TOWARDS A BETTER UNDERSTANDING OF VECTOR-FREE JAPANESE ENCEPHALITIS VIRUS TRANSMISSION

A Thesis Submitted to the
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In Partial Fulfillment of the Requirements
For the Degree of Masters of Science
In the Department of Veterinary Microbiology
University of Saskatchewan
Saskatoon, Canada

By
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Japanese encephalitis virus (JEV) is the emerging and geographically expanding flavivirus and the major causative agent of encephalitis in humans in Asia. There are risks of JEV introduction into the Americas given a large population of amplifying hosts—pigs and wild boars, and insect vectors—Culex mosquitoes. There are emerging concerns about vector-free routes of flavivirus transmission, for example sexual and transplacental Zika virus transmissions, which may change flavivirus epidemiology and expand the geographical range, even to territories with no insect vectors. It is unknown whether JEV has tropism in the female lower reproductive tract and the potential for sexual transmission in humans. While clinical outcomes of transplacental JEV infection are described in humans and pigs, cellular targets and tissue tropism in the upper reproductive tract are also unknown. Here, I studied JEV infection phenotypes and host transcriptional responses in human reproductive epithelial cells. I found that JEV causes infection and cytopathology in the vaginal epithelium, endometrial epithelium, and trophoblast. Human vaginal epithelial cells infected with JEV had altered transcriptional responses associated with inflammation and epithelial barrier disruption. Also, using pigs—the native amplifying host for JEV—as a model, I confirmed JEV tropism in the female reproductive tract. JEV persisted in the vaginal mucosa for at least 28 days, and pigs shed the virus in vaginal secretions. I also found JEV persistence at the maternal-fetal interface with transplacental and fetal infections. Altogether, I discovered that JEV targets the vaginal epithelium and has the potential for sexual transmission in humans. My thesis also contributed to a better understanding of JEV pathogenesis during transplacental infection. Further studies are needed to better understand the interactions of JEV with reproductive tissues, how persistent infection affects female reproductive functions, and the risks for non-vector transmission.
At the outset, I would like to express my sincere gratitude to my supervisor Dr. Uladzimir Karniychuk for his continued support, guidance, and mentorship during my study. His motivation, constructive criticism, attention to detail, and openness to discussion encouraged my critical thinking and gave me the right tools to deal with complicated research problems. Working and studying under his guidance was a great privilege and rewarding experience.

I am grateful to the members of my advisory committee, Dr. Darryl Falzarano, Dr. Maarten Voordouw, Dr. Janet Hill (temporary Chair), and Dr. Emily Jenkins (Chair), for providing constructive feedback and suggestions.

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I owe the deepest gratitude to my parents and my sister for their love, support, kindness and upbringing, which shaped my character and much of my life. Finally, the experiments presented in this thesis would not have been possible without financial support from funding agencies. Therefore, big thanks to all the funding agencies.
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<th>Description</th>
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<tbody>
<tr>
<td>Abs</td>
<td>Antibodies</td>
</tr>
<tr>
<td>AEC</td>
<td>3-Amino-9-Ethylcarbazole</td>
</tr>
<tr>
<td>AME</td>
<td>Acute meningoencephalitis</td>
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<tr>
<td>BBB</td>
<td>Blood-brain barrier</td>
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<tr>
<td>CCL-2</td>
<td>Chemokine ligand 2</td>
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<tr>
<td>CCL-3</td>
<td>Chemokine ligand 3</td>
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<tr>
<td>CD</td>
<td>Cluster of differentiation</td>
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<tr>
<td>cDNA</td>
<td>Complementary DNA</td>
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<tr>
<td>CNS</td>
<td>Central nervous system</td>
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<td>CSF</td>
<td>Cerebrospinal fluid</td>
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<td>DC</td>
<td>Dendritic cells</td>
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<td>DC-SIGN</td>
<td>Dendritic cell-specific intercellular adhesion molecule 3-grabbing nonintegrin</td>
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<td>DENV</td>
<td>Dengue virus</td>
</tr>
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<td>DMEM</td>
<td>Dulbecco's Modified Eagle Medium</td>
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<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>DPBS</td>
<td>Dulbecco's phosphate-buffered saline</td>
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<tr>
<td>ELISA</td>
<td>Enzyme-linked immunosorbent assay</td>
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<tr>
<td>FBS</td>
<td>Fetal bovine serum</td>
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<td>FFU</td>
<td>Focus-forming units</td>
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<td>GO</td>
<td>Gene ontology</td>
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<td>HRP</td>
<td>Horseradish peroxidase</td>
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<tr>
<td>IFN</td>
<td>Interferon</td>
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<tr>
<td>Acronym</td>
<td>Definition</td>
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<tr>
<td>IgM</td>
<td>Immunoglobulin M</td>
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<td>IL-1β</td>
<td>Interleukin 1 beta</td>
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<td>IL-2</td>
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<tr>
<td>IL-17</td>
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</tr>
<tr>
<td>IPMA</td>
<td>Immunoperoxidase monolayer assay</td>
</tr>
<tr>
<td>ISG</td>
<td>Interferon-stimulated gene</td>
</tr>
<tr>
<td>JAK</td>
<td>Janus kinase</td>
</tr>
<tr>
<td>JEV</td>
<td>Japanese encephalitis virus</td>
</tr>
<tr>
<td>MDA</td>
<td>Melanoma differentiation associated protein</td>
</tr>
<tr>
<td>MDSC</td>
<td>Myeloid-derived suppressor cell</td>
</tr>
<tr>
<td>moDC</td>
<td>Monocyte-derived dendritic cell</td>
</tr>
<tr>
<td>MOI</td>
<td>Multiplicity of infection</td>
</tr>
<tr>
<td>mRNA</td>
<td>Messenger Ribonucleic acid</td>
</tr>
<tr>
<td>MVEV</td>
<td>Murray valley encephalitis virus</td>
</tr>
<tr>
<td>NS</td>
<td>Non-structural</td>
</tr>
<tr>
<td>NGS</td>
<td>Next generation sequencing</td>
</tr>
<tr>
<td>NHPs</td>
<td>Non-human primates</td>
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<tr>
<td>OAS3</td>
<td>2′-5′-Oligoadenylate Synthetase 3</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>ORF</td>
<td>Open reading frame</td>
</tr>
<tr>
<td>PAMP</td>
<td>Pathogen-associated molecular patterns</td>
</tr>
<tr>
<td>PCV2</td>
<td>Porcine circovirus 2</td>
</tr>
<tr>
<td>PCV3</td>
<td>Porcine circovirus 3</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate-buffered saline</td>
</tr>
<tr>
<td>PBMC</td>
<td>Peripheral blood mononuclear cells</td>
</tr>
<tr>
<td>PPV</td>
<td>Porcine parvovirus</td>
</tr>
<tr>
<td>prM</td>
<td>pre-membrane</td>
</tr>
<tr>
<td>PRR</td>
<td>Pathogen recognition receptors</td>
</tr>
<tr>
<td>RANTES</td>
<td>Regulated on activation, normal T cell expressed and secreted</td>
</tr>
<tr>
<td>PRRSV</td>
<td>Porcine reproductive and respiratory virus</td>
</tr>
<tr>
<td>RIG-1</td>
<td>Retinoic acid-inducible gene I</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
</tr>
<tr>
<td>RT-qPCR</td>
<td>Reverse transcription-quantitative polymerase chain reaction</td>
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<tr>
<td>SARS-CoV</td>
<td>Severe acute respiratory syndrome coronavirus</td>
</tr>
<tr>
<td>SLEV</td>
<td>St. Louis encephalitis virus</td>
</tr>
<tr>
<td>STAT</td>
<td>Signal transducer and activator of transcription</td>
</tr>
<tr>
<td>TBEV</td>
<td>Tick borne encephalitis virus</td>
</tr>
<tr>
<td>TCID</td>
<td>Tissue culture infectious dose</td>
</tr>
<tr>
<td>TIM</td>
<td>T cell/transmembrane, immunoglobulin, and mucin</td>
</tr>
<tr>
<td>TLR</td>
<td>Toll-like receptor</td>
</tr>
<tr>
<td>TNF-α</td>
<td>Tumor necrosis factor alpha</td>
</tr>
<tr>
<td>UTR</td>
<td>Untranslated region</td>
</tr>
<tr>
<td>WHO</td>
<td>World Health Organisation</td>
</tr>
<tr>
<td>Code</td>
<td>Description</td>
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<tr>
<td>-------</td>
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</tr>
<tr>
<td>WNV</td>
<td>West Nile virus</td>
</tr>
<tr>
<td>YFV</td>
<td>Yellow fever virus</td>
</tr>
<tr>
<td>ZIKV</td>
<td>Zika virus</td>
</tr>
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</table>
CHAPTER 1: LITERATURE REVIEW

1.1 General introduction

1.1.1 Flaviviruses
Within the *Flaviviridae* family there are four distinct genera: *Hepacivirus*, *Pegivirus*, *Pestivirus*, and *Flavivirus* [1]. The *Flavivirus* genus comprises more than 70 viruses replicating in a diverse range of invertebrates and vertebrate organisms [2]. Flaviviruses have single-stranded RNA, and they infect rodents, pigs, birds, non-human primates, humans, and other vertebrate hosts. Several flaviviruses are transmitted by arthropod vectors and cause human diseases. Dengue virus, West Nile virus (WNV), yellow fever virus (YFV), Zika virus (ZIKV), tick-borne encephalitis virus (TBEV), and Japanese encephalitis virus (JEV) are among the most clinically significant flaviviruses [3].

Flaviviruses were initially grouped among the togaviruses based on early serological assessments. After 1984, they were separated from the togaviruses and placed in the *Flaviviridae* family based on differences in their structure, gene sequence, and replication strategy [4]. Phylogenetic analysis of the *Flavivirus* genus shows that this group of viruses might have evolved from an ancestral virus in Africa thousands of years ago. Within this time span, flaviviruses underwent significant ecological diversification and gave rise to different lineages that adapted to different arthropod vectors and modes of transmission. Accordingly, flaviviruses have evolved unique strategies to evade the innate and adaptive immunity of the vertebrate host [5].
Most flaviviruses that cause disease in humans are zoonotic, and they have a complex transmission cycle involving insect vectors and amplifying in vertebrate reservoir hosts. There has been a remarkable spread of these viruses during the last seven decades [3]. Flaviviruses are distributed worldwide in tropical and subtropical regions, with some viruses like WNV spreading into temperate zones. Factors that have led to the dispersal of flaviviruses into previously non-endemic regions include viral genome evolution, global mobility, rapid urbanization, an increase in global temperatures due to climate changes, and increased deforestation [6].

Flaviviruses are important and emerging pathogens that have affected humanity throughout history, accounting for millions of deaths worldwide. Disease outbreaks related to flavivirus infections (though the concept of viruses was not prevalent then) can be traced back to the mid-1600s when “Blood Vomit” was a known disease in the Caribbean and the Americas. Since then, multiple outbreaks, chiefly of yellow fever (YF)-like nature, have been documented. In one of the largest YF outbreaks in America, with an estimated 120,000 cases, more than 20,000 people died [4]. Yellow fever virus currently causes approximately 200,000 infections and 30,000 deaths annually all over the world [7]. Dengue fever is another disease caused by flavivirus infection, historically referred to as “break-bone fever.” Dengue virus is transmitted by female mosquitoes belonging to the species *Aedes aegypti* and, to a lesser extent, via *Aedes albopictus*. Though the disease was limited to 9 countries before 1970, it is now endemic in more than 100 countries globally. As of 2019, the World Health Organization (WHO) reported 5.9 million annual dengue cases [8].

Due to the cosmopolitan distribution of competent arthropod vectors, flaviviruses have a high potential for causing epidemics. The recent Zika virus epidemic across South America was an
example of the increasing global public health risk and pandemic potential of flaviviruses. According to WHO, in 2019, 87 countries reported evidence of mosquito-borne transmission of ZIKV [9]. West Nile virus, another pathogenic flavivirus, has caused outbreaks since it was first identified in a febrile patient from the West Nile district of Northern Uganda in 1937. More recently, WNV caused a large epidemic that spread throughout the continental United States of America (USA) and led to several human deaths after being imported to New York in 1999 [10]. Tick-borne encephalitis virus (TBEV) and Powassan virus (POWV) are two tick-borne flaviviruses that have the potential for (re)emergence and outbreaks [11–13]. The most recent example of the public health threat posed by flaviviruses is the first outbreak of JEV in Australia in February 2022, where the virus spread quickly among the swine herds and humans. At least 37 human cases were confirmed, and five human deaths were attributed to JEV infection [14]. Similarly, JEV is currently (July 2022) causing an outbreak in Assam, India, where hundreds of cases are reported, with at least 38 deaths caused by infection [15]; this pattern is repeated annually during the mosquito season.

The constant danger of flavivirus (re)emergence requires a more fundamental understanding of the biology of these viruses, their interactions with existing and potential hosts, the host immune response that can neutralize them, and the possible interventions that can minimize their impact on global public health. The best methods to control flaviviruses include limiting the insect vector population through pesticide treatments (i.e., that target mosquito larvae and preventing human contact with the vectors [16]. A combinatorial approach of proactive immunization and post-symptomatic treatment is crucial to limit human infections and further complications related to flavivirus infections.
1.1.2 Heterogeneity and geography of JEV

Before the availability of genetic sequencing, flavivirus classification was based on antigenically distinct serological complexes or serocomplexes based on serum cross-neutralization assays. According to this classification, JEV belonged to the Japanese encephalitis serocomplex, which included Murray Valley encephalitis virus, St. Louis encephalitis virus, West Nile virus, Yaounde virus, Cacipacore virus, Koutango virus, and Usutu virus. The pathogenic members of this serocomplex have enzootic cycles involving arthropods, birds, and mammals, and cause diseases in humans and other mammalian hosts [17].

Molecular genetic analysis of JEV isolates from different geographic regions of the world has revealed at least five distinct clusters of the virus [18]. Genotyping of JEV was initially based on the differences in the nucleotide sequence of the genomic region encoding a pre-membrane protein (prM), a 240 nucleotide sequence (between nucleotides 456 and 695) that is conserved [18]. Later, the envelope (E) glycoprotein gene was promoted as a better candidate for phylogenetic analyses because the E gene is the important structural gene of JEV and it provides similar results as the prM gene [19,20].

Based on the nucleotide sequence of the E gene, JEV is divided into five genotypes (genotype I, II, III, IV, and V; Figure 1.1) [21]. Generally, the divergence of 10% in nucleotide sequences is used to distinguish between the genotypes of flaviviruses [22]. Genotypes I-IV were isolated from mosquito vectors, bats, and human patients in Asia and Australia, respectively. For several decades, the only example of genotype V JEV was the Muar strain isolated from brain tissues of patients in Malaysia in 1952. Later in 2009-2010, genotype V JEV was isolated from Culex triateniorhyncus mosquito collected in China [23] and South Korea [24]. Among the five
genotypes of JEV, genotype I is divided into two large clades, GI-a and GI-b, as reported in a study published in 2014 [25]. In terms of evolutionary history, genotype IV represents the oldest lineage of JEV [21]. Most naturally circulating JEV isolates belong to GI-b and III [21]. It was generally accepted that all genotypes of JEV except IV are capable of causing disease in humans [21,26]. However, the most recent outbreak in Australia (February 2022) with human deaths was caused by genotype IV JEV [27]. Moreover, the direct relationship between genotype and virulence in human patients is controversial [22].

Isolates from epidemic outbreaks in temperate zones mainly belonged to GI-b and III, while GI-a, II, and IV are usually associated with endemic isolates observed in the tropical areas [24]. Genotype V has reemerged in China and Korea after a nearly 60-year-long hiatus since its first emergence in 1952 from a Malaysian patient [23,28]. Genotype I represents isolates from northern Thailand, Cambodia, and Korea. More recently, the genotype I strain was identified in Northern Australia [29]. Genotype II includes isolates from southern Thailand, Malaysia, Indonesia, and Northern Australia. Genotype III isolates are mainly detected in temperate regions, including Japan, China, Taiwan, the Philippines, and the Indian subcontinent. Genotype IV includes isolates from Indonesia and the isolates from the recent outbreak in Australia [27].

Before the 1970s, most human JE outbreaks were associated with genotype III, but a considerable rise in GI-b was reported in the last few decades [26]. For instance, genotype I was introduced in Korea in 1993 and gradually replaced genotype III as the dominant lineage [30]. Similarly, genotype I constitutes the majority of samples collected in Japan after 1994, Thailand
after 2000, and China after 2001 [30–32]. Genotype I displaced genotype III within a year after first being detected in Taiwan in 2008 [33]. An epidemiological study found a correlation between genotype and host: most isolates identified as genotype I were from mosquitoes and pigs, while human isolates were associated with genotype III [34]. Still, this observation in host preference does not imply that humans in genotype I-dominated areas are safer because many cases of acute encephalitis due to genotype I have been reported in Japan, China, and India [26,35].

**Figure 1.1. Geographic distribution of JEV.** World geographic distribution of JEV genotypes (I to V) with colors representing countries of reporting. Image taken from [36] and adapted for updated information. *No human cases are reported in Italy to date.*
Several studies questioned why GI-b took over genotype III and emerged as the dominant lineage. A study from 2012 showed that experimental infection of several avian species with GI-b caused higher and more prolonged viremia than GIII [37]. Another group investigated the difference in infection dynamics between the two genotypes using infection in vectors and host cells. By orally infecting Culex tritaeniorhyncus with a blood meal containing the virus, the authors found that GI-b and G-III had similar infection rates with comparable replication titers. Interestingly, in the cells derived from amplifying hosts, GI-b viruses generated higher viral titres than G-III, especially at elevated temperatures. Further, GI-b produced an earlier and higher viremia when pigs, ducklings, and young chicken were experimentally infected [38]. Mutations in viral NS2B/NS3 protease may have enhanced the infectivity of genotype I [38]. On the other hand, some studies have demonstrated no difference between viral genotypes in the magnitude and duration of viremia in chicks, ducklings [39], and pigs [40]. Hence, more studies are needed to better understand why GI-b JEV replaced G-III.

The geographical range of JEV has expanded since the first JE epidemic was reported in Japan in the 1930s. Japanese encephalitis virus is endemic to large parts of the Asian-Pacific region, covering vast areas from southeastern Russia to Japan, Eastern China, Southeast Asia and India, stretching down to Australia [41]. Though the JEV burden throughout Asia has decreased due to aggressive mosquito control (via pesticide usage) and JEV vaccines, the virus still dominates as the region's most prominent cause of human encephalitis. For instance, in a study conducted in Vietnam, 52% of acute meningoencephalitis (AME) cases from 1998 to 2007 were caused by JEV [42]. Another similar study in Cambodia confirmed that 35% of AME patients had JEV infection, highlighting the need for public health measures to control this pathogen [43].
Japanese encephalitis is considered a rural disease, although cases in urban and peri-urban zones are reported [44]. Historically, JE has two distinct epidemiological patterns depending on the climate in the region [45]: JE occurs as a summer epidemic in northern temperate zones including Korea, Japan, China, and Nepal. Tropical areas such as Vietnam, Thailand, Malaysia, and the Philippines have year-round occurrences with a peak during the rainy season [46]. The incidence of JE ranges from 0.003 per 100,000 people in countries like Japan and Korea, where successful vaccination programs are established, to 3.7 per 100,000 people in countries like Cambodia, Indonesia, and Malaysia. The numbers, however, are likely to be underestimated due to underreported and asymptomatic JEV cases [44].

Multiple JE cases are identified in non-endemic regions. For example, when local birds were sampled from 1996 to 2000 in Italy, JEV RNA and antibodies against JEV and were detected; viral RNA was also detected in field-collected *Culex pipiens* mosquitoes in 2011 [47]. Furthermore, the JEV genome was detected in a yellow fever human patient who did not travel anywhere in 2016 in Angola, suggesting the possibility of local transmission in Africa [48].

Human and bird migration, accidental transport of mosquito vectors, climate change, altered distributions of the vector and vertebrate host, all influence the broader dispersal of JEV worldwide. Hence, new territories are constantly prone to emerging JEV, which can be further aggravated by the presence of susceptible avian and mammalian hosts and competent vectors in the new region. An example of such a threat was recently observed in Australia. Wind-blown mosquitoes were likely the cause of JEV introduction in Northern Australia from Papua New
Guinea [46]. Also, after the flooding season, the influx of migratory water birds was linked to the first JEV outbreak infecting swine herds and humans with fatal outcomes [49].

1.1.3 Clinical representations of JEV infection

Japanese encephalitis virus is a pathogen of humans as well as animals. It is the most prominent causative agent of viral encephalitis in humans in Asia [50]. Humans are the dead-end hosts of JEV, where the viremia after infection is insufficient for further transmission to mosquitoes. Though only a small fraction of individuals infected with the virus develop severe clinical complications, the case fatality rate of JEV infection can be high with a range of 15-30%. Up to 50% of surviving encephalitis individuals experience long-term neurological sequelae. JEV causes at least 68,000 infections annually; estimates, however, suggest that the number of infections is underestimated because of many asymptomatic infections [51].

Clinical outcomes in human patients

Japanese encephalitis virus affects the central nervous system (CNS) causing clinical and fatal infections, and it remains the most important cause of viral encephalitis in Asia. The virus particularly affects immunologically naïve individuals, and young children are infected more frequently than adults [52,53]. Clinical signs range from generic flu-like symptoms with headache, high fever, and lethargy to severe symptoms like motor and memory dysfunction, paralysis, and seizures [54,55]. The average incubation period of JEV ranges from 5 to 15 days [56]. Clinical manifestation starts with a high fever, mental status change, and the gradual decline of speech and motor function. In infants and children, initial symptoms may also include anorexia, nausea, abdominal discomfort or pain, vomiting, diarrhea, and acute convulsions [56].
In early JE patients, generalized weakness, hypertonia and hyperreflexia are among the most common motor abnormalities [56]. Symptomatic infections are rare, yet mortality rates can reach 30%, causing 10,000 to 15,000 deaths yearly [52]. In addition, up to 50% of the survivors may suffer from permanent neurological damage such as cognitive, motor, and coordination abnormalities [57]. In some cases, poliomyelitis-like acute flaccid paralysis is observed. These patients demonstrate markedly reduced motor amplitudes, and electrophysiological studies have confirmed severe damage to anterior horn cells in these individuals [58,59]. The poor prognosis is associated with clinical features like a short prodromal period (time between initial symptoms to full disease), deep obtundation, prolonged fever, respiratory dysfunction, and status epilepticus. Fatal outcomes are often associated with high virus loads and low antibody titres in the cerebrospinal fluid, which indicate uninhibited viral proliferation in the brain [56].

Infection with JEV has also been associated with pregnancy-related abnormalities in humans [60]. In a series of JEV outbreaks in Uttar Pradesh, India, between 1978 and 1980, women who became infected with JEV in the first or second trimesters miscarried, and the virus was isolated from the placenta and fetal brain. Interestingly, the women who were infected with JEV in the third trimester did not show pregnancy-related adverse outcomes [56].

Clinical outcomes in infected pigs

Besides being a human pathogen, JEV is an agricultural and veterinary pathogen with the potential for severe impact on the swine and pork industry [61]. Viremia is observed after JEV infection, and the virus causes age-specific clinical disease in pigs. In sexually mature pigs, JEV infection can result in reproductive failure like abortions and transient infertility [62–64]. If
pregnant pigs become infected before 60 to 70 gestation days, abortions, abnormal farrowing, mummified fetuses, and weak piglets are observed [65–67].

JEV invades the central nervous system and causes nonsuppurative meningoencephalitis in young pigs, similar to human cases [68]. Still, natural infection and disease in young piglets are not widely reported in endemic areas, probably because of maternal antibodies that can protect for up to 6 months of age [65,66]. Early nonspecific signs in young pigs include fever, anorexia, and depression. After five days post-infection, neurological symptoms such as hind limb tremors and ataxia can develop. Wasting-like syndrome is observed in a fraction of infected pigs [67]. Despite such clinical outcomes, JEV infection is not considered a lethal swine disease because most pigs survive with seroconversion after infection. These survivors may develop secondary JEV infections when experimentally infected with a different JEV genotype [69]. Altogether, pigs are the most critical amplifying hosts because of their capability to develop high viremia that contributes to the maintenance of the transmission cycle of JEV to humans. Also, pigs can be used as an experimental tool for JEV pathogenesis and transmission studies.

1.1.4 JEV Vaccines

*Human Vaccines*

There are no specific therapies and approved antivirals to treat JEV infection. Hence, prevention via vaccination is essential, and WHO recommends integrating JEV vaccines into the national immunization schedules in endemic countries [70]. Currently, there are three types of licensed vaccines available for JEV which are considered safe and efficient: 1) inactivated Vero cell-derived vaccines; 2) a live attenuated vaccine; and 3) a live chimeric vaccine [57,70,71]. The available vaccines are summarized in Table 1.
Table 1. Human vaccines against JEV.

<table>
<thead>
<tr>
<th>Type</th>
<th>Vaccine name (virus strain)</th>
<th>Dose regimen</th>
</tr>
</thead>
<tbody>
<tr>
<td>Inactivated Vero cell-derived vaccines (JE-VC)</td>
<td>JEIMMUNUGEN, TC-JEV, or JE-BIK-V (Beijing-1)</td>
<td>Three doses (days 0, 7, and 28) at 12-24 months of age. A booster after 12-14 months and every 3-5 years.</td>
</tr>
<tr>
<td></td>
<td>IXIARO, JESPECT, or JEEV (SA14-14-2)</td>
<td>Two doses (days 0 and 28) as early as 2 months of age. A booster after 1 year.</td>
</tr>
<tr>
<td>Live attenuated vaccine</td>
<td>CDJEVAX (SA14-14-2)</td>
<td>Single dose at 8-9 months of age. A booster after 3-12 months and at 6-7 years of age.</td>
</tr>
<tr>
<td>Live chimeric vaccine</td>
<td>IMOJEV (ChimeriVax-JE)</td>
<td>Single dose with a booster after 5 years for those who are 18 years and older. Single dose with a booster at 12-24 months for those between 9 months to 18 years old.</td>
</tr>
</tbody>
</table>

Swine vaccines

There is a potential for reproductive diseases in JEV-infected pigs, and pigs can serve as a source for zoonotic spillovers. Despite these risks, there are currently no licensed JEV vaccines for pigs. Regionally approved vaccines, for example, live attenuated at222, ML17, and anyang300, are available for local use in Japan, China, and Korea [72–74]. The live attenuated SA14-14-2 human vaccine, though not licensed for swine use, is adopted in China to immunize pigs [75–77]. Despite having an excellent safety record for human use, there are concerns about reversion to virulence in amplifying hosts like pigs because isolates closely related to SA14-14-2 were detected from the cerebrospinal fluid of aborted fetuses and stillborn piglets from some vaccinated pigs in China [78]. Moreover, though live attenuated vaccines elicit robust immune responses, adequate protection requires boosters [30] which is an expensive strategy for swine production. Due to these reasons, the vaccination of pigs against JEV is hard to attain because of associated costs. Hence, more research is required to develop cheaper options to protect the naïve population of pigs against JEV infection.
1.2 JEV Biology

1.2.1 JEV genome
Japanese encephalitis virus has a single-stranded, positive-sense RNA genome of approximately 11kb, flanked by 5’ and 3’ untranslated regions [79] (Figure 1.2). The genome has a 5’ cap but lacks a 3’ poly-A tail. The genomic RNA has a single open reading frame (ORF) that codes for a polyprotein of 3,400 amino acids. Viral and host proteases cleave this polyprotein into structural and nonstructural proteins. There are three structural genes; proteins encoded by these genes are involved in antigenicity and capsid formation: core (C), pre-membrane (prM), and envelope (E) proteins. The remaining seven genes encode nonstructural proteins: NS1, NS2a, NS2b, NS3, NS4a, NS4b, and NS5. The key functions of each protein encoded by the JEV genome are listed in Table 2.

Figure 1.2 JEV genome.
<table>
<thead>
<tr>
<th>Type of protein</th>
<th>Viral protein</th>
<th>Major function</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Structural proteins</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Capsid</td>
<td>• Forms the ribonucleoprotein by binding with the viral RNA.</td>
</tr>
<tr>
<td></td>
<td>Pre-membrane/Membrane (prM/M)</td>
<td>• Prevents premature viral fusion by blocking the E protein of immature virions.</td>
</tr>
<tr>
<td></td>
<td></td>
<td>• M protein acts as a chaperone for E protein folding.</td>
</tr>
<tr>
<td></td>
<td>Envelope (E)</td>
<td>• Viral attachment, membrane fusion, neuroinvasion.</td>
</tr>
<tr>
<td></td>
<td></td>
<td>• Domain I – consists of the N-terminus signal peptide sequence to direct the endoplasmic reticulum (ER) membrane topology of the structural polyprotein; the structurally central domain of E protein.</td>
</tr>
<tr>
<td></td>
<td></td>
<td>• Domain II – consists of the dimerization domain and hydrophobic fusion loops needed for virus-host membrane fusion for cytoplasmic release of the viral genome.</td>
</tr>
<tr>
<td></td>
<td></td>
<td>• Domain III – plays a role in virus attachment and induces potent neutralizing antibodies.</td>
</tr>
<tr>
<td><strong>Nonstructural proteins</strong></td>
<td>Nonstructural protein 1 (NS1)</td>
<td>• ER-lumen component of the virus replication complex.</td>
</tr>
<tr>
<td></td>
<td></td>
<td>• Highly immunogenic and is conserved among flavivirus; used as a diagnostic marker.</td>
</tr>
<tr>
<td></td>
<td></td>
<td>• Triggers endothelial hyper permeability.</td>
</tr>
<tr>
<td></td>
<td></td>
<td>• Immune evasion: inhibition of complement activation, TLR3 inhibition, ROS and JAK-STAT pathway suppression in mosquito midgut.</td>
</tr>
<tr>
<td></td>
<td>NS1' (52-amino acid C-terminal extension of NS1, produced as a result of −1 programmed ribosomal frameshifting at the conserved slippery heptanucleotide and 3′-adjacent pseudoknot near)</td>
<td>• Substitute of NS1 in the virus replication complex.</td>
</tr>
<tr>
<td></td>
<td></td>
<td>• Neuroinvasion and neurovirulence.</td>
</tr>
<tr>
<td></td>
<td></td>
<td>• Immune evasion: IFN-β antagonism by MAVS targeting.</td>
</tr>
<tr>
<td>Protein</td>
<td>Description</td>
<td></td>
</tr>
<tr>
<td>---------</td>
<td>-------------</td>
<td></td>
</tr>
</tbody>
</table>
| **NS2A** | - Transmembrane component of the virus replication complex.  
- Generates virus-induced membranes for virus assembly in the ER.  
- Immune evasion: suppression of RNA interference, suppression of IFN-β transcription. |
| **NS2B** | - Transmembrane component of the virus replication complex.  
- Forms the viral protease with NS3 as an essential co-factor. |
| **NS3** | - Cytoplasmic component of the virus replication complex.  
- N-terminal serine protease with NS2B.  
- C-terminal helicase and nucleoside 5’-triphosphatase. |
| **NS4A** | - Transmembrane component of the virus replication complex.  
- Remodels ER membranes to create the replication site.  
- Immune evasion: inhibition of dsRNA-activated protein kinase R. |
| **NS4B** | - Transmembrane component of the virus replication complex.  
- Immune evasion: interferes with Type-I IFN signaling, RNAi, formation of stress granules, and unfolded protein response. |
| **NS5** | - Cytoplasmic component of the virus replication complex.  
- RNA methyltransferase and RNA-dependent RNA polymerase.  
- Immune evasion: inhibition of IFN-β-induced apoptosis, suppression of Type-I IFN production, interferes with dsRNA-induced nuclear translocation of IRF3 and NF-kB, blocks IFN-Stimulated JAK-STAT signaling. |
1.2.2 Viral structure

Japanese encephalitis virus is an enveloped, spherical virus with an icosahedral geometry [79] (Figure 1.3). The virus particle has a diameter of 40 nm; the viral surface is covered by 180 heterodimers of the E and M glycoproteins antiparallel to each other. The envelope encapsulates the RNA genome in a polyhedral capsid [80]. The nucleocapsid is a complex of the genomic RNA with multiple copies of highly-basic C proteins enveloped by a host-derived lipid bilayer consisting of 2 membrane-anchored surface proteins, prM/M and E [81]. The seven C-terminal nonstructural proteins (NS1 to NS5) play multiple roles in the viral life cycle, RNA replication, virus assembly, and innate immunity evasion [82]. An extended form of NS1 protein is formed by a classical -1 ribosomal frameshifting between codons 8 and 9 of NS2A, adding 52 extra amino acids. Site-directed mutagenesis showed that the extended form of NS1 (also known as NS1’) is involved in neuroinvasiveness [81].

**Figure 1.3. Structure of JEV.** Image from the Research Collaboratory for Structural Bioinformatics Protein Data Bank (RCSB PDB [rcsb.org]) [83]. Data files contained in the archive are free of all copyright restrictions.
1.2.3 JEV life cycle

Entry of JEV into a host depends on several interactions between the virus and the host cell, starting with the nonspecific binding of the viral glycoprotein E to one or more cellular attachment factors on the cell surface [84]. The initial nonspecific attachment concentrates the virions at the cell surface and facilitates specific interactions. Attachment to specific host cell receptors such as heparin sulfate, dendritic cell-specific intercellular adhesion molecule 3-grabbing nonintegrin (DC-SIGN) or CD29, and mannose receptor directs the classical clathrin-dependent endocytosis or non-classical clathrin-independent endocytosis pathways depending on the cell type [84–89]. Like most other flaviviruses, the JEV E glycoprotein undergoes low pH-induced conformational changes following endocytosis that triggers the fusion of viral and host endosomal membranes [90–94]. Membrane fusion results in the release of the positive-sense genomic RNA into the cytoplasm, where it is translated into precursor polyproteins that are post-transcriptionally cleaved into the structural and nonstructural viral proteins [95,96]. The viral RNA serves as a template for the synthesis of a double-stranded RNA at the surface of the endoplasmic reticulum mediated by viral RNA-dependent RNA polymerase. The double-stranded RNA is further transcribed and replicated, resulting in viral mRNAs/new positive single-stranded RNA genomes.

The newly translated seven nonstructural proteins and several host factors are then involved in the genomic RNA replication that occurs in the virus-induced endoplasmic reticulum (ER)-derived unique membranous structure called organelle that serves as a site for replication complexes [97–100]. NS3 and NS5 catalyze viral RNA replication [101]. Immediately after RNA replication, two viral glycoproteins, E and prM, envelope the newly synthesized genomic RNA and C proteins on the ER membrane to produce the immature virion of approximately 60 nm in diameter. The virion has 60 protruding spikes composed of three parallel prM:E
heterodimers [102,103]. The immature virion then passes through the constitutive trans-Golgi secretory pathway, where viral maturation occurs through the furin-mediated cleavage of the prM protein to M [104–106]. Structural rearrangement of M and E proteins generates the mature virion of 50 nm, covered by 30 flat lipid rafts, each composed of three parallel E:M:M:E heterotetramers [102,107]. Mostly, viral replication takes place entirely in the cytoplasm. However, two viral proteins, C and NS5, have also been detected in the nucleus [108]. The role of their nuclear localization in viral replication and pathogenesis is still not fully understood and needs further investigation.

1.3 Immune responses to JEV infection

During natural vector-borne JEV infection, the virus initially replicates in skin resident dermal cells like keratinocytes, fibroblasts, endothelial cells, and various immune cells [109–111]. Then, the viral replication occurs in the peripheral system, including peripheral blood mononuclear cells (PBMCs), where macrophages, dendritic cells (DCs), and monocytes become infected [112]. Subsequently, over the incubation period, the virus can be cleared through an effective peripheral immune response or can infiltrate further into the central nervous system (CNS) [113].

1.3.1 Innate immune response to JEV

After becoming infected, host cells produce various cytokines that induce an inflammatory response and inhibit viral replication. Innate immune responses against viral infections depend on the recognition of pathogen-associated molecular patterns (PAMPs) by the host pattern recognition receptors (PRRs) [114]. Infection with JEV upregulates PRRs like retinoic acid-inducible gene I (RIG-1), melanoma differentiation-associated protein 5 (MDA-5), and toll-like
receptor 3 (TLR-3). Cells deficient in these receptors have increased viral loads and lower levels of chemokines and cytokines [115,116]. Toll-like receptor-3 and TLR-7 play an important role in sensing JEV and mounting anti-viral immune responses [115,117].

Microglial cells (the brain resident macrophages) produce many anti- and pro-inflammatory cytokines in response to the virus. Studies have confirmed the upregulation of pro-inflammatory molecules like RANTES, TNF-α, IL1-α, IL-6, IL-12, IL-8, IL-β, CCL2, CXCL9, CXCL10, CXCL11, and pro-inflammatory enzymes like cyclooxygenase-2 and iNOS during JEV infection [118–123]. In contrast, IL-10, an anti-inflammatory cytokine, is reduced as a result of JEV infection [124]. Astrocytes in the CNS also produce type I interferons (IFNs) and inhibit JEV replication, reducing virus-induced cytopathic effects.

Interferon-induced activation of JAK-STAT signaling results in the expression of different interferon-stimulated genes (ISGs) that establish an anti-viral state in the host cell. The ISGs network is differentially modulated in different cell lineages [125]. Interferon-stimulated genes like PKR, OAS, TRIM21, ISG15, and MX1 are induced at transcriptional and translational levels during JEV infection. When interferon-alpha (IFN-α) stimulated genes like ISG15, MX2 and OAS-L are overexpressed, strong anti-JEV effects are observed [126–128]. Defective type I IFN response leads to higher viral loads and JEV-induced death in RIG-1, MDA5 and MyD88 knockout mice [129].

1.3.2 Adaptive immune response to JEV
The humoral and cellular arms of adaptive immune responses are implicated during JEV infection.
**Humoral arm of adaptive immunity**

The immune response to JEV is well characterized. Antibodies protect the host by restricting JEV replication during the viremic phase—the phase before the virus crosses the blood-brain barrier (BBB) and reaches the CNS [130,131]. Effective monotypic immunoglobulin M (IgM) response can be detected in the serum and cerebrospinal fluid (CSF) of infected individuals [125]. Production of IgM peaks within seven days of infection; failure to mount a significant IgM response often results in fatal outcomes [132–134]. Increased IgM levels are observed in sera from asymptotically infected individuals, indicating a strong protective role [135]. In human patients that fully recovered from the infection, class switching of antibodies from IgM to IgG occurs, and most patients are positive for JEV-specific IgG within 30 days after recovery.

Neutralizing antibodies that recognize epitopes in the viral E protein determine protection against JEV. A neutralizing antibody response to JEV infection can be detected as early as 5 days post-infection in pigs; and early and high neutralizing antibody titers are needed to prevent viral neuroinvasion and lethal outcomes in mice [109,136]. Neutralizing antibody titers of ≥ 1:10 are accepted as evidence of protection against JEV infection [109,137]. Neutralizing antibodies against the viral E, NS1, and prM antigens mediate protective immunity against JEV infection and prevent lethality in mice [138–141].

**Cellular arm of adaptive immunity**

Dendritic cells are essential for the initiation of the adaptive immune response and T-cell activation during JEV infection [142,143]. In mouse models of JEV infection, dendritic cells regulate monocyte differentiation in lymphoid tissue and CNS, leading to reduction of pro-
inflammatory Th17 cells and increasing anti-inflammatory Treg cells [144]. These events may protect the immune-privileged CNS in case of severe neuroinflammation [144]. Infection of human monocyte-derived DCs (moDCs) with JEV results in their maturation and upregulation of CD274/PD-L1, which is implicated in Tregs cells expansion [144]. A more recent transcriptomic-based study showed that JEV infection of human moDCs activates IFN and NF-kB mediated antiviral and inflammatory pathways and causes expansion of Tregs in an allogenic response [145]. Tregs exert a protective effect during acute viral infections by balancing pathogen clearance and excessive inflammation [146].

Activation of T cells provides either partial or complete protection against JEV infection in mouse models [147,148]. JEV-specific CD4+ T cells are involved in B cell development, antiviral cytokine production, and T cell memory response [149–151]. Enhanced myeloid-derived suppressor cell (MDSC) populations in JEV-infected mice suppress CD4+ T cells, resulting in reduced B cells and blood plasma cells and eventually decreased total IgM and JEV-specific neutralizing antibodies [152]. Memory T cell response plays a role in protection against JEV infection [150]. For instance, several studies have demonstrated that adaptive transfer of JEV-primed T cells can protect mice from lethal JEV challenges [148,153].

### 1.4 JEV persistence

Persistent and latent infections of JEV are described in cell lines such as neuroblastoma, murine microglial cells, in mouse models, and in T-lymphocytes and the nervous system of human
patients [154–157]. Studies in pigs have found more discrete evidence of JEV persistence. One of the recent novel findings on JEV persistence was observed in porcine tonsil tissues at least after the acute phase of infection [136]. Viral RNA was also detected in the brain of pigs at 21 days post-inoculation following an oronasal challenge. Persistence of JEV in the tonsils of infected pigs was detected for up to 46 days post-infection, based on RT-qPCR [69]. A recent study demonstrated that JEV persistence in porcine tonsils was associated with a weak innate immune response, absence of IFN-gamma mRNA expression, and a decreased frequency of CD4+CD8+ T double-positive T cells [158]. The combined findings from all these studies suggest that apart from tropism for the central nervous system, JEV might have a tropism for the tonsils in the pigs. This lymphoid organ might hence act as a viral reservoir, as suggested by the presence of high infectious viral titers at least 11 days after infection [159].

Apart from the tonsils and the central nervous system, studies investigating the persistence of JEV in other tissues are still lacking. Further in vivo and in vitro studies are necessary to identify the tissues and organs that might be the sites of JEV persistence.

1.5 Flavivirus and specifically JEV interactions with the epithelium
Epithelial cells line the body surfaces and protect against the invasion of pathogens by forming a selective barrier. These cells are present on the skin, blood vessels, and the mucous membranes of the nose, vagina, respiratory tract, etc. The epithelial lining of different organs is susceptible to infection by flaviviruses.
**Skin epithelium**

The skin constitutes the first line of defense against pathogens. Skin is permissive to flavivirus infections, however. The skin’s epidermis and dermis are considered the cellular targets for flavivirus infection. The plasma membrane of epithelial cells has two surfaces, i.e., apical and basolateral, displaying specialized proteins and receptors. The epithelial cells are tightly packed at cell junctions and act as diffusion barriers for pathogens. However, flavivirus receptors are present on apical and basolateral cell surfaces [160]. A wide array of receptors that may serve as a port of entry for flaviviruses are expressed in the skin epithelium: C-type lectin receptors, T-cell immunoglobulin and mucin domain, TYRO3, AXL, and MER, etc. [161,162]. During bites and feeding, the mosquitoes inoculate flaviviruses through epithelial cells to other skin cells, such as dendritic cells. Experimental and clinical studies reported that viruses, including JEV, Zika virus, and dengue virus, infect the skin’s dendritic cells. Once infected, these cells migrate to lymphoid organs where viruses continue to replicate. The viruses then spread to other internal organs [163].

**Nasal epithelium**

Vielle et al. used human nasal epithelial cells (NECs) to evaluate their susceptibility to several flaviviruses, including JEV, Zika virus, West Nile virus, and Usutu virus. The authors found that all these flaviviruses infect NECs without affecting the integrity of the epithelium. After replication in the cytoplasm, the live flaviviruses are actively released through the basolateral epithelial surface [160]. For JEV, the apical and basolateral shedding was reported in NECs [164].
Vaginal epithelium

The human vaginal epithelium is the first barrier against sexually transmitted pathogens. However, Zika virus can replicate in the epithelium of the vaginal tract. For example, intravaginally inoculated guinea pigs shed the virus in vaginal secretion [165]. Another study identified tyrosine-protein kinase receptor UFO as the Zika entry point in human vaginal epithelial cells [166]; this receptor is encoded by the AXL gene. Thus, the vaginal epithelium plays an important role in mediating sexual transmission of the Zika virus in humans [165]. To my knowledge, interactions of other flaviviruses, including JEV, with the vaginal epithelium have not been studied.

1.6 Transmission of JEV

Understanding how a virus is transmitted and maintained in the environment is essential to disease control. Flaviviruses have complex transmission cycles due to the involvement of hematophagous vectors, vertebrate reservoirs, and amplifying hosts. Environmental and climatic factors may also facilitate virus transmission.

A representative scheme of JEV transmission is summarized in Figure 1.4. Depending on the type of hosts (i.e., wild, domestic, or human) and vectors (i.e., urban, primary, or accessory), different types of transmission cycles can occur: enzootic or sylvatic cycle in the wild; epizootic or rural cycle; and epidemic or urban cycle [167,168]. The sylvatic (or enzootic) cycle maintains and amplifies the virus in nature by allowing natural transmission of the virus between the wild vertebrate hosts and primary arthropod vectors [167]. The epizootic (or rural) cycle involves the transmission of the virus between domestic animals and primary or accessory vectors.
Figure 1.4. Transmission of JEV.

Often, these epizootic cycles lead to viral outbreaks in domestic animals, and humans get infected via competent arthropod vectors and zoonotic spillovers [169]. For example, the emergence of SARS-CoV in humans in 2002/2003 and the MERS-CoV in 2012 were likely from viral strains found in bats; palm civets and dromedary camels potentially acted as intermediate hosts [169]. Currently, it is believed that in the case of JEV, only enzootic and epizootic transmission cycles exist because humans are dead-end hosts, meaning that they cannot produce sufficient viremia capable of infecting mosquitoes. However, vector-free JEV transmission, specifically contact transmission, is an emerging concern [136].
1.6.1 Vector-borne transmission of JEV
Flaviviruses are arthropod-borne viruses or arboviruses. As defined by WHO, arboviruses are “viruses which are maintained in nature principally or to an important extent through biological transmission between susceptible vertebrate hosts by hematophagous arthropods; they multiply and produce viremia in the vertebrates, multiply in the tissues of arthropods, and are passed on to new vertebrates by the bites of arthropod after a period of extrinsic incubation” [170].

Japanese encephalitis virus has been isolated from *Culex, Aedes, Anopheles*, and *Mansonia* species of mosquitoes, and in some cases, from midges (*Lasiohela taiwana*) and ticks (*Haemaphysalis japonica*) [171]. However, to have ecological and epidemiological importance, a vector needs to be competent for transmission and detecting JEV in arthropod vectors does not necessarily mean they act as vectors for transmission. The arthropod vector must be susceptible to arboviral infection and virus replication, dissemination to the salivary glands, and eventual virus shedding with saliva and transmission back to the vertebrate host [172]. The *Culex* family of mosquitoes with *Culex tritaeniorhynchus, Culex gelidus, Culex vishnui, Culex annulirostris* species meet these criteria. *Culex tritaeniorhynchus* is identified as the principal vector of JEV due to its high susceptibility, high virus transmission rate, and wide geographical distribution [172,173]. *Culex* mosquitoes breed in ground pools, shallow waters, and rice paddies, which they use as egg-laying and larval habitats [137]. They are primarily active during dusk, and are exophilic and zoophilic; *Culex* mosquitoes prefer animals to humans for feeding. *Culex tritaeniorhynchus*, in particular, prefer to feed on pigs, supporting the role of pigs as critical amplifying hosts [174].
On average, the saliva of infected *Culex tritaeniorhynchus* has 100,000 focus-forming units per ml (FFU/ml) of JEV, when allowed to salivate for an hour [175]. Once a mosquito feeds on a viremic blood meal, the virus titer in the mosquito midgut lumen needs to be high enough to overcome the midgut barrier and immune system, infect the gut epithelial cells, and disseminate into the haemocoel [176]. Next, the virus spreads to salivary glands, replicates there, and ultimately gets excreted with salivary compounds when the mosquito feeds upon the host [176]. However, that mosquitoes can transmit the virus simply by probing, and successful blood feeding is not an absolute requirement for transmission [177]. Additionally, vertical transmission of JEV from an adult female mosquito to developing eggs has been documented in experimental settings for *Culex pseudovishnui*, *Culex tritaeniorhynchus*, *Culex annulus*, *Culex quinquefasciatus*, *Armigeres subalbatus*, *Aedes albopictus*, and *Aedes togoi* [178–180]. To summarize, the enzootic transmission of JEV is determined by the presence of both competent vectors and amplifying hosts; the vectors drive the disease transmission, and the susceptible hosts sustain the viral cycle in nature.

**Birds as amplifying hosts**

Birds play an important role in the transmission of JEV because they develop high viremia and because of their changing migratory behavior, birds play an important role in the transmission of JEV. More than 90 bird species may potentially amplify JEV [171]. Water-wading birds from the *Ardeidae* family, like egrets and herons, are highly susceptible to JEV infection and other flaviviruses, for example WNV and Usutu virus [21]. Several experimental infection studies of wild birds with different strains of JEV showed that birds develop sufficient viremia to transmit JEV via mosquito vectors to mammalian hosts, specifically to pigs and humans [181,182]. Other
bird groups like raptors, including the crested goshawk, honey buzzard, and white-bellied sea eagle, contained JEV-specific neutralizing antibodies in their sera, as reported in a recent study in Singapore [183]. Birds migrate between the northern and southern hemispheres each year [184,185]. They have wide distributions, hence they can potentially disperse virus I to new geographic regions [37,186]. In addition to flaviviruses, migratory birds can act as vehicles for other zoonotic pathogens like influenza viruses and enteropathogens [185,187]. In seroprevalence studies, poultry also contain JEV-specific antibodies [188]. The viremia in poultry is low and may not cause viral transmission [189]. However, a recent study showed that ducklings and chicks can contribute to the JEV maintenance cycle [190]. Therefore, the role of poultry in JEV epidemiology needs reevaluation.

Pigs as amplifying hosts

Pigs and wild boars are the most important vertebrate hosts for JEV transmission to humans. Domesticated pigs have a special role in JEV transmission because they can act as the amplifying hosts, having sufficient viremia in the blood to infect feeding mosquitoes [159]. Additionally, proximity to pig farms in endemic regions is a major risk factor for JEV transmission to humans [191]. Several JEV isolates were detected in infected pigs [64,192,193]. Unlike birds, pigs can develop pathological symptoms after infection. Infection with one genotype of JEV in the past may not entirely prevent secondary infection with another genotype, as observed in experimental settings. Heterologous infection was confirmed by the detection of viral RNA in lymphoid or nervous tissues and oronasal swabs within 10 days post-infection [69,136]. Pigs with JEV-neutralizing antibodies are still susceptible to JEV infection via mosquito bites and develop sufficient viremia to infect a fraction of feeding mosquitoes [194].
Wild (or feral) boars are a group of *Sus scrofa* biotypes, including feral or escaped domestic pigs, Eurasian or Russian wild boars, and cross-bred hybrids [195]. Most of the modern breeds of pigs are derived from the wild Eurasian boar and are also considered important drivers of JEV outbreaks in endemic regions [195]. JEV antibodies were detected in seroprevalence studies of wild boars in Korea and Japan. In some studies, antibodies were found in 66 to 83% of the boars sampled [74,196–198]. JEV RNA was also detected in wild boar populations[199]. Even though wild boars are believed to be the key enzootic hosts of JEV, there are many knowledge gaps about their pathology and clinical signs, hence requiring for more research in this field.

*Other vertebrate hosts*

Horses and cattle can be infected with JEV, but the viremia in these hosts is not high enough for efficient transmission to mosquito vectors. Before the introduction of a vaccine, JEV affected thousands of horses in Japan, leading to multiple epidemics [200]. However, after the vaccine introduction, only two cases of infection in horses were reported [201,202]. In another study, live JEV was isolated from the blood of asymptomatic horses in India [203].

Cattle, like horses, can also be infected with JEV, and there are reports of mild to moderate symptoms along with reproductive losses [192,204]. Seroprevalence studies showed that JEV can subclinically infect other species of animals like goats, dogs, cats, raccoons, raccoon dogs, and meerkats [197,205,206]. Though these species do not suffice as reservoirs for the maintenance of viral existence in the environment, they may serve as sentinel species to indicate the seasonal occurrence of JEV [61]. Further, in 2017, two speckled seals in the Weihai Aquarium in Shandong Province in China were discovered to have a lethal JEV infection.
Infection was confirmed in these two seals by virus isolation, immunostaining, and electron microscopy [207].

Bats are reservoir hosts for many pathogenic viruses like Ebola, SARS-CoV, MERS-CoV, Nipah, and Hendra [23]. Regarding JEV, the virus was isolated in bats of various species in multiple locations [208,209]. More recently, two bat JEV isolates—one from Leschenault’s rousette, a fruit bat, and the another from a little tube-nosed insectivorous bat—were identified when archived samples were analyzed using genome sequencing [210]. This indicates that bats might serve as reservoirs for JEV and other flaviviruses.

1.6.2 Oronasal transmission
Evolutionary phylogenetic analysis of the Flaviviridae family suggests the emergence of non-vectored transmission before vector-borne transmission [211]. Sequence analysis and phylogenetic investigation of the Flaviviridae family have found that flaviviruses may be able to lose or acquire a variety of genetic traits in response to selection pressures associated with host availability and can support host switching through a selection process via gain and/or loss of genetic material over time to achieve optimal fitness [212–214]. These observations suggest that the association of flaviviruses with arthropod vectors like ticks and mosquitos could be an acquired trait from a non-vectored ancestral virus [215,216].

If this hypothesis is correct, the transmission of flaviviruses can be expected to deviate from traditional routes within certain ecological contexts and viral characteristics. With an abundant vector population and high infection rates, it has been demonstrated that co-feeding facilitates the transmission of the virus from one vector to another in the absence of host viremia [217,218].
Vertebrates may contract viral infections intranasally, orally, venereally, or through contact with the cornea, skin abrasions, or mucosal tissues. Food, contaminated water, aerosol, body secretions (urine, fecal matter, saliva, milk, mucus), hair, feathers, or skin can be the source of viruses. Animal behavior like eating, drinking, sniffing, licking, aggression, and insectivorous tendencies might support such infectious events [219].

Unconventional routes of transmissions without the involvement of arthropod vectors are reported for flaviviruses in several cases. Vertical transmission in humans has been seen for dengue virus [220–222], Zika virus [222,223], West Nile virus [224], yellow fever virus [225] and JEV [60]. Moreover, transmission through needle stick injuries and other blood exposures are reported for dengue virus, West Nile virus, yellow fever virus, and Zika virus [226,227]. Zika virus, the causative agent of microencephaly, is transmitted sexually [228–230]. Zika virus was also detected in the saliva, urine, breast milk, and amniotic fluid of infected individuals [231]. Thus, direct transmission of flaviviruses that were thought to be strictly vector-borne has become a subject of great concern from the public health perspective and demands experimental studies.

Although the typical JEV infection is mosquito-borne, oronasal infection and contact transmission have been confirmed. For example, intranasal inoculation of rhesus monkeys with JEV results in clinical features comparable to natural disease in humans [232]. More recently, high viral loads were detected in the lungs of sentinel mice as confirmed by plaque assay [233]. Furthermore, Garcia-Nicolas et al. used porcine nasal mucosa tissue explants and three-dimensional porcine nasal epithelial cell cultures and to demonstrate that JEV effectively replicates in the nasal mucosa
targeting porcine epithelial cells [164]. Lyons et al. found JEV RNA in oral fluids of intradermally inoculated domestic pigs up to two weeks after inoculation.

A more detailed description of vector-free transmission and persistence of JEV in pigs is described in another experimental study [136]. Sentinel pigs that were in contact with needle-infected pigs showed viremia for 2-4 days. They shed live virus 6-10 days after contact; most oronasal swabs become positive for viral RNA about 4-7 days after infection. Histopathological CNS lesions were demonstrated for both needle-infected and contact-infected pigs. At 25 days post-injection, virus RNA was still found in the tonsils of contact-infected pigs.

Epidemiological studies also confirm the importance of vector-free, possibly oronasal JEV transmission in pigs. For example, mathematical modeling data from JEV outbreaks on swine farms in Cambodia supports efficient virus maintenance and transmission beyond mosquito seasons [234]. Epidemiological data from Japan also confirm this scenario [62].

### 1.6.3 Sexual transmission

There are emerging concerns about sexual transmission of flavivirus in humans which may change flavivirus epidemiology and expand the geographical range to areas with no insect vectors. The first case of Zika virus sexual transmission was reported in 2008 [228]. Afterward, during the Zika outbreak in 2015-2017 in the Americas, many cases of sexual transmission of Zika virus were described, including male-to-male, male-to-female, and female-to-male transmissions [235–237]. Fetal infection with congenital Zika syndrome after sexual transmission in mothers was also reported [238]. In humans, Zika virus has been detected in vaginal secretions [239–241] with replication in vaginal epithelial cells [242]. Rodent and non-human primate models support
replication in reproductive tissues and sexual transmission of Zika virus [242–248]. Human sexual transmission of other flavivirus has not been documented yet. It is unknown whether JEV has tropism in the vaginal epithelium and the potential for sexual transmission in humans.

1.6.4 Transplacental transmission
Another example of vector-free flavivirus transmission is transplacental Zika virus transmission. Zoonotic flaviviruses were thought to primarily impact non-reproductive tissues in human patients. However, Zika virus replicates in the maternal-fetal interface breaching the placental barrier and infecting the fetal tissues [249–251]. Zika virus crosses the placental barrier to reach the intrauterine cavity with the fetus and replicates in placental trophoblasts. Virus infection during all three trimesters of pregnancy may result in fetal infection and congenital abnormalities in newborns; however, severe clinical disease was often attributed to infection in early pregnancy [223]. While clinical outcomes of transplacental JEV infection are described in humans [60] and pigs [252], cellular targets and tissue tropism in the upper reproductive tract are unknown.
CHAPTER 2: HYPOTHESIS AND RESEARCH OBJECTIVES

2.1 Rationale

There are emerging concerns about flavivirus transmission routes other than the known, classical vector-borne route of flavivirus transmission. Such alternative transmission routes may change flavivirus epidemiology and expand the geographical range to areas with no insect vectors. For example, it was thought that JEV transmission occurred only through mosquitoes, but a recent study demonstrated that JEV can be transmitted from pig to pig, suggesting a vector-free route for the virus to spread [136]. Further studies confirmed efficient JEV replication in porcine and human nasal epithelial cells [160,164] and virus oronasal shedding in pigs [253,254] that could enable contact transmission. Another example of vector-free flavivirus transmission is sexual Zika virus transmission [235–237]. It is unknown, however, whether JEV also has tropism in the vaginal epithelium and the potential for sexual transmission in humans. To investigate this possibility, I studied JEV infection phenotypes and host transcriptional responses in human primary vaginal epithelial cells. I also studied JEV infection in vaginal tissues and vaginal virus shedding in pigs, the native amplifying host for JEV.

Zoonotic flaviviruses were thought to primarily impact human non-reproductive tissues. However, Zika virus replicates in the maternal-fetal interface breaching the placental barrier and infecting the fetal tissues [249–251]. Zika virus crosses the placental barrier to reach the intrauterine cavity with the fetus and replicates in placental trophoblasts. While clinical outcomes of transplacental JEV infection are described in humans [60] and pigs [252], cellular targets and tissue tropism in the upper reproductive tract are unknown. To better understand JEV pathogenesis during transplacental infection, I studied JEV infection phenotypes in human
primary endometrial cells and trophoblast. In pigs, I studied infection in the endometrium, placenta, and fetuses.

2.2 General hypothesis

I hypothesize that JEV has tropism in the primary human vaginal epithelium and the porcine vaginal tissues, suggesting potential risks for sexual transmission.

I also hypothesize that JEV has tropism in the primary human endometrial epithelium and trophoblast, and in the porcine endometrium and fetal implantation sites, posing risks for transplacental transmission and fetal infection.

2.3 Research objectives

1. To identify whether JEV causes infection and altered gene expression in the human vaginal epithelium, and persists in vaginal tissues of pigs (Subchapter 4.1).

2. To identify whether JEV causes infection in the human endometrial epithelium and trophoblast, and persists in the endometrium and fetal implantation sites of pigs (Subchapter 4.2).
CHAPTER 3: MATERIALS AND METHODS

3.1 Ethics Statement

We followed the Canadian Council on Animal Care guidelines and Animal Use Protocol #20200106 was approved by the University of Saskatchewan's Animal Research Ethics Board and Animal Care and Use Review Office (ACURO) of the US Army Medical Research and Development Command. All efforts were made to minimize animal suffering. Pigs were euthanized with an anesthetic overdose followed by exsanguination.

3.2 Cells

Mosquito (Aedes albopictus) C6/36 cells (ATCC, CRL-1660) were cultured in a minimum essential medium (MEM; Sigma M4655) supplemented with 10% FBS and 1x penicillin-streptomycin. VERO E6 cells (ATCC CRL-1586) were cultured in DMEM supplemented with 3% FBS, 1x penicillin-streptomycin, and 2.67 mM sodium bicarbonate (Gibco 25080-094). Human vaginal epithelial cells from two donors (ATCC, PCS-480-010 and Lifeline Cell Technology LLC, FC-0083) were cultured in a vaginal epithelial cell basal medium (ATCC, PCS-480-030) with a Vaginal epithelial cell growth kit (ATCC, PCS-480-040). The commercial supplier confirmed the phenotype by staining cells with epithelium-specific (Pan-CK) and fibroblast-specific (TE-7) antibodies. Human primary endometrial epithelial cells (Lifeline Cell Technology, FC-0078) were cultured in ReproLife female reproductive epithelial cell culture media with supplements (Lifeline Cell Technology; LL-0068). HTR-8/SVneo trophoblast cells (ATCC CRL-3271) were cultured in Roswell Park Memorial Institute 1640 Medium (RPMI; Gibco 11875119) supplemented with 5% FBS and 1x Penicillin-Streptomycin. VERO E6, human reproductive epithelial cells, and HTR-8/SVneo trophoblast were cultured at +37°C and
C6/36 at +28°C in a 5% CO₂ humidified incubator. All cells were mycoplasma free as confirmed by LookOut Mycoplasma PCR Detection Kit (Sigma-Aldrich).

3.3 Viruses

The JEV Nakayama strain (GenBank EF571853) stock was initially produced at the World Reference Center for Emerging Viruses and Arboviruses, the University of Texas Medical Branch at Galveston, and transferred to our facility through the Public Health Agency of Canada. I inoculated VERO E6 cells and harvested media 9 days after inoculation to produce the working stock. Culture media containing JEV was centrifuged (12,000g, 20 min, +4°C); the supernatant was collected, aliquoted, and frozen at -80°C. The virus stock was mycoplasma free as confirmed by LookOut Mycoplasma PCR Detection Kit (Sigma-Aldrich).

3.4 Reverse transcription-quantitative polymerase chain reaction (RT-qPCR)

I used QIAamp Viral RNA Mini Kit (QIAGEN) according to the manufacturer’s instructions to purify JEV RNA from 140 µl of virus stock, maternal and fetal blood plasma, maternal vaginal and nasal swabs, and the supernatants of different cell cultures. Maternal and fetal tissue samples were dissected and weighed on analytical balances. One ml of TRI Reagent Solution (Thermo Fisher Scientific) was added to 80-100 mg of tissues before homogenization (5 min at 25 Hz) with RNase-free stainless-steel beads and TissueLyser II (QIAGEN). Then, RNA extraction was performed with PhaseMaker tubes (Thermo Fisher Scientific) and PureLink RNA Mini Kit (Thermo Fisher Scientific) according to the manufacturer’s instructions.
For JEV RNA quantification, I used the previously described probe-based one-step RT-qPCR assay [255]. All RT-qPCR reactions were conducted on the StepOne Plus platform (Life Technologies, USA) and analyzed using StepOne software version 2.3. The reaction mixture (20 μl) for RT-qPCR (Bioline) consisted of 10 μl 2x SensiFAST Probe One-Step Mix, 0.4 μl RiboSafe RNase Inhibitor, 0.2 μl reverse transcriptase, 1 μl (500 nM) of forward (Universal-JEV-F: 5’-GCCACCCAGGAGGTCTT-3’) and reverse (Universal-JEV-R: 5’-CCCCAAAACCCGAGGAAT-3’) primers, 0.5 μl (250 nM) probe (Universal-JEV-Probe: 56-FAM-CAAGAGGTG /ZEN/ GACGGCC-3IABkFQ), 1.9 μl nuclease-free water and 4 μl of sample RNA. A reverse transcription step of 10 min at 48 °C and an enzyme activation step of 2 min at 95 °C were followed by 40 amplification cycles (10s at 95°C and 20s at 60°C). RNA extracted from a stock of JEV was used to generate standard curves with known RNA concentration determined by Qubit that had a wide dynamic range (10^{2.5}-10^{12.5} RNA copies/ml) with a high linear correlation; r^2 = 0.99 between the cycle threshold (Ct) value and template concentration. The standard curve was used to find the detection limit at Ct 40. Assay values were corrected for fluid volumes or tissue weights and upon logarithmic transformation expressed as JEV RNA genome copies per ml or gram.

Productive infection in tissues was confirmed with JEV negative-strand-specific RT-PCR: cDNA was synthesized with SuperScript III First-Strand Synthesis System (Invitrogen) using 10 pmole of the JEV-MinusStr forward primer 5-GGTCAGGAACCACACTGACAGT-3. Afterward, cDNA was amplified using the primers Universal-JEV-F and Universal-JEV-R (500 nM of each) and Universal-JEV-Probe (250 nM) described above. An enzyme activation step of 2 min at 95°C was followed by 60 amplification cycles (10s at 95°C and 20 s at 60°C).
In all RNA extraction and PCR assays, I used VERO E6 cell culture media containing JEV as a positive PCR control. As a negative control, I used samples from non-manipulated control animals from previous studies [256,257]. Strict precautions were taken to prevent PCR contamination. Aerosol-resistant filter pipette tips and disposable gloves were always used. Kit reagent controls were included in every RNA extraction and PCR run.

### 3.5 Detection and quantification of infectious virus

I used the endpoint dilution assay in VERO E6 cells to isolate and quantify infectious titers in the JEV stock, blood plasma (maternal and fetal), nasal, and vaginal swabs (maternal) [256–261]. Fluids were serially diluted five-fold in four replicates starting from 1:10 in DMEM media (Thermo Fisher Scientific) supplemented with 5% FBS, a mixture of antibiotics (1,000 IU/ml penicillin and 1 mg/ml streptomycin, Gibco), and 2.25 g/l Sodium Bicarbonate (Thermo Fisher Scientific). Fifty μl of each dilution was added to confluent VERO E6 cells cultured in 96-well plates. After 2 hours of incubation at 37°C, 150 μl of fresh media was added to each well. The cells were incubated for seven days at 37°C. After washing and then drying for at least 4 h, the plates were kept at -20°C for at least 2 hours or until use. Anti-pan flavivirus E protein monoclonal antibodies clone D1-4G2-4-15 (ATCC; HB-112) [262] were used for immunohistochemistry staining [256–259,261] to detect JEV-infected cells. Fifty percent endpoint titers were calculated by the Spearman-Kärber formula and expressed in a decimal logarithm of a 50% infection dose for cell cultures (log_{10} TCID_{50}) per ml. Media from mock-inoculated cells were used as negative controls.
Maternal blood, nasal swabs, and vaginal swabs which were negative or caused cytotoxic effects on VERO E6 cells, were used to inoculate C6/36 cells for virus isolation. Cells in 96-well plates were inoculated with undiluted or 1:10 diluted blood plasma or swabs in MEM media supplemented with 10% FBS, 1,000 IU/ml penicillin, 1 mg/ml streptomycin, 1x Gentamicin/Amphotericin Solution (Thermo Fisher Scientific), and 2.25 g/l Sodium Bicarbonate. After 12 hours of incubation at 37 °C, fluids were removed, replaced with media, and cells were incubated for seven days at 28 °C. Afterward, plates were fixed and stained with D1-4G2-4-15 antibodies as described above.

To make 10% suspension for titration, maternal tissues were homogenized in media with TissueLyser II (QIAGEN) for 3 min at 20 Hz. Samples were centrifuged at 2,000g for 10 minutes, and after two hours of incubation on C6/36 cells at 37°C, 150 μl of fresh media was added to each well, and cells were incubated for seven days at 28°C. Afterward, plates were fixed and stained with D1-4G2-4-15 antibodies as described above.

3.6 In vitro infection phenotypes in human reproductive epithelial cells and trophoblast

I used human primary vaginal epithelial cells from two healthy deceased female donors: a 24-year-old African American donor (ATCC, PCS-480-010; Lot Number 80924222; 3rd passage) and an 18-year-old Caucasian donor (Lifeline Cell Technology FC-0083; Lot Number 04033; 3rd passage). Also, I used primary human endometrial epithelial cells from a healthy 13-year-old African American donor (Lifeline Cell Technology FC-0078; Lot Number 09953; 3rd passage). Information on the menstrual phase of human donors was not available, as the samples were de-identified. Cells were free of bacteria, yeast, fungi, mycoplasma, hepatitis B, hepatitis C, HIV-1,
and HIV-2, as confirmed by manufacturers with sterility tests and PCR. In JEV studies, three technical replicates were included for cells from each biological donor. Twenty-five thousand vaginal or endometrial epithelial cells were seeded in 96-well plates in appropriate media. The next day, cell monolayers were inoculated with JEV at MOI of 0.1 or 10 in 100 μl of the same media. Plates were incubated at +37°C for 2 hours. Afterward, cells were washed three times with sterile PBS and covered with 200 μl media. Mock-infected cells were included as controls in each plate. Infected plates were incubated (5% CO₂, +37°C) for 0, 3, 5, and 7 days when supernatants were collected, clarified (2,000 g, 5 min), and frozen (-80°C) for subsequent JEV load quantification. After the supernatant collection, plates with cell monolayer were dried for at least 4 hours and frozen (-80°C). Plates were stained with flavivirus-specific D1-4G2-4-15 antibodies as described above, and infected cells were visualized with a bright-field microscope. The same protocol was used to determine JEV infection kinetics in HTR-8/SVneo trophoblast cells (ATCC CRL-3271).

3.7 RNA-seq and bioinformatics

Primary vaginal epithelial cells from two human donors were seeded into 24-well plates with 10⁵ cells per well. On the following day, cells in four wells representing technical replicates were inoculated with MOI 10 of JEV prediluted in DMEM media, and four wells were mock-inoculated with virus-free media from VERO E6 cells prediluted in the same way. At 48 hours after inoculation, cells were homogenized in 1 ml of TRI Reagent Solution (Thermo Fisher Scientific), and RNA was extracted according to the manufacturer’s protocol. RNA was assessed on a bioanalyzer and all samples had RNA Integrity Number (RIN) values above 8.0. DNA from samples was removed with TURBO DNA-free Kit (Thermo Fisher Scientific). mRNA with
intact poly(A) tails were enriched with NEBNext Poly(A) mRNA Magnetic Isolation Module (New England Biolabs) and used for library construction with NEBNext Ultra II Directional RNA Library Prep Kit for Illumina and NEBNext Multiplex Oligos for Illumina (96 Unique Dual Index Primer Pairs; New England Biolabs).

Libraries were sequenced on the NextSeq as paired-end reads using the NextSeq 500/550 High Output Kit v2.5 (150 cycles) (Illumina). FASTQ files were trimmed for adaptor sequences and filtered for low-quality reads using Trimmomatic. On average, 22.1 million reads per sample were generated. RNA-seq analysis was performed as we previously described [256]. Briefly, a complete transcriptome database was generated from ENSEMBL Homo sapiens GRCh38.p13 (GCA_000001405.28). Sequencing data were mapped and quantified using kallisto [263]. Then counts were analyzed using R BioConductor packages tximport, edgeR and limma. The voom function from the limma package was used for differential expression analysis. Gene set enrichment analysis was performed with camera function in limma using the GMT file (version 7.5.1) containing symbols of gene sets derived from the Gene Ontology Biological Process Ontology of the Gene Set Enrichment Analysis (GSEA) Molecular Signatures Database (MSigDB).

The set enrichment results from camera were graphed in Cytoscape using the EnrichmentMap plugin [256,264]. All networks were generated using a Jaccard + Overlap with a cutoff of 0.375 and a Combined Constant of 0.5. Sub-networks were discovered using GLay cluster and annotated using the WordCloud plugin of the top 4 words with a bonus of 8 for word co-occurrence. An accession number for RNA-seq data is PRJNA823367 in NCBI BioProject.
3.8 Animal experiment

**Figure 3.1 A schematic diagram of the animal experiment.** Pigs at early pregnancy (30 days of pregnancy), pigs at mid pregnancy (54 days), pigs at late pregnancy (86 days), and non-pregnant pigs were inoculated intradermally and intravenously with $10^7$ TCID$_{50}$ of JEV. The duration of pregnancy in pigs is 114 days.
Eight female Landrace-cross pigs (gilts; on average 35-weeks-old) were purchased from the high-health status herd free from clinical symptoms caused by porcine reproductive and respiratory virus (PRRSV), porcine parvovirus (PPV), congenital porcine circovirus 2 (PCV2), and porcine circovirus 3 (PCV3), which can cause fetal infection in pigs. Accordingly, maternal and fetal samples were negative for PRRSV, PPV, PCV2, and PCV3 in virus-specific PCR assays [257,265]. Before delivering to containment, six pigs (A to F) were synchronized and bred with semen from a single donor to reduce biological variability; two pigs (G and H) remained non-pregnant. Insemination was scheduled to ensure that at the time of pig inoculation with JEV, three stages of pregnancy (the duration of pregnancy in pigs is 114 days) were represented: two pigs at early pregnancy (inoculation at 30 days of pregnancy; pigs A and B), two pigs at mid-pregnancy (54 days; C and D), and two pigs at late pregnancy (86 days; E and F) (Figure 3.1). The pregnancy was confirmed with ultrasound, and all pigs were delivered to Vaccine and Infectious Disease Organization, University of Saskatchewan biosafety level 3 containment facility. Animals were housed in two identical rooms in individual pens and had no contact with each other. After seven days of acclimatization in containment, all pigs were sedated and inoculated with $10^7$ TCID$_{50}$ of JEV intradermally (ear skin, 1 ml) + intravenous (ear vein, 1 ml); this inoculation dose and routes were previously used for JEV inoculation in young piglets [136,159]. Clinical signs, including appetite, activity, and rectal temperature, were recorded before and after JEV inoculation.

We collected blood from the jugular vein with BD Vacutainer Plastic Blood Collection EDTA tubes; nasal and vaginal swabs were also collected (Figure 3.1). Samples were collected before JEV inoculation and at 1-7, 14, 21, and 28 days after virus inoculation. After blood centrifugation
(2,000g, 20 min, +4 °C), plasma was aliquoted and frozen at -80°C. For nasal and vaginal swabs, swabs were inserted into the top of the nose or vagina and rotated to obtain secretions. Afterward, a swab was placed into a tube containing 500 µl sterile media, the handle was broken one centimeter from the top of the swab, and the tube was stored at -80°C.

Adult pigs were euthanized and sampled 28 days after JEV inoculation. In all pigs, I sampled and froze maternal tonsils, mesenteric lymph nodes, brains, nasal mucosa, and vaginal mucosa with individual sterile instruments. In two non-pregnant pigs, uterine walls with endometrium were sampled and frozen. In six pregnant pigs, uteri with fetuses were removed to sample each fetus (14-15 fetuses per pig) with individual sterile instruments. First, a uterine wall with the placenta was collected from each conceptus (a fetus with fetal membranes) and frozen. Second, umbilical cord blood was aspirated from each fetus with sterile syringes and needles, centrifuged, and plasma was aliquoted and frozen at -80°C. Finally, fetuses were removed, inspected for gross pathology, and whole fetal brains were collected and frozen.

3.9 Serology

I used an adapted virus-neutralizing assay to quantify JEV-neutralizing antibodies in maternal blood plasma [257,259]. Briefly, 50 µl of JEV (10⁴ TCID₅₀/ml) were mixed with equal volumes of two-fold serially diluted plasma (in two replicates) and incubated at +37°C for 1 h before inoculation in VERO E6 cells in 96-well plates. After 2 h, 100 µl/well of fresh DMEM supplemented with 1% FBS, 1x Penicillin-Streptomycin and 2.67 mM Sodium Bicarbonate were added. After 7 days, cells were fixed and stained with D1-4G2-4-15 antibodies as described
for virus titration. The neutralizing antibody titers were calculated as the highest plasma dilution inhibited JEV infection in 50% of the inoculated wells.

I also quantified JEV-specific IgG antibodies in maternal blood plasma with the immunoperoxidase monolayer assay (IPMA) [257,259]. Briefly, VERO E6 cells in 96-well cell culture plates were inoculated with 50 μl media containing 10^4 TCID_{50}/ml of JEV and incubated for 2 h (+37°C, 5% CO₂). Then 100 μl of the culture medium (DMEM supplemented with 5% FCS, 1x Penicillin/Streptomycin, 2.67 mM Sodium Bicarbonate) was added. After 7 days of incubation at +37 °C, 5% CO₂, the plates were dried for at least 4 hours and stored at -20 °C until use. Plates with cells were thawed, dried, and fixed in 10% buffered formalin for 1 hour, and washed twice with 1x DPBS (pH 7.2). Afterward, fixed cells were incubated with 100% methanol in the presence of 0.3% H₂O₂ for 10 min. Then plates were washed with DPBS, and two-fold serial dilutions of tested blood plasma were added, followed by 1 hour incubation at +37 °C. Plates were washed three times with DPBS containing 0.05% Tween-80 and 50 μl/well of rabbit anti-pig IgG (1:400, Abcam, ab136735) conjugated to horseradish peroxidase were added. After incubation for 1 hour at +37 °C and washing, a color reaction was initiated by adding substrate solution: 1 mM 3-amino-9-ethylcarbazole, 5% N,N-dimethylformamide, 50 mM Sodium Acetate (pH 5.0), and 10 mM H₂O₂ (H₂O₂ was added just before placing on cells). The reaction was stopped by replacing the substrate with an acetate buffer, and JEV-specific staining was determined by examination with a microscope. The titers were defined as the log reciprocal of the highest serum dilution. Blood plasma samples of mock-inoculated control animals from our previous studies were used as a negative control.
3.10 Interferon-alpha quantification

To quantify interferon-alpha (IFN-α), maternal and fetal blood plasma samples were diluted 1:2 and tested with Invitrogen Porcine IFN-alpha ELISA Kit (Thermo Fisher Scientific) according to the manufacturer’s instructions.

3.11 Immunohistochemistry

I used Mouse and Rabbit Specific HRP/DAB IHC Detection Kit - Micro-polymer (Abcam, ab236466) to identify JEV antigen in fetal brains. Fetal brain cryosections of 10 μm were fixed in 10% buffered formalin for 15 min at +4 °C. After treatment with 0.3% H₂O₂ and 1% Triton X-100 for 15 min and protein block for 10 minutes, tissue sections were washed in PBS and incubated with mouse monoclonal antibodies D1-4G2-4-15 (1:10) against flavivirus E protein for 1 hour at +37 °C. Afterward, the sections were washed and incubated with mouse specifying reagent, goat anti-rabbit HRP-conjugate, DAB chromogen, and DAB substrate according to the kit’s instructions. Subsequently, tissues were counterstained with hematoxylin and analyzed with a light microscope.

For JEV antigen identification in human reproductive epithelial cells and trophoblast, cells were fixed and stained with D1-4G2-4-15 antibodies as described above in the detection and quantification of infectious JEV.

3.12 Statistical analysis

I used GraphPad PRISM 8 software to analyze data. A p-value (p) < 0.05 was considered statistically significant. Viral loads and immunology variables were expressed as individual
values and mean ± standard deviation (M ± SD). Japanese encephalitis virus RNA loads and infectious titers in fetal blood plasma collected at euthanasia were compared between fetuses from early, mid, and late pregnancy subgroups using one-way ANOVA; Tukey’s multiple comparison test was used for multiple comparisons between fetuses of different subgroups. The same statistical tests were used to compare JEV RNA loads in the placenta and fetal brains. The number of fetuses with pathology was compared between subgroups with the Yates-corrected χ²-test. I used the Spearman correlation to evaluate relationships between fetal IFN-α levels in blood plasma and JEV loads. For maternal samples statistics were not done because of only two adult pigs per subgroup.
CHAPTER 4: RESULTS

4.1 JAPANESE ENCEPHALITIS VIRUS INFECTION AND MOLECULAR PATHOLOGY IN THE HUMAN VAGINAL EPITHELIUM AND IN VAGINAL TISSUES OF PIGS

4.1.1 Japanese encephalitis virus causes infection and molecular pathology in human vaginal epithelial cells

It is unknown whether JEV has tropism in the vaginal epithelium and the potential for sexual transmission in humans. Here, I assessed virus infection kinetics in human vaginal epithelial cells and transcriptional responses to infection.

I inoculated primary vaginal epithelial cells from two donors and quantified viral loads in the cell culture supernatant on different days. The virus caused infection with very high loads at 3, 5, and 7 days after inoculation (Figure 4.1A). The virus loads were similar in the vaginal epithelium of two donors and at different MOIs (Figure 4.1A). A productive persistent infection, at least for 7 days, was confirmed by immunohistochemistry specific for the JEV envelope protein (Figures 4.1B and 4.2). Interestingly, I observed the combination of virus-positive and virus-negative cells near each other at each sampling time (Figure 4.1B).
Figure 4.1 Japanese encephalitis virus causes persistent infection in human primary vaginal epithelial cells. (A) JEV infection kinetics in human primary vaginal epithelial cells. The dotted line is the detection limit. (B) Immunohistochemistry specific for the JEV envelope protein in human vaginal epithelial cells at 7 days after inoculation (MOI 10). The combination of virus-positive and virus-negative cells in close proximity was observed. The insert shows mock-inoculated cells.
Figure 4.2 Immunohistochemistry specific for the JEV envelope protein in primary human vaginal epithelial cells. Red staining shows JEV-positive cells. MOI: multiplicity of infection.

Next, to better understand molecular pathology induced by JEV infection in the vaginal epithelium, I quantified whole-genome expression in vaginal epithelial cells. Gene expression
differed considerably between control and JEV-infected cells with 1584 upregulated and 286 downregulated genes (FDR-adjusted p < 0.05; log₂ fold change (FC) > 1; Figure 4.3A; Supplementary Table 1A; the Supplementary Table 1A containing all raw gene counts and statistics can be downloaded from the Supporting information in my recent publication [266]). Among the top ten upregulated genes with 10-13 log₂ fold change were genes encoding interferon lambda 1, 2, 3 and interferon beta 1 (IFNL1, 2, 3 and IFNB1; Figure 4.3A). In accordance with previous findings where treatment of human vaginal epithelial cells with interferon lambda and beta induced a strong interferon-stimulated gene (ISG) response [242], I also found overexpression of 23 canonical ISGs (Figure 4.3A). In addition, among the top ten upregulated genes were genes encoding proinflammatory chemokines CCL5, CXCL10, and CXCL11, which recruit leukocytes to the site of inflammation [267,268]. Fifteen genes encoding gap junction and adhesion proteins were also affected (Figure 4.3A), suggesting changes related to epithelial barrier function. Accordingly, enrichment of Gene Ontology (GO) biological processes showed significant effects in the JEV-infected epithelium with 255 upregulated and 78 downregulated processes (FDR-adjusted p < 0.05; Figure 4.3B, Supplementary Table 1B, the Supplementary Table 1B containing all raw gene counts and statistics can be downloaded from the Supporting information in my recent publication [266]). Specifically, genes with altered expression were positively enriched for processes related to type I, II, and III interferon responses (12 GO processes FDR-adjusted p < 0.05), inflammatory responses (10 GO processes), and virus-host interactions (5 GO processes) (Figure 4.3B; Supplementary Table 1B). Interestingly, “negative regulation of morphogenesis of an epithelium” and “negative regulation of bicellular tight junction assembly” GO processes were also enriched.
Figure 4.3 Cellular transcriptional responses during JEV infection in human primary vaginal epithelial cells. (A) Cellular transcriptional responses in human primary vaginal epithelial cells during JEV infection. Plots of the upregulated (red) and downregulated (blue) genes. All affected genes, top 10 affected genes, interferon-stimulated genes, and genes encoding gap junction and adhesion proteins with FDR-adjusted p < 0.05 and log2 fold change (FC) > 1 are shown. (B) Molecular pathology network in human primary vaginal epithelial cells during JEV infection. An enrichment map of significantly altered GO biological processes is shown. Red are pathways with positive and blue are with negative enrichment. All subnetworks with FDR-adjusted p < 0.05 and at least three connected nodes are shown.
In accordance with infection kinetics and molecular pathology, JEV caused cytopathology in vaginal epithelial cells with more prominent cell rounding and detachment compared to control cells (Figure 4.4). Cell death started at 3 days after inoculation, increased at 5 and 7 days after inoculation, and was dose-dependent. In conformity with immunohistochemistry where the combination of virus-positive and virus-negative cells was observed (Figure 4.1), the
combination of dead and live cells was also observed even at 7 days after inoculation (Figure 4.4).

In summary, I discovered that JEV causes persistent infection in the human vaginal epithelium and therefore might have the potential for sexual transmission in humans. Also, RNA-seq analysis in human vaginal epithelial cells infected with JEV provides evidence for molecular pathology that leads to inflammation and disruption of epithelial barrier function. In support, the vaginal epithelium showed progressive cytopathology during JEV infection.

4.1.2 Japanese encephalitis virus persists in vaginal tissues of the native amplifying host
To further explore the potential of JEV for sexual transmission, I studied virus persistence and shedding in vaginal tissues of the native amplifying host—pigs.

One non-pregnant pig showed elevated body temperature 39.3-39.4 °C (the baseline temperature was 38.4-38.5 °C) and decreased appetite at 3-4 days after JEV inoculation. Other animals did not show clinical signs. As previously demonstrated in young piglets [136], adult pigs also had JEV RNAemia (Figure 4.5A), shedding in nasal secretions (Figure 4.5A), and virus-specific antibody responses (Figure 4.6B). As a host response to infection, during RNAemia and JEV shedding in body fluids, pigs showed decreased IFN-α concentrations (Figure 4.6C). Afterward, on days 14, 21, and 28, during JEV clearance in blood and body fluids, IFN-α concentrations were close to or exceeded the initial base concentrations.

Interestingly, in contrast to a previous study in young piglets [136], I discovered that adult pigs shed JEV RNA in vaginal secretions. Non-pregnant pigs shed JEV within 3-7 days after inoculation except on day 4. Pregnant pigs continually shed virus RNA within 3-7 days, and
even at 14 days after inoculation (Figure 4.5A). Virus loads in non-pregnant pigs peaked at 4 log<sub>10</sub> RNA copies/ml. In pregnant pigs, JEV loads peaked at 6 log<sub>10</sub> RNA copies/ml (Figure 4.5A). Vaginal mucosa sampled at 28 days after inoculation was also positive for JEV RNA in two pigs (Figure 4.12G).

**Figure 4.5** Japanese encephalitis virus loads in blood, nasal secretions, and vaginal secretions in non-pregnant and pregnant pigs. (A) JEV RNA loads determined by virus-specific RT-qPCR in blood plasma, nasal swabs, and vaginal swabs. The dotted line is the detection limit. Columns represent mean values with standard deviations. (B) VERO E6 cells inoculated with blood plasma collected from a pig before JEV infection. (C) JEV-positive staining (red) in VERO E6 cells inoculated with blood plasma collected from a mid-pregnancy pig two days after JEV inoculation. In accordance with the highest viral RNA loads identified in pigs from the mid pregnancy subgroup at 2-3 days after inoculation, I isolated infectious JEV in VERO cells at the same time points. (D) Cytotoxicity in VERO E6 cells inoculated with maternal blood plasma. Isolation and titration of infectious flaviviruses from samples of immunocompetent mammalians is challenging, most probably because of insufficient sensitivity of cellular assays [244,246,261,269–271]. Most PCR-positive plasma samples did not show infectious JEV, indicating infectious titers below the detection limit of the VERO-based endpoint dilution assay. In addition, maternal blood plasma samples, even after 1:10 dilution, caused cytotoxicity. Similarly, inoculation of mosquito C6/36 cells, which can be more sensitive than VERO cells for flavivirus isolation [259], with undiluted or 1:10 diluted blood plasma led to strong cytotoxicity preventing interpretation of results. Nasal
and vaginal swabs with initial sampling dilution caused cytotoxicity in VERO and C6/36 cells that halted the assay, and with subsequent higher dilutions, I did not isolate infectious JEV.

![Figure 4.6 Antibody and IFN-α responses in blood plasma of adult pigs.](image)

**Figure 4.6 Antibody and IFN-α responses in blood plasma of adult pigs.** Neutralizing (A) and IgG virus-binding (B) antibody titers in non-pregnant and pregnant pigs. (C) Individual IFN-α concentrations in the blood plasma of non-pregnant and all pregnant pigs. Dots represent individual pigs. The dotted line is the detection limit. Columns represent mean values with standard deviations. **Ab**: Antibodies. **IPMA**: Immunoperoxidase monolayer assay.

Altogether, I confirmed previous findings where pigs had JEV RNAemia and shedding in nasal secretions [136] and I discovered that pigs shed JEV in vaginal secretions. Viral RNA was detected in vaginal secretions 2-10 days after it was cleared from blood plasma suggesting local and prolonged JEV replication in vaginal tissues.
4.2 JAPANESE ENCEPHALITIS VIRUS INFECTION IN THE HUMAN ENDOMETRIAL EPITHELIUM AND TROPHOBLAST, AND IN THE ENDOMETRIUM AND FETAL IMPLANTATION SITES OF PIGS

4.2.1 Japanese encephalitis virus causes infection and cytopathology in human endometrial epithelial cells and trophoblast

While clinical outcomes of transplacental JEV infection are described in humans [60], cellular targets in the upper reproductive tract are unknown. To better understand JEV pathogenesis during transplacental infection, I assessed virus infection kinetics in human endometrial epithelial cells and trophoblast.

In primary endometrial epithelial cells, JEV kinetics depended on the initial inoculation dose. At MOI 0.1, high viral loads in the supernatant and virus-positive cells were detected only in one well replicate at 5 days after inoculation (Figure 4.7A). In contrast, at MOI 10, high viral loads and virus-positive cells were detected from 3 to 7 days (Figures 4.7A-B and 4.8). I did not observe distinct cytopathology in infected cells; both control and infected epithelium had detached rounded cells (Figure 4.9).

In human trophoblast, JEV kinetics also depended on the initial inoculation dose. At MOI 0.1, JEV loads in supernatants gradually increased from 3 to 7 days after inoculation (Figure 4.7C). At MOI 10, high viral loads were detected at 3, 5, and 7 days after inoculation (Figure 4.7C). Immunohistochemistry showed highly intensive staining with many virus-positive cells on all sampling days for MOI 0.1 and 10 (Figures 4.7D and 4.10). In cells inoculated with MOI 10, gradually aggravating cytopathology with focal and diffuse cell detachment and disruption of the
cell monolayer was observed from 3 to 7 days after inoculation. However, at MOI 0.1, I did not observe distinct cytopathology (Figure 4.11).

Figure 4.7 Japanese encephalitis virus causes persistent infection in human primary endometrial epithelial cells and trophoblast. (A) JEV infection kinetics in human primary endometrial epithelial cells. (B) Immunohistochemistry specific for the JEV envelope protein in human primary endometrial epithelial cells at 3 days after inoculation (MOI 10). The insert shows mock-inoculated cells. (C) JEV infection kinetics in human trophoblast—HTR-8/SVneo cells. (D) Immunohistochemistry specific for the JEV envelope protein in human trophoblast at 7 days after inoculation (MOI 10). The insert shows mock-inoculated cells. The dotted line on graphs represents the limit of detection.
Figure 4.8 Immunohistochemistry specific for the JEV envelope protein in primary human endometrial epithelial cells. Red staining shows JEV-positive cells. MOI: multiplicity of infection.
Figure 4.9 Cytopathic effect in primary human endometrial epithelial cells infected with JEV. MOI: multiplicity of infection.
Figure 4.10 Immunohistochemistry specific for the JEV envelope protein in human trophoblast. Red staining shows JEV-positive cells. MOI: multiplicity of infection.
Figure 4.11 Cytopathic effect in human endometrial trophoblast infected with JEV. MOI: multiplicity of infection. Arrowheads show focal trophoblast detachment, diffuse trophoblast detachment, and disrupted trophoblast monolayer.

In summary, I discovered that JEV causes infection and cytopathology in the human endometrial epithelium and trophoblast, possibly contributing to transplacental JEV transmission.
4.2.2 Japanese encephalitis virus persists in the endometrium and fetal implantation sites of native amplifying hosts and causes transplacental infection

To better understand the pathogenesis of JEV transplacental transmission, I studied infection in the maternal-fetal interface dissected from the uterus after sampling/freezing (for convenience, I call it placenta in the text below) and fetuses of the native amplifying host—pigs.

As previously demonstrated in young piglets [136,158], adult pigs also had JEV persistence in tonsils, lymph nodes, brain, and nasal mucosa collected 28 days after inoculation (Figure 4.12). I also identified JEV persistence in one endometrial tissue sample out of two tested (Figure 4.12G; the endometrium from two non-pregnant pigs was tested). Virus persistence in the endometrium may lead to infection in the adjacent placenta and afterward in fetuses. Indeed, I found high JEV RNA loads in the placenta from nearly all (pig A, early pregnancy) or all (pig C, mid pregnancy) fetuses (Figure 4.13A). Accordingly, fetuses from pig A and pig C had high JEV RNA loads in their blood plasma as determined by PCR (Figure 4.13B). Two fetuses from pig A and six from pig C also had high infectious JEV titers in blood plasma as determined by virus isolation and titration (Figure 4.13C). Infectious JEV titers were significantly higher in fetuses from pig C inoculated at mid pregnancy than from pig A (p=0.01) inoculated at early pregnancy (Figure 4.13C). Only one pregnant pig (50%) in early and mid-pregnancy subgroups showed transplacental and fetal infections suggesting the stochastic nature of JEV transmission from dam to fetuses or unknown factors that may affect the virus spread through the placenta.
Figure 4.12 Japanese encephalitis virus persists in different porcine tissues of dams sampled at 28 days after inoculation. (A) JEV RNA loads determined in tonsils by virus-specific RT-qPCR. (B) JEV negative-strand RNA PCR values in tonsils. (C) Infectious JEV titers in tonsils determined by the endpoint dilution assay in C6/36 cells. (D) Mock-inoculated control C6/36 cells with no staining; (E-F) JEV-positive staining (red) in C6/36 cells inoculated with tonsils collected from an early pregnancy pig. (G) JEV RNA loads determined by virus-specific RT-qPCR in lymphoid, nervous (in the brain, replicate tissues collected from two anatomical locations—frontal and occipital lobes—were tested in each pig), respiratory, and reproductive tissues. JEV negative-strand RNA was identified in mesenteric lymph nodes. In all graphs, dots represent individual pigs. The dotted line (except in JEV negative-strand RNA PCR) is the detection limit. Columns represent mean values with standard deviations. NA: not available.

Fetuses from pigs A and C also had JEV RNA in their brains (Figure 4.13D). Three fetuses from pig A inoculated at early pregnancy had JEV RNA in their brains. Twelve out of thirteen fetuses (92.3%) from pig C inoculated at mid pregnancy had JEV RNA in their brains with high titers ranging from 4.6 to 11.7 log\textsubscript{10} RNA copies/g (Figure 4.13D). Virus RNA loads in brains were significantly higher (p=0.001) in fetuses from pig C inoculated at mid pregnancy than in fetuses from pig A inoculated at early pregnancy. I also sectioned selected frozen fetal brains from pig A (early pregnancy) and pig C (mid pregnancy), and stained tissues with antibodies specific to the JEV envelope protein. In accordance with extremely high viral loads in the brain of fetus #10

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(11.7 $\log_{10}$ RNA copies/g) from pig A, its brain contained intensive diffuse JEV-specific staining (Figure 4.13E). However, I did not find JEV antigen in the brain of fetus #1 that had almost twice lower JEV loads (6.8 $\log_{10}$ RNA copies/g), suggesting more focal antigen localization or insufficient sensitivity of the immunohistochemistry assay. Fetuses #6 (11.7 $\log_{10}$ RNA copies/g) and #14 (11.4 $\log_{10}$ RNA copies/g) from pig C also had intensive JEV-specific staining in brain tissues.

Figure 4.13 Japanese encephalitis virus infection in fetuses. (A) JEV RNA loads in the placenta. JEV RNA loads (B) and infectious titers (C) in fetal blood. (D) JEV RNA loads in fetal brains. (E) JEV-specific immunohistochemistry in the brain from a virus-negative fetus (insert) and in the brain from the fetus positive for JEV; brown staining represents cells positive for the JEV envelope protein. (F) IFN-α concentrations in fetal blood plasma. Control blood plasma samples were from fetuses of two healthy pigs sampled at 78 days of pregnancy in previous studies [257]. Dots represent individual pigs or fetuses. The dotted line is the detection limit—36 pg/ml. In fetuses, the short horizontal line represents mean values. In all graphs, dots represent individual fetuses. The dotted line is the detection limit. The short horizontal line represents mean values. *: JEV loads were significantly different.
In accordance with JEV infection in fetuses, pigs A and B had one fetus with pathology (7.1% and 6.3% of the total number of fetuses) (Figure 4.14A); specifically, fetuses had edema and hemorrhages (Figure 4.15). Pigs C and D inoculated at mid pregnancy had four and one fetuses (23.5% and 6.3%) with edema, tissue autolysis, and mummification (Figures 4.14A and 4.15). Pigs E and F sampled at late pregnancy did not have fetuses with visible pathology; only one (6.3%) mummified fetus was found in pig F.

A fetus from pig A inoculated at early pregnancy, and many fetuses from pig C inoculated at mid pregnancy had brain lesions (Figure 4.14B). Fetuses with and without gross pathology had brain lesions (Table 3, Figure 4.14) suggesting the fetal brain is a primary and vulnerable target for JEV. The fetus from pig A (early pregnancy) had focal brain hemorrhage (Figure 4.14 C-D). Eight fetuses from pig C (mid pregnancy) had complete brain autolysis, partial brain autolysis, diffuse brain hemorrhages, and focal brain hemorrhages with less defined sulcus and gyrus structures (Figure 4.14 E-L). Fetuses from other pigs did not have brain lesions. The number of fetuses with brain lesions was significantly higher in the mid pregnancy subgroup than in early pregnancy (p < 0.016) and late pregnancy (p < 0.0039) subgroups. These data obtained from only one pregnant pig with transplacental infection per subgroup, and experiments with the larger number of animals are needed to validate the data.
Figure 4.14 Fetal brain pathology. (A) The number of fetuses with gross pathology. (B) The number of fetuses with brain pathology. *: The number of fetuses with brain lesions was significantly higher in the mid pregnancy subgroup than in early pregnancy (p < 0.016) and late pregnancy (p < 0.0039) subgroups. The fetal brain with no lesions (C) and focal hemorrhage (D) from early pregnancy pig A. Mid pregnancy pig C: The fetal brain with no lesions (E and I). (F) The fetal skull with the completely autolyzed brain. (J) The fetal brain with complete autolysis. (G and K) The fetal brain with partial autolysis and diffuse hemorrhages. (H and L) The fetal brain with focal hemorrhages and less defined sulcus and gyrus structures.
Figure 4.15 Fetal gross pathology. (A) A fetus with no gross pathology from early pregnancy pig A. (B) A fetus with edema from early pregnancy pig A. (C) A fetus with mild edema from mid-pregnancy pig C. (D) A fetus with severe pathology from mid-pregnancy pig C.

 Previous studies from our and other groups suggest that type I interferon in utero responses contribute to fetal pathology and death during congenital viral infections [256,260,261,272]. In the present study, two JEV-positive fetuses from early pregnancy pig A showed increased IFN-α levels (Figure 4.13F). In mid pregnancy, nine out of 13 fetuses of pig C showed increased IFN-α levels (Figure 4.13F); accordingly, interferon levels in blood plasma positively correlated with JEV loads in blood plasma (p=0.019, r=0.68).
Collectively, I showed that JEV persists in the endometrium of non-pregnant pigs for at least 28 days. Also, I discovered high virus loads in the placenta that allowed the virus to cross the placental barrier causing severe lesions in the fetal brain, fetal interferon-mediated immunopathology, and fetal death.
CHAPTER 5: DISCUSSION AND FUTURE DIRECTIONS

I studied the potential of JEV for sexual transmission in humans. I also studied infection in endometrial and placental cells to better understand the pathogenesis of transplacental JEV transmission. For the sexual transmission potential, I discovered that JEV causes persistent infection in the human vaginal epithelium leading to altered expression of genes related to inflammation and epithelial barrier function. And that pigs—the native amplifying host for JEV—shed the virus in vaginal secretions. For transplacental transmission, I discovered that JEV causes infection and cytopathology in the human endometrial epithelium and trophoblast. And that JEV persists in the endometrium and placenta of pigs with subsequent transplacental infection and fetal death.

There are emerging concerns about sexual flavivirus transmission. These concerns emerged during the recent Zika epidemic where many cases of sexual transmission in humans have been reported including male-to-male, male-to-female, and female-to-male transmissions [235–237]. Zika virus has been detected in vaginal secretions [239–241] replicating in human vaginal epithelial cells [242]. However, it was unknown whether JEV—another flavivirus related to Zika virus, also has tropism for the human reproductive epithelium and the potential for sexual transmission. This knowledge is important because almost a half of the world’s population lives in territories where JEV is permanently circulating with an estimated 68,000 cases reported annually [273]. Because many JEV-infected humans do not develop apparent clinical signs, sexual JEV transmission may be underestimated. Similarly, Zika virus sexual transmission in sustaining the virus in the human population during the epidemic was most probably underestimated [274].
I showed that JEV causes infection in the human vaginal epithelium with high loads for at least 7 days (Figure 4.2). Transcriptional responses identified in human vaginal epithelial cells during JEV infection showed evidence of potential inflammation, altered bicellular junctions, and disruption of epithelial barrier function (Figure 4.3). The vaginal epithelium is the first line of defense against sexually transmitted infections in women and the disrupted epithelial barrier may facilitate the sexual transmission of JEV as well as other pathogens, for example human immunodeficiency virus 1 and chlamydia. High JEV titers and intensive antigen staining in infected cells persisted for at least 7 days despite type I and III interferon transcriptional responses, including interferon lambda—an antiviral cytokine that functions at barrier surfaces [242,275,276]. Interestingly, at both low (MOI 0.1) and high (MOI 10) inoculation doses and at each sampling time point, I observed many virus-positive cells with intensive red staining and virus-negative cells tightly adjacent to each other (Figures 4.1B and 4.2). I hypothesize that antiviral responses in JEV-infected epithelial cells will differ from responses in uninfected bystander cells. Thus, it will be interesting to assess transcriptional responses in infected and virus-free bystander epithelial cells to identify reasons for such phenotypical heterogeneity and better understand mechanisms employed by flaviviruses to subvert epithelial immunity.

Next, I discovered that non-pregnant and pregnant pigs shed JEV in vaginal secretions collected by swabs (Figure 4.5). I also found virus persistence in vaginal mucosa for at least 28 days (Figure 4.12). These findings are unexpected because in a previous study the authors did not find JEV in vaginal secretions [136]; however, the vaginal swabs were collected in young, sexually immature, 7-week-old piglets (pigs reach sexual maturity at 20-24 weeks). Here, JEV-inoculated pigs were on average 35-weeks-old. Thus, age and probably age-dependent hormonal
changes determine JEV tropism in vaginal tissues and vaginal shedding. In support, hormonal changes in mice and non-human primates affect Zika virus replication in the female reproductive tract and transgenital transmission to internal organs [242–244,247].

Interestingly, infected pigs had considerably longer JEV shedding with higher titers in vaginal secretions than in nasal secretions (Figure 4.5) supporting the previous suggestion that the dampened innate response to RNA viruses in the lower female reproductive tract is an exceptional feature of the vaginal mucosa which may not extend to other mucosal surfaces [277].

Vaginal JEV shedding and persistence in vaginal mucosa were detected after the virus was cleared from blood plasma, suggesting local JEV replication in reproductive tissues (Figure 4.5). Isolation and titration of infectious flaviviruses from samples of immunocompetent mammalians is challenging [261,269–271], and similar to the previous Zika studies in non-human primates [244,246], I did not recover infectious JEV in vaginal tissues. Additional studies are needed to identify whether the JEV RNA that persists in vaginal tissues imposes the potential for virus sexual transmission. Interestingly, it has been reported that Zika virus preferentially replicates in the female reproductive tract of non-human primates after vaginal inoculation but not after peripheral inoculation [244]. The data from the present study with peripheral inoculation and a previous JEV study where pigs showed systemic infection after artificial insemination with virus-positive semen [278] suggest that JEV has strong tropism to female reproductive tissues independently of the route of infection.
My study also contributed new knowledge for JEV pathogenesis during transplacental infection. I discovered dose-dependent JEV infection in the primary human endometrial epithelium (Figure 4.7A-B). The endometrial epithelium is the vital maternal point of first contact for an embryo during implantation and early development [279,280]. Thus, it will be important to identify whether silent JEV infection may impact interactions between embryo and endometrial epithelial cells and evoke early embryonic loss. Also, JEV persistence in the endometrium may lead to infection in the adjacent placenta and afterward in fetuses. Indeed, I discovered aggressive JEV infection and cytopathology in the trophoblast (Figures 4.7C-D, 4.10 and 4.11). The trophoblast is a specialized epithelium vital for placental function in supporting fetal development [281]. Virus replication in the trophoblast is the primary step toward fetal infection. Human HTR-8/SVneo cells used in the present study were derived from chorionic villi explants of the human first-trimester placenta. Aggressive JEV infection in the early HTR-8/SVneo trophoblast supports previous clinical findings in humans where JEV infection in at least five pregnant women was described during an extensive outbreak in India. In addition, abortions occurred after infection with JEV early in pregnancy, while newborns of women infected near term did not show obvious congenital pathology [60].

Supporting the role of the pregnancy stage in infection phenotypes in reproductive tissues, JEV crossed the placental barrier in pigs in only early and mid-pregnancy (Figure 4.13) and did not cause placental and fetal infections in late pregnancy. My findings of JEV infection in the porcine placenta and fetal brains at early pregnancy are consistent with a historical study where pigs infected at early pregnancy delivered dead or abnormal piglets [252]. However, the new finding of transplacental infection at mid gestation with a higher number of infected and
damaged fetuses (Figures 4.14) suggests a broader period of susceptibility. More efficient transplacental spread at earlier pregnancy stages may be the common feature of different flaviviruses in mammalian species because the discovered JEV phenotype is similar to the known Zika phenotype where the virus more readily crosses the placental barrier at early pregnancy in humans and experimental animals [275,282]. The limitation in the present study is that all pig subgroups representing different pregnancy stages had only two animals, and I could not increase the sample size in this study. Comparative experiments with larger animal groups are needed to confirm and better understand the kinetics of transplacental JEV infection at different stages of pregnancy.

To summarize, there are emerging concerns about vector-free sexual and transplacental flavivirus transmission, which may change flavivirus epidemiology and expand the geographical range to territories with no insect vectors. Japanese encephalitis virus is an emerging and geographically expanding flavivirus with a history of transplacental infections in humans. Here, I discovered that JEV persists in the vaginal epithelium and has the potential for sexual transmission in humans. I also contributed to a better understanding of JEV pathogenesis during transplacental infection and showed cellular targets and persistence in the endometrium and placenta. Further studies are needed to better understand the interactions of JEV with reproductive tissues, how persistent infection affects female reproductive functions, and the risks for sexual and transplacental transmission.
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APPENDIX

A. Publication during my study


Subash Chapagain made major contributions to the study design, practical experiments, data analysis, and manuscript preparation.

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B. Presentations given during my studies

Subash Chapagain. Japanese encephalitis virus persists in reproductive tissues of amplifying and dead-end hosts. Graduate Student Poster Day, Western College of Veterinary Medicine, University of Saskatchewan Canada, March 24, 2022. Poster presentation