting the viral RNA, and quantifying viral loads by RT-qPCR it was determined that genomic fragments of JEV can be shed in fecal excrement of pigs. This is the first study to provide evidence confirming the presence of JEV in rectal swabs of ID inoculated pigs.

Of the ten ID inoculated domestic pigs, five produced rectal swabs with positive detection of JEV genome. It is possible that four other pigs may have developed fecal shedding as well, however they were euthanized on day three to characterize acute pathology and tissue tropism. Shedding appeared as early as 3 dpi. and persisted for three to five days over the course of the seven-day study. As hypothesized, fecal shedding profiles seem to mimic that of oral shedding, as described by Ricklin et al (2016) and Lyons et al. (2018). According to recent studies, the nasal epithelium of pigs is susceptible to infection at as low as 10 $TCID_{50}$/mL (Ricklin, García-Nicolás, et al. 2016). All five pigs had viral loads above the limit of detection for the RT-qPCR assay, which was 14.86 $TCID_{50}$/ml. The average viral load shed in three of the five infected pigs was above 2 logs. This indicates that fecal shedding of JEV has potential to be directly transmitted to naïve pigs via direct contact with the nasal epithelium.

It is important to note that the nucleic acid detected in rectal swabs could be artifact from JEV infected lymphoid tissue in the gastro-intestinal tract of the infected pigs. Previous studies

have demonstrated JEV tropism in the lymph tissues, which are abundant in the gastrointestinal system of pigs (Park et al. 2018; Ricklin, Garcìa-Nicolàs, et al. 2016).

While this study provided a more complete picture of JEV shedding in pigs, there were some limitations. There were only ten pigs challenged with JEV, half of which were euthanized on day three post challenge, preventing complete analysis of all pigs through seven days. It is also important to note that the 10^7 $TCID_{50}$/mL dose that pigs were ID inoculated with is a higher load than natural infection. This has the potential to alter the viral dissemination and tissue tropism which may have an impact on the viral shedding profile, producing results that may not be representative of a natural infection.

Future studies should explore this same idea in feral pig populations which are abundant in JEV endemic regions and in regions susceptible to JEV emergence. It is also important to evaluate the infectivity of this shed virus, perhaps by exposing naïve pigs to solutions containing excrement from JEV infected pigs. Demonstrating infectivity in tissue culture would be challenging given the high concentration of contaminants associated with fecal matter. A larger data set would be necessary to assess the viability of using rectal swabs as an effective sampling method for JEV surveillance. The idea is appealing because rectal swabs are less invasive and less costly than serum collection and provides a record of JEV infection in individual pigs.

As pig rearing and rice cultivation continue to expand in JEV endemic regions the necessity to investigate shedding profiles and subsequent transmission of JEV in swine is imperative. Direct transmission allows for JEV to be maintained in the absence of a mosquito vector and in swine populations throughout temperate regions during inter-epidemic periods. This could create situations where outbreaks of JEV occur earlier in the rainy season when mosquitoes become active. Identifying surveillance techniques that are less costly and involve less invasive methods

of sampling could assist with increased surveillance which may improve epidemiological modeling and inform more effective control measures in JEV endemic regions.

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