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Review

Flavivirus infection—A review of immunopathogenesis, immunological response, and immunodiagnosis

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ABSTRACT

Flaviviruses are group of single stranded RNA viruses that cause severe endemic infection and epidemics on a global scale. It presents a significant health impact worldwide and the viruses have the potential to emerge and outbreak in a non-endemic geographical region. Effective vaccines for prophylaxis are only available for several flaviviruses such as Yellow Fever virus, Tick-borne Encephalitis Virus, Dengue Virus and Japanese Encephalitis Virus and there is no antiflaviviral agent being marketed. This review discusses the flavivirus genome, replication cycle, epidemiology, clinical presentation and pathogenesis upon infection. Effective humoral response is critical to confer protective immunity against flaviviruses. Hence, we have also highlighted the immune responses elicited upon infection, various diagnostic facilities available for flaviviral disease and monoclonal antibodies available to date against flavivirus infection.

1. Introduction

The Flavivirus genus belongs to the family *Flaviviridae* which is comprised of several different flavivirus members. All flaviviruses are important human pathogens capable of generating high morbidity and mortality rates. As of 2015, dengue virus (DENV,) West-Nile virus (WNV) and Japanese Encephalitis virus (JEV) account for most of the recorded flavivirus infections (Daep et al., 2014). In addition, the Zika virus (ZIKV) has recently emerged as one of the most prevalent flaviviruses resulting in a significant increase in ZIKV infections since the time the first virus prototype was discovered in 1947 (Weaver et al., 2016). Flaviviral infections may be asymptomatic or can progress into serious illness such as hemorrhagic fever, meningoencephalitis and neurological complications.

According to WHO, DENV is endemic in more than 100 countries. The virus is found globally throughout tropical and sub-tropical regions especially in South-East Asia and the Western Pacific (Chaturvedi and Nagar, 2008). Two-fifths of the world's population are at risk of developing dengue infection and a 30-fold increase of dengue cases have been reported over the past 50 years (Kyle and Harris, 2008). Dengue infection can lead to dengue hemorrhagic fever (DHF) or dengue shock syndrome (DSS) with 20,000 fatalities recorded annually (Webster et al., 2009). JEV is endemic in South-East Asia and the Western Pacific with an estimate of 68,000 cases reported annually. Although the endemic regions of both JEV and DENV overlap, they are transmitted by different mosquitoes species involving different amplifying hosts (Gould and Solomon, 2008). Japanese encephalitis (JE) is the main form of viral encephalitis in Asia. JE may present as either asymptomatic or symptomatic infections with a 20–30% fatality rate (Solomon, 2004). WNV was initially isolated in 1937 in Uganda (Chancey et al., 2015). From the 1950s to 1970s, WNV caused infrequent outbreaks associated with febrile illness (Narat, 2003). To date, WNV is predominantly reported in Africa, Asia, Europe and Australia. However in 1999, WNV caused a massive outbreak in New York and rapidly spread over North-America to Central-America and finally to South-America (Gubler, 2007) while in 2003, WNV infection peaked with 9862 cases and 264 deaths being recorded in the US (CDC statistic). As such, it is recognized that WNV is capable of emerging in new and unaffected geographical regions (Heinz and Stiasny, 2012). ZIKV which was previously considered insignificant in Africa and Asia recorded an epidemic in 2007 on the Yap Islands, Micronesia and Gabon followed by an unexpectedly massive outbreak in Latin America in 2016 (Duffy et al., 2009; Grard et al., 2014; Colón-González et al., 2017). It was since declared as a global health emergency as ZIKV infection has been linked to severe neurological diseases such as Gullian-Barré Syndrome (GBS) and congenital Zika syndrome (Baud et al., 2017). According to WHO, approximately 4 million ZIKV infection have been recorded in America alone in 2016 (Samarasekera and Triunfol, 2016). Since then,

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ZIKV is spreading rapidly with outbreaks occurring in French Polynesia, South America, Africa and Asia (Ioos et al., 2014). More recently, ZIKV infections were also reported in Southeast Asia and Africa (Heinz and Stiasny, 2017).

Considering the disease severities of DENV, WNV, JEV and ZIKV flavivirus infections coupled with high infection occurrences which are expected to increase anually, this review aims to provide a comprehensive and updated overview of these particular flavivirus infections. The genomic organization, epidemiologies, clinical presentations and molecular pathogenesis of these highly prevalent flaviviruses will be described succinctly with the hope to provide researchers in the field with up-to-date information on these flaviviruses and their associated diseases. In addition, the current understanding of the adaptive and innate immune responses towards flaviviral infections will also be discussed. Current developments of various immunodiagnostic and immunotherapeutic platforms to detect and treat these highly prevalent flaviviral infections are further described.

2. Flavivirus genomic organization

All flaviviruses are enveloped viruses with a positive sense (+) ssRNA genome size of approximately 10–11 kb. The RNA genome of a flavivirus virion consists of a long open reading frame (ORF) flanked by 5' and 3' untranslated regions with a cap at 5'-end of the genome (Fig. 1). The single ORF is translated into a polyprotein comprising of three structural proteins (C, prM and E protein) and seven non-structural proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B and NS5) (Tijssen, 1985). The structural and non-structural proteins play major roles in virion assembly, cell receptor binding and entry, viral polyprotein processing and viral replication. These are summarized in Table 1.

2.1. Flavivirus replication and transmission cycle

2.1.1. Flavivirus replication

Flaviviruses infect monocytes, macrophages and dendritic cells (Marianneau et al., 1999; Tassaneetrithep et al., 2003; Krishnan et al., 2007). The first stage of infection involves virus attachment to the cell surface followed by cellular entry of the virus through receptor-mediated endocytosis. The low pH of the endosomal compartment triggers the fusion of the virus particle and the host cell membrane, leading to the release of nucleocapsid and viral RNA genome into the host cell's cytoplasm. The viral RNA replicates in the rough endoplasmic reticulum (ER) and the double membrane vesicle packets (VP) derived from the Golgi-apparatus (Mackenzie, 2005). The non-structural protein and double stranded RNA are concentrated in the VP, constituting the site for viral RNA synthesis (Westaway et al., 1999, 1997). The newly synthesized RNA is exported to the intermembrane space of the VP and subsequently exits into cytoplasm (Uchil and Satchidanandam, 2003). This is followed by viral assembly in the rough ER. The newly synthesized genome is packaged by a viral capsid protein (C) which surrounded by a lipid bilayer embedding E (envelope) and prM (precursor of membrane) proteins (Khromykh and Westaway, 1996). During virion assembly, the E protein complexes with prM proteins in the ER to form prM-E heterotrimeric complexes which are embedded into the lipid bilayer that envelopes the nucleocapsid to form an immature virus particle (Mukhopadhyay et al., 2005). The immature virions are spiky, measuring 60 nm in diameter (Lindenbach et al., 2013). The immature virions then bud off from the lumen of the rough ER and transported to the trans-Golgi network (TGN) for maturation process to take place (Mackenzie and Westaway, 2001). The acidic pH in TGN induces conformational changes of the immature particles, leading to reorganization of the envelope protein and cleavage of prM into pr and M by the cellular protease furin (Yu et al., 2008; Stadler et al., 1997). Under acidic conditions, the cleaved pr fragment remains associated with the virions in the TGN but dissociate at the neutral pH of the extracellular environment, generating a mature and infectious virus particle. At this stage, the E protein has adopted a metastable conformation before being released by exocytosis (Heinz and Stiasny, 2017). Subviral particles (SVPs) lacking the viral genome and capsid while consisting of only the lipid membrane with bound prM-E complexes are produced as by-products during the virion assembly process (Wang et al., 2009). The SVPs are transported, processed and released from the ER as whole, non-infectious virion (Mazeaud et al., 2018).

2.1.2. Flavivirus transmission cycle

Mosquitoes are the most important vectors associated with flavivirus infections. Conditions promoting mosquito breeding significantly increase the risk of flavivirus infections (e.g. agricultural irrigation, flooding). The virus is maintained in an enzootic cycle between mosquitoes as the principal vector while mammals and avians are amplifying hosts (Pandit and Doyle, 2018). Mosquitoes acquire the virus during a blood meal of an infected host. The virus replicates in the

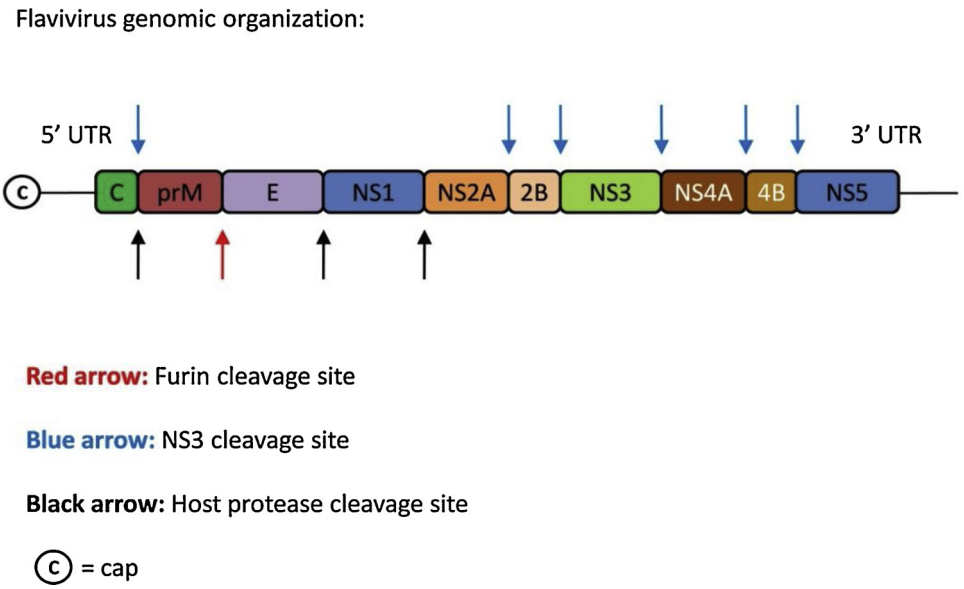

Fig. 1. The genomic organization of flavivirus with three structural protein; C, prM and E followed by seven non-structural protein in the order of NS1, NS2A, NS2B, NS3, NS4A, NS4B and NS5.

Table 1
Summary of the currently up-to-date and known functions of flaviviral structural and non-structural proteins.

Structural proteins		
Protein	Function	Reference
C	capsid associating with RNA genome	(Murray et al., 2008)
prM (pr and M)	protects E protein from pH-induced conformational changes in immature virion	(Heinz et al., 1994; Wang et al., 1999)
E	cell receptor binding and entry	(Murray et al., 2008; Wang et al., 1999; Zhang et al., 2017)

Non-structural proteins		
Protein	Function	Reference
NS1	virus genome replication, immune system evasion, may facilitate antibody-dependent enhancement (ADE) of infection	(Akey et al., 2014; Falconar, 2008)
NS2A	Component of the replicase complex, virion assembly, immune system modulation and evasion	(Murray et al., 2008; Xie et al., 2015; Tu et al., 2012)
NS2B	Cofactor for NS3 protease	(Murray et al., 2008)
NS3	Protease responsible for polyprotein cleavage, multifunctional enzyme involved in viral genome replication (helicase,NTPase)	(Murray et al., 2008; Bollati et al., 2010)
NS4A	NS3 cofactor, component of the replicase complex	(Murray et al., 2008; Zou et al., 2015)
NS4B	component of the replicase complex, immune system modulation and evasion	(Zou et al., 2015; Zmurko et al., 2015; Muñoz-Jordán et al., 2005)
NS5	component of the replicase complex, RNA-dependent RNA polymerase (RdRp) for viral genome synthesis, RNA methyltransferase (MTase), immune system modulation and evasion	(Murray et al., 2008; Bollati et al., 2010; Lin et al., 2006)

amplifying host and is incidentally transmitted to humans. Humans are often regarded as dead-end hosts such as in the case of WNV infections where low level viremia in the body prevents the virus from being transmitted to another host (Colpitts et al., 2012). However, this presumption is not always true in cases of DENV and ZIKV infections or WNV infections of immunocompromised individuals (Vicenzi et al., 2018; Bowen and Nemeth, 2007).

Although mosquitoes remain as the principal vector for in the transmission cycle, other routes of transmission have been reported. For instance, WNV can be transmitted among humans through blood transfusions, organ transplants or transplacental transmission to the newborn (Iwamoto et al., 2003; Alpert et al., 2003; Control, 2002) whereas oral transmission of WNV has been demonstrated in hamster, birds and mice (Komar et al., 2003; Odelola and Oduye, 1977; Sbrana et al., 2005). Human-to-human DENV transmissions via blood transfusions have been reported (Sabino et al., 2016; Slavov et al., 2019). JEV can be transmitted transplacentally from and infected mother to the fetus in the first and second trimester (Chaturvedi et al., 1980) and in infected pregnant mice (Mathur et al., 1982). Seminal transmission of JEV in pigs resulting in abortion of the embryo has also been documented (Guerin and Pozzi, 2005). Similarly, ZIKV has been shown to persist in bodily fluids implicating a route of horizontal transmission (Paz-Bailey et al., 2018).

3. Clinical presentations and pathogenesis of flaviviral infections

3.1. Dengue virus (DENV)

The four well-identified dengue virus serotypes are DENV-1, DENV-2, DENV-3 and DENV-4. Infection with one dengue serotype provides lifelong immunity to the particular virus but does not confer immunity to the virus of different serotype. Hence, humans living in DENV endemic regions can be infected with all four different serotypes in their lifetime (Gubler, 1988). *Aedes aegypti* is the principal vector in the enzootic transmission cycle of DENV (Gubler, 1998). After an infectious mosquitoes bite, the virus undergoes an incubation period of 3–14 days. Thereafter, the infected person experiences an onset of fever. This initial phase, also known as the febrile phase typically presents with symptoms such as high fever ($> 38.5\,^{\circ}C$), headache, vomiting, myalgia and joint pain. Thrombocytopenia, leucopenia and moderate elevation of hepatic aminotransferase levels are commonly noted. The febrile phase typically lasts for 3–7 days and patients generally recover without any complications.

In some cases, dengue fever may progress into dengue hemorrhagic fever (DHF). DHF can occur in both children and adults. Patients with DHF develop systemic vascular leakage syndrome with increased hemoconcentration, hypoproteinemia, pleural effusion and ascites accumulation (Simmons et al., 2012). In response to vascular leakage, physiological feedback mechanisms responsible for maintaining adequate circulation to critical organs are upregulated. This narrows the pulse pressure and when the pulse pressure reaches a minimum of 20 mm Hg or less with signs of peripheral vascular collapse being observed, dengue shock syndrome is diagnosed (Simmons et al., 2012). At this stage, patients may appear deceptively well with a normal systolic pressure however, once hypotension develops, the systolic pressure may drop rapidly, causes irreversible shock and ultimately death. Hence, during the transition of febrile to critical phase (between day 4 and day 7), it is important for clinicians to monitor for any significant vascular leakage, persistent vomiting, increased hematocrit levels and mucosal bleeding. In children, significant bleeding is usually only observed in patients with profound and prolonged shock. In contrast, major skin bleeding or mucosal bleeding may occur in adults with only minor plasma leakage. Moderate to severe thrombocytopenia, coagulation disorders, vascular changes are commonly observed in the critical phase. Patients may have disseminated intravascular coagulation as shown by concomitant thrombocytopenia, an increase in partial thromboplastin time and decrease in fibrinogen level (Simmons et al., 2012). The altered vascular permeability persists for approximately 48–72 hours and reverts to its normal state followed by rapid improvement of symptoms.

During the recovery phase, mild maculopapular rash to severe itchy lesions may appear, suggesting leukocytoclastic vasculitis that resolves over 1–2 weeks. Autopies revealed that the central nervous system (CNS) of dengue-infected patients undergo neuropathological changes including edema, vascular congestion, perivascular lymphocytic infiltration. Neuronal abnormalities such as acidophilic neurons or cytoplasm shrinkage were also reported (Bhamarapravati et al., 1967; Chimelli et al., 1990). Infiltration of DENV-positive macrophages in the gray matter and white matter was also reported. In addition, DENV antigens were detected in neurons, astrocytes, microglia, endothelial, perivascular cells while brain tissue samples were found to be positive for viral RNA by RT-PCR (Nogueira et al., 2002).

Epidemiologic studies revealed that young age, female sex, high body-mass index, virus strain and genetic variants of human MHC class

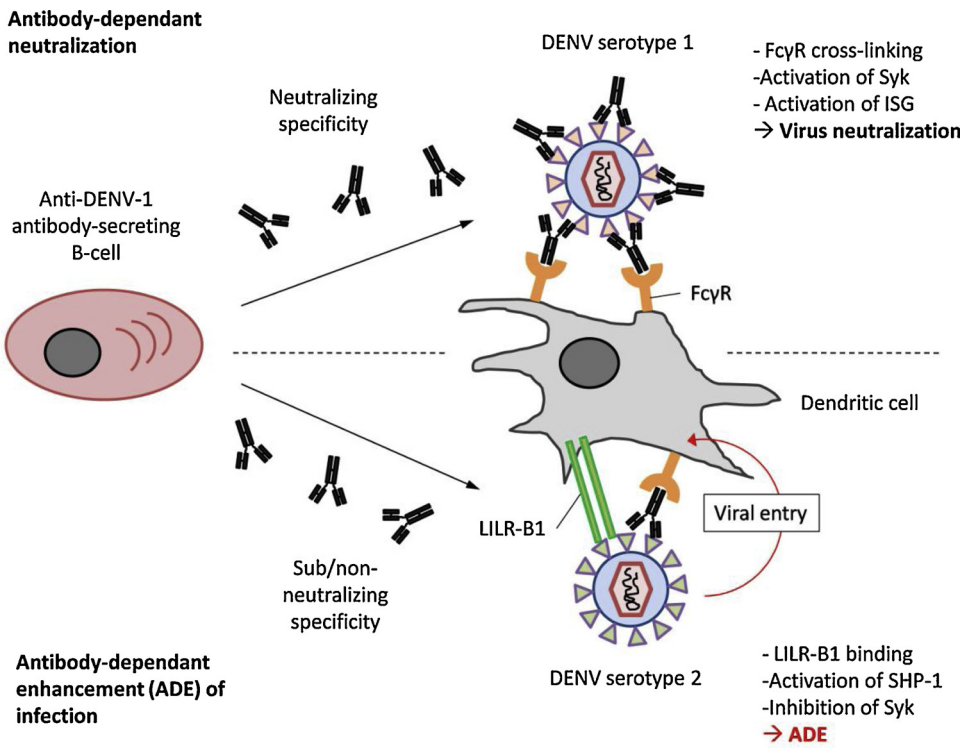

Fig. 2. The mechanisms of antibody-dependant neutralization and enhancement of infection (ADE). Using DENV infection as an established example, a B-cell producing highly specific antibodies towards DENV serotype 1 may produce sub/non-neutralizing antibodies towards DENV serotype 2. The result is inhibition/reduction of FcγR cross-linking to activate the antiviral response via activation of spleen tyrosine kinase (Syk)/IFN-stimulated genes (ISG). This is represented in the bottom-half of the figure; reduced FcγR cross-linking is mediated through DENV-LILR-B1 binding which inhibits Syk via dephosphorylation by Src-homology phosphatase-1 (SHP-1) resulting in ADE entry and infection of the dendritic cell.

I-related sequence B and phospholipase C epsilon 1 genes are the risk factors correlated to severe dengue disease (Khor et al., 2011; Anders et al., 2011). Secondary infection with heterologous dengue serotypes is also a known risk for developing severe dengue disease (Halstead, 1988). In secondary infection, pre-existing dengue antibodies with sub-neutralizing specificities form antigen-antibody complexes with the infecting virus. These immunocomplexes bind to the immunoglobulin Fc-gamma receptor (FcγR) on the cell membrane of the macrophage or dendritic cells via the antibodies' Fc region. Due to the sub-neutralizing nature of the antibodies, FcγR-binding does not result in receptor cross-linking to initiate the antiviral response. Instead the virus particle is brought closer to the cell membrane allowing binding to leukocyte immunoglobulin-like receptor B1 (LILR-B1); recently implicated in antibody-dependent enhancement (ADE) by downregulation of IFN-stimulated genes (ISG) (Fig. 2) (Chan et al., 2014). ADE results in enhanced internalization of the virus and allows the virus to replicate in the host cell. In response to dengue infection, cells secrete vasoactive mediators, causing increased vascular permeability leading to hypovolemic shock (Ohlson et al., 1997; Halstead and O'rourke, 1977; Morens et al., 1987).

It has been suggested that dengue virus entry into the CNS is mediated by cytokine-mediated breakdown of the blood-brain barrier (Miagostovich et al., 1997; Chaturvedi et al., 1991). Infection with a single dengue serotype induces both serotype-specific and serotype cross-reactive dengue virus-specific memory of $CD4^+$ $CD8^-$ and $CD4^-$ $CD8^+$ T lymphocytes (Kurane et al., 2011). $CD4^+$ T lymphocytes secrete cytokines such as gamma interferon (IFN-γ), IL-2, IL-4, IL-5, IL-6, IL-10, and TNF-β (Azeredo et al., 2001; Gagnon et al., 2002). Macrophages infected by dengue virus produce TNF-α, IL-1, IL1B, IL-8, IL-12 and platelet-activating factor (PAF) (Chaturvedi et al., 2000; Chen and Wang, 2002; Yang et al., 1995). In addition, altered immunomodulation of the complement pathway particularly through increased levels of the complement factors C3a, C4a and C5a increase the vascular permeability in DHF and DSS (Nascimento et al., 2009). Collectively, cytokine cascade activation coupled with altered immunomodulation synergistically enhance their effects resulting in increased vascular permeability (Kurane, 1997).

3.2. Japanese Encephalitis Virus (JEV)

JEV is the primary cause of mosquito-borne encephalitis affects the pediatric group and non-immune adults. Symptomatic infections initially present with mild febrile illness such as headache, high fever, nausea, vomiting, Parkinsonian movement disorders and muscle aches. These may eventually progress into multifocal CNS pathologies such as encephalitis, meningitis and poliomyelitis-like paralysis after the incubation period of 5–15 days (Lundqvist et al., 2006). Neurological manifestations depend on which part of the nervous system is infected; meninges (meningitis), parenchyma of brain (encephalitis) and spinal cord (myelitis) (Solomon and Vaughn, 2002). JE has a mortality rate of ~25% and 50% of surviving patients suffer from permanent neurological disorders while 25% recover fully from the disease (Leighton et al., 2015).

Although the precise mechanisms of JEV entry into CNS remain unclear, three major routes of virus entry into CNS have been documented. These include direct entry across the blood-brain barrier, entry via the peripheral nervous system and entry via leukocytes (King et al., 2007). Upon transmission, the virus subsequently escapes the bloodstream and infects the neuronal cells. JEV has shown to develop a particular tropism towards growing neurons and neuroprogenitor cells as evidenced by severity of JEV infection and their outcome in children (Ogata et al., 1991); an observation correlated to growth stage-specific membranous vimentin expression (Shen et al., 2014). The virus amplifies peripherally in lymph nodes and dermal tissues, causing transient viremia before crossing the blood-brain barrier to enter the CNS. It has been suggested that passive transfer of JEV across endothelial cells, transcellular transport and infected monocytes could be the possible routes for crossing the blood-brain barrier into the CNS (German et al., 2006; Myint et al., 1999; Liu et al., 2008). An EM study indicated that JEV particles bind to the endothelial surface and were internalized, trancystosed and transported via the endocytic vesicles to the parenchyma side of the blood-brain barrier (Liou and Hsu, 1998). Chances of JEV neuroinvasion also increase with meningitis, head injuries or neurocysticercosis co-infections. JEV neuroinvasion is followed by infiltration of inflammatory cells and phagocytosis of infected cells (Singh

et al., 2004; Klaus and Mitchell, 1974).

Following JEV infection, secreted proinflammatory cytokines and chemokines cause neuronal injury and trigger host defenses against JE. Microglia and astrocytes are first activated. This is followed by secretion of chemotactic cytokines which attract inflammatory cells. The presence of cytokines such as IFN-α, IFN-β and IFN-γ activate the transcription of host IFN-inducible genes and lead to induction of intracellular antiviral pathways upon binding to specific receptors on the surface of infected cells (Leighton et al., 2015). IFN-α is important in the activation of monocytes, enhancement of chemokine expression and MHC class I and MHC class II induction (Lee et al., 2016). Antiviral activity induced by IFN-α is mediated by nitric oxide radicals which are synthesized by monocytic phagocytes. It was also observed that inhibition of nitric oxide synthase led to increased mortality of JEV-infected mice (Nagase et al., 1983). Moreover, microglia and JEV-infected leukocytes have been identified as possible viral reservoirs responsible in the pathogenesis of subacute and chronic infection as well as development of neurological disorders following JEV infection (Thongtan et al., 2010; Yang et al., 2004).

A number of studies have been performed to further understand and identify specific viral determinants that govern neuroinvasiveness and neurovirulence of JEV. These studies demonstrated that the area within the lateral surface of domain III of E protein plays a prominent role in cellular receptor binding whereas the base of domain II of E protein is responsible for fusion with the target cells (McMinn, 1997; Lee et al., 2004; Lefranc, 2001). C and prM structural proteins have also shown to be involved in neurovirulence as indicated by numerous studies in which mutations in the viral capsid and premembrane proteins drastically decreased the viral neurovirulence in mice models and mammalian cells *in vitro* (Kim et al., 2008; Mori et al., 2005). Also, the production of JEV NS1' protein due to ribosomal frame-shift mutations in members of the JE-serocomplex was shown to play a role in viral neuroinvasiveness (Melian et al., 2010).

3.3. West Nile Virus

West Nile Virus (WNV) is a member of the JE-serocomplex and is a major cause of arboviral encephalitis. It is estimated that 80% of WNV infections are asymptomatic whereas symptomatic infections may vary from the development of West Nile fever (WNF), flu-like malaise to severe neurological disorders (Stils, 2005). Among the severe forms of WNV disease, 50–71% develop WN encephalitis, 15–35% develop meningitis, and 3–19% develop acute flaccid paralysis (AFP) (Narat, 2003; Lindsey et al., 2010). Approximately 3–19% of severe WN encephalitis cases are fatal while in survivors, the encephalitis-associated physical and mental impairments generally resolve within a year (Loeb et al., 2008). Persistent WNV symptoms of more than 6 months were largely reported in patients with West Nile neuroinvasive disease (WNND), hypertension and diabetes (Cook et al., 2010). WNND is also a risk factor for development of chronic kidney disease as reported in a long term follow-up study of WNV patients (Nolan et al., 2012). Elderly and immunocompromised groups were reported to be more susceptible to WNV infection (Nielsen and Marks, 2004). While the three main clinical syndromes are meningitis, encephalitis and AFP, neuromuscular weakness is also reported as one of the symptoms in 50% of WNND patients (Nielsen and Marks, 2004). Patients with AFP typically present with monoplegia, generalized asymmetric tetraplegia or quadriplegia. For patients who demonstrate AFP symptoms, the involvement of one or more cranial nerves necessitate intubation or artificial ventilation due to respiratory failure (Kramer et al., 2007). Patients with WNV encephalitis without focal neurological deficits often recover fully (Nielsen and Marks, 2004). The relapsing form of AFP and long term persistence of WNV as evidenced from viral shedding in urine was also reported (Benhar, 2007).

Immunohistochemical studies revealed that WNV has a specific viral predilection for gray matter areas of the brainstem such as medulla, spinal cord, cerebellum, temporal lobes, basal ganglia and thalamus (Sampson and Armbrustmacher, 2001; Shieh et al., 2000). The virus primarily targets pyramidal motor neurons of anterior horns and cerebellar Purkinje cells and occasionally, astroglial and monocytic cells (Guarner et al., 2004; Ge et al., 2018). Similar to JEV, WNV enters the CNS by crossing the blood-brain barrier. Increasing the permeability of the blood-brain barrier could facilitate viral entry into the CNS. It was reported that the induction of TLR3-mediated release of TNF-α, macrophage migration inhibitory factor (MIF), intracellular adhesion molecule-1 (ICAM-1) and matrix metallopeptidase 9 (MMP9) could increase the brain endothelial capillary permeability (Arjona et al., 2007; Dai et al., 2008; Wang et al., 2008). Following this, the virus crosses the blood-brain barrier via transcellular transport, paracellular transport, direct infection of endothelial cells or retrograde axonal transport through peripheral motor nerves (Samuel et al., 2007).

Upon entry into the CNS, WNV selectively infects anterior horn neurons. Once infected, bystander damage occurs due to immunopathological effects of the CD8 + T-cell response, recruitment of inflammatory monocytes and apoptosis of the neural cells (King et al., 2007; Getts et al., 2008). Just like JEV, E protein is responsible in the neuroinvasiveness and neurovirulence of WNV (Beasley et al., 2004; Shirato et al., 2004). Genetic mutations in the C-C chemokine receptor type 5 (CCR5) and 2–5 oligoadenylate synthetase (OAS) gene as well as T-cell defects in both CD4 and CD8 increase the host susceptibility towards severe and neuroinvasive WNV infection (Brien et al., 2009).

3.4. Zika virus

ZIKV infection was considered insignificant with sporadic cases affecting a small number of the world's population (Plourde and Bloch, 2016). Eighty percent of ZIKV infections are asymptomatic but ZIKV infection can also present with a broad range of clinical symptoms (Brandon and Adams, 2015). The incubation period of ZIKV is approximately 3–12 days. Thereafter, ZIKV-infected patients will present with symptoms such as arthralgia, edema, mild fever, headaches, conjunctivitis, vertigo and myalgia. The recent epidemic showed that ZIKV infection has been associated with serious clinical outcomes such as multi-organ failure, thrombocytopenia, thrombocytopenic purpura (Swaminathan et al., 2016; Karimi et al., 2016). ZIKV causes uveitis and conjunctivitis in adults due to direct infection of the eyes (Sun et al., 2016; Furtado et al., 2016). However, the greatest concern is that ZIKV infection has been linked to more severe neurological disorders such as Gullian-Barré Syndrome, microcephaly, meningitis, meningoencephalitis and congenital CNS malformations (Simmins, 2016; Munoz et al., 2016). A plausible explanation for causal association between CNS anomalies and ZIKV is that the virus hijacks the autophagy machinery during viral replication (Tetro, 2016). Cellular proteins aim to maintain the normal number of centrosomes for brain development (Theodoridis et al., 2002). It was hence postulated that the interference of ZIKV in autophagy leads to an increase in chromosome number which results in microcephaly in mice.

In ZIKV-infected pregnant cases, congenital ZIKV infection such as cerebral calcifications, microcephaly, intrauterine growth restriction and fetal demise were reported (Brasil et al., 2016). Magnetic resonance imaging revealed that the brain of infected neonates demonstrated cerebellum and brainstem hypoplasia, ventriculomegaly, delayed myelination, enlarged cisterna magna, corpus callosum abnormalities, calcifications, and cortical malformations (Aragao et al., 2016). As ZIKV preferentially infects progenitor cells, it injures the neural progenitor cells and impairs the neurodevelopment of the brain (Tang et al., 2016). Studies have shown that direct intraventricular inoculation of the brain of a wild type mouse fetus with ZIKV has resulted in cortical infection and thinning, inhibition of neural progenitor cell differentiation and microcephaly (Li et al., 2016). Since the study was conducted in mice, the findings confirmed the ability of ZIKV to cause cell death and cerebral cortex disease in animal models. In another study performed by

Huang et al., direct intracranial inoculation of ZIKV in postnatal mice resulted in depletion of proliferating cells in the ventricular zone of the stem cell compartment and disruption of the corticospinal pyramidal neurons (Huang et al., 2016). Similarly, *in vitro* human neurosphere organoid cultures infected with ZIKV showed alterations in cell division and induction of cell death (Garcez et al., 2016). Taken together, direct infection of neural progenitor cells could be the sole factor for ZIKV-induced microcephaly disease.

In humans, ZIKV RNA was detected in both maternal and fetal tissues including cord blood, placental cells, amniotic fluid as well as in developing fetal and neonatal brains (Bhatnagar et al., 2017). *in vitro* infection studies on placental cells revealed that ZIKV replicates in placental macrophages, trophoblasts and fetal endothelial cells and expression of antiviral genes were simultaneously induced (Quicke et al., 2016; Tabata et al., 2016). However, the vulnerability of placental cells to ZIKV infection depends on gestational age and genetic variations in host factors (Wang et al., 2015).

The precise mechanism for the entry of ZIKV into humans, however, is poorly understood. One of the factors suggested for ZIKV entry into humans is the membrane protein, AXL. AXL is a member of the Tyro3 Axl Mer (TAM) family; a group of tyrosine kinase receptors which are responsible for clearance of apoptotic cells and regulation of innate immunity (Lemke and Rothlin, 2008; Rothlin et al., 2007). Merteens et al., showed that AXL is expressed in human microglial and astrocytes and mediates the ZIKV infection of glial cells (Meertens et al., 2012). ZIKV enters the glial cells through Gas6 ligands. Gas6 recognizes and interacts with the phosphatidylserine exposed on the surface of the viral envelope and AXL on the surface of the cell, thereby bridging the viral particle to the AXL receptor (Meertens et al., 2012). After fusion, ZIKV is internalized by clathrin-mediated endocytosis and is trafficked to the endosome to initiate infection. During viral entry, AXL kinase activity was activated by the ZIKV/Gas6 complex (Meertens et al., 2017). This negatively regulates the type I interferon signalling and facilitates infection (Meertens et al., 2017). Hence, AXL has dual functions during ZIKV infection; first, it promotes viral entry and second, it modulates the innate immune response (Moussavou et al., 2015).

4. Response to flavivirus infection of the infected cell

The earliest host cell responses against flavivirus infection involve secretion of type I interferons (IFN) which comprise of IFN-α and IFN-β (Thurmond et al., 2018). Interferons are cytokines with anti-proliferative, antiviral and immunomodulatory properties. They are the main components in the innate immune system against viral infection (Haller et al., 2006). All the secreted type I interferons bind to type I IFN receptors found on the surface of human cells. The type I receptor is formed from two subunits, IFNAR1 and IFNAR2, which are associated with Janus-activated kinases (JAKs), tyrosine kinase 2 (TYK2) and JAK1. The binding of the type I IFNs to the type I IFN receptor causes rapid autophosphorylation and activation of the receptors associated to JAKs TYK2 and JAK1 (Kotenko et al., 2003; Silvennoinen et al., 1993) which in turn results in phosphorylation and activation of STATs (Darnell et al., 1994). After phosphoryation by JAKs, the activated STATs form homodimers/heterodimers which translocate to the nucleus and then associate with interferon regulatory factor 9 (IRF9). The hexotrimeric complex is formed, and interferon stimulated gene factor 3 (ISGF3) binds to the *cis*-acting interferon stimulated response element (ISRE), resulting in the transcription of IFN-inducible genes including interferon stimulated genes (ISG), the double-stranded-RNA (dsRNA)-activated protein kinase (PKR) and 2′,5′-OAS ultimately leading to induction of antiviral pathways.

It was reported that mice deficient in type I IFN or type I IFN receptors showed an increased mortality rate upon challenge with MVEV and WNV (Samuel and Diamond, 2005; Lobigs et al., 2003). In addition, PKR and 2′,5′-OAS are capable of inhibiting WNV replication (Kajaste-Rudnitski et al., 2006). PKR inhibits both viral and host cell protein synthesis upon activation by replicated viral dsRNA via inhibition of eIF2α which is responsible for translation initiation ultimately leading to apoptosis of the infected cell (Langland et al., 2006). 2′,5′-OAS is responsible for the production of unusual multimeric oligoadenylate nucleic acids (known as 2-5A) which then activate RNAse L leading to degradation of viral and host RNA (Silverman, 2007).

With the induction of antiviral pathways, flaviviruses are still capable of proliferating, suggesting that there are virus-specific mechanisms in which the induction of IFN pathways can be altered, inhibited or bypassed. For example, NS2A of WNV interacts with STAT1 and STAT2 to prevent its phoshorylation, translocation to the nucleus and subsequent transcription of downstream antiviral target genes (Liu et al., 2006). Tu et al. also showed that A549 cells induced to co-express a JEV NS2A construct had significantly reduced progression into the apoptotic stage upon stimulation with polyinosic:polycytidilic acid (poly I.C.) (Tu et al., 2012). Further investigations on the mechanism of reduced apoptosis showed that JEV NS2A could bind directly with PKR which may prevent PKR-mediated apoptosis (Tu et al., 2012). Also, IFN-α showed reduced antiviral activity during JE infection *in vitro* due to the presence of NS5 that blocks activation of TYK2, thereby preventing the subsequent phosphorylation of STAT1 (Lin et al., 2006). Consistently, flaviviral NS proteins seem to be the main antagonists of the IFN antiviral pathways (Tu et al., 2012; Muñoz-Jordán et al., 2005; Lin et al., 2006). DENV and ZIKV have also shown to bypass the PKR-eIF2α-induced global translational blockade by hijacking the alternative translational pathway involving eIF4 dedicated for the translation of the cellular stress response genes during infection (Roth et al., 2017).

It should be noted however, that IFN-induced cellular stress responses have been observed to be inconsistent such as in the case of ZIKV infections. Factors such as the infecting ZIKV strain of a specific cell type may result in upregulation or inhibition IFN antiviral pathways resulting in reduced or increased viral replication respectively (Colavita et al., 2018) (See ZIKV subsection). Fig. 3 summarizes IFN-induction pathway during flavivirus infection.

4.1. Innate immunological response to flavivirus infection

For the innate immune response, microglial and astrocytes in the CNS respond to the flavivirus infection. Microglial are resident macrophages in the CNS, and express TLRs which are responsible in pathogen recognition, processing and presentation of antigen to the T cells. Astrocytes on the other hand are important trophic support cells in the CNS, express TLRs and secrete cytokines as an antiviral response in the CNS (Carpentier et al., 2005). Microglial and astrocyte activation during flavivirus infection is common in mouse models and human tissues infected with JEV or WNV (King et al., 2007). Microglial and astrocytes upregulate chemokine production such as CCL2 and CCL5 (Chen et al., 2000). These secreted chemokines in turn stimulate other leukocytes in the brain in an autocrine manner. This results in cellular migration and secretion of other potentially neurotoxic mediators.

In response to TNF secretion, chemokines are often secreted during the early phase of infection. For example, in mice models infected with MVEV, TNF stimulation of astrocytes and microglial triggers the production and secretion of neutrophil-attracting chemokine N51/KC (Andrews et al., 1999). The expression of adhesion molecules coupled with the secreted chemokines cause a large influx of neutrophils into the CNS. Large numbers of activated neutrophils produce high levels of nitrogen oxide which results in oxidative bystander damage. While chemokines play an important role in immunopathogenesis during the course of flavivirus infection, chemokines are also important in non-pathological immune-mediated viral clearance. For example, CCR5 is important for the survival of WNV-infected mice as WNV-infected CCR5-deficient mice have shown enhanced brain viral loads with reduced infiltration of T cells, macrophages and NK cells into the CNS resulting in increased mortality (Glass et al., 2005).

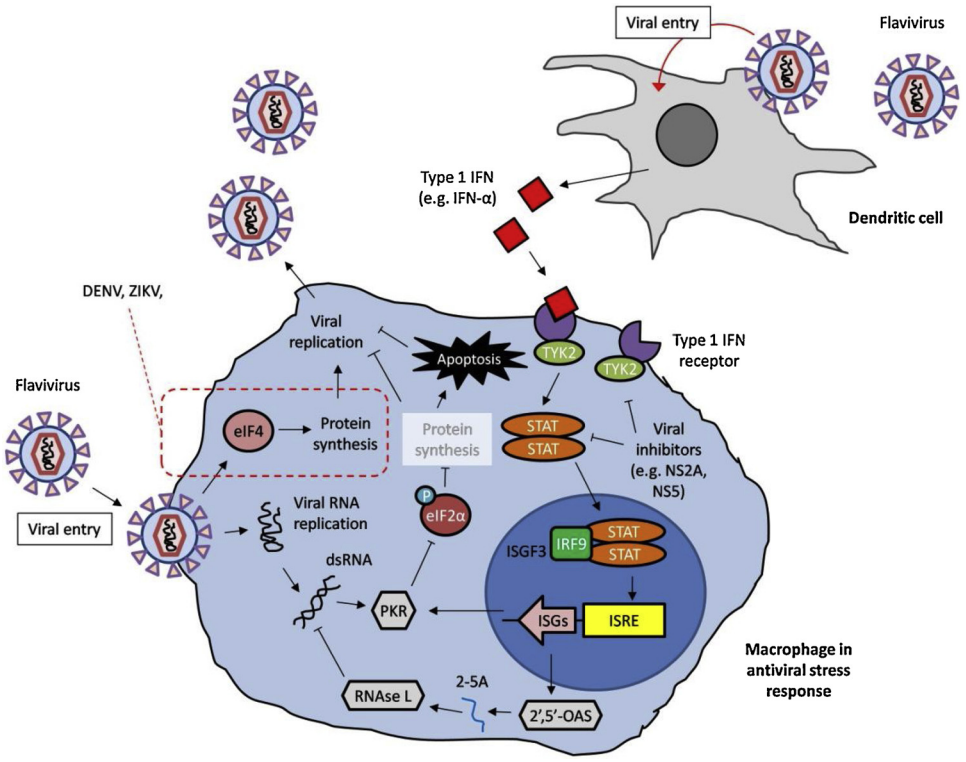

Fig. 3. Graphical representation of a typical flavivirus-induced IFN antiviral stress response. Infection of a dendritic cell triggers the release of Type 1 IFNs (e.g. IFNα) which bind to Type 1 IFN receptors on a macrophage which in turn triggers the IFN-induced antiviral signal cascade in the cell. Upregulation of ISGs result in expression of key components of the antiviral response (e.g. PKR, 2′,5′-OAS). If the macrophage is infected, PKR and 2′,5′-OAS are activated to inhibit viral replication via a few pathways. The dotted red box shows how flaviviruses such as DENV and ZIKV bypass the antiviral mechanisms in an infected cell. Solid lined arrows = inducing interactions, solid T lines = inhibiting interactions.

4.2. Adaptive immunological response to flavivirus infection

Flaviviruses induce both the humoral and cellular immune response for protection against flavivirus infection. In humoral immunity, neutralizing antibodies are produced in response to the invasion of flavivirus into host cell. Neutralizing antibodies primarily target epitopes located on the E glycoprotein, although antibodies specific against prM and non-structural (NS) protein have also been reported (Vázquez et al., 2002; Falconar, 1999; Chung et al., 2007; Mayo et al., 2009; Stettler et al., 2016). These antibodies inhibit viral attachment, internalization, or replication within cells. As E protein is primarily involved in the interaction of the virus with the host cell to initiate infection, multiple researches have been conducted to isolate antibodies against the infective E protein. Of these, more than 12 distinct epitopes were identified on the surface of E protein that are capable of eliciting antibodies with different degrees of neutralization potency (Oliphant et al., 2005; Crill and Chang, 2004; Roehrig et al., 1998; Heinz et al., 1983). The crystal structure of E protein showed that E protein was folded into three antigenic domains; domain I, domain II and domain III (Kolaskar and Kulkarni-Kale, 1999). Various potent neutralizing antibodies characterized to date target the upper lateral surface of domain III (DIII) which protrudes off the surface of the virion (Beasley and Barrett, 2002; Mackenzie et al., 2002).

The non-structural 1 (NS1) protein which is another common target against flavivirus infection is found to be highly conserved among flaviviruses and is detected at high levels in serum during flavivirus infection (Young et al., 2000). As NS proteins are not incorporated into the virion, antibodies specific for NS protein do not directly neutralize the virus infectivity. Instead, they protect the host via other effector mechanisms. Anti-NS1 antibodies elicit protection to the host via Fc-gamma receptors and/or complement activation pathways depending on the NS1 regions at which a particular antibody is reactive to (Chung et al., 2006).

It is important to note that different flaviviruses induce different humoral responses in the host. For instance, WNV-infected mice lacking functional B-cells showed an increase in disease severity whereas dengue-infected mice lacking functional B-cells showed no change in disease severity or increase in viral load (Shresta et al., 2004). Although antibodies display potent neutralizing abilities against flavivirus infection *in vivo* and *in vitro*, antibodies in the humoral response could also result in ADE of infection as mentioned earlier. ADE is not only limited to a particular flavivirus with different serotypes. *in vitro* studies showed that cross-reactive anti-DENV-antibodies can enhance ZIKV infection and vice versa (Charles and Christofferson, 2016; Priyamvada et al., 2016). This is attributed to the highly conserved nature among the members of flaviviruses, therefore, antibodies produced against these flaviviruses can cross-react with one another (Melian et al., 2010). In some circumstances, it was reported that passive transfer of antibodies caused a significant increase in viral titer in animal models infected with DENV and WNV (Goncalvez et al., 2007; Halstead, 1979). Rapid progression of the disease was also observed in mice models infected with JEV and MVEV (Wallace et al., 2003; Broom et al., 2000). Other mechanisms described include more efficient post-attachment steps in the viral replication following Fc-gamma receptor mediated signalling, direct alteration in the viral fusion process, delivery of antibody-virus complex to a more favourable location in the endocytic compartment and antibody-dependent release of chemokines, cytokines, nitric oxide and other mediators that modulates viral replication (Chareonsirisuthigul et al., 2007; Suhrbier and La Linn, 2003).

Apart from the humoral immune response, flavivirus infections also induce cellular immunity in the infected host, e.g: T-cell responses. WNV infection induces the production of CCL5 in the CNS, which subsequently recruits CCR5-expressing T cells and macrophages to the CNS in mice models and humans (Lin et al., 2008; Glass et al., 2006). In some studies, it was demonstrated that T cells play a major role in viral clearance of flavivirus infections. In WNV-infected mice, the virus-specific $CD8^+$ T cells proliferate in response to viral infection and migrate to the CNS to control viral replication through cytokine and lysis-dependent mechanisms (Brien et al., 2008) CXCL10 was produced in order to attract $CD8^+$ cells to the CNS. In regards to this, studies revealed that WNV-infected mice that lack $CD8^+$ T cells or lack CXCL10-CXCL3 interactions are less likely to survive the viral challenge as compared to wild-type mice (Klein et al., 2005; Shrestha and Diamond, 2004). Although T-cell responses are robust in viral load clearance, they

can be damaging to host. Mice lacking functional $CD8^+$ T-cells challenged with a low dose of WNV Sarafend strain showed increased mortality but survived longer than the wild-type mice, suggesting that $CD8^+$ T-cells have both protective and immunopathological responses in mice (Wang et al., 2003). In contrast, high doses of virus caused 100% mortality in wild-type mice with minimal cellular infiltration into the brain whereas $CD8^+$-deficient mice showed 80% of mortality with extended survival periods.

In summary, the immunity be it humoral or cellular immune response induced in response to flavivirus infection is complex. During dengue infection, mice that lack $CD4^+$ or $CD8^+$ T cells do not show altered disease phenotypes or an increase in viral load compared to wild-type mice but mice which lack T-cells and B-cells are more likely to develop dengue-virus induced disease (Cecilia and Gould, 1991). Humoral immune responses generated confer protective immunity to the host although in some cases it may enhance viral replication through ADE. The cellular immune response elicited by virus-specific $CD8^+$ T cells which migrate to the site of infection and control viral replication, in some instances however, cause damage to the infected tissue, particularly to the vulnerable CNS. Mortality rates of flavivirus-induced encephalitis are significant as a result of the inability of the immune response to control viral replication, viral infection and immunopathogeneis. Hence, survival from flavivirus infection is dependent on a balance between successful elimination of the virus (conferred by the robust adaptive immunity) and minimal tissue damage (well-timed suppression of adaptive response) (Xu and Davis, 2000).

5. Diagnosis of flavivirus infection

As flavivirus infections bring huge implications to public health worldwide, it is important to have comprehensive diagnostic facilities for accurate, specific and easier detection of flavivirus infections in both urban and remote settings. Early and accurate diagnosis of flavivirus infections are important for clinical care, surveillance support, pathogenesis studies and timely treatments to be administered to patients before the infection exacerbates. Flavivirus infection can be diagnosed by virus isolation, detection of viral antigens, virus genome sequence characterization and serological assays. Of various diagnostic tests available, serological assays are routine methods that are widely applied in various laboratories and hospitals (Guzmán and Kourı, 2004). Each diagnostic assay has its advantages and limitations that will be further reviewed below.

5.1. Virus isolation

Upon infection, samples for virus isolation must be obtained as soon as possible (no later than 5 days) since viremia is usually short starting three to four days before onset of fever and lasting only four to five days (Haymaker and Sabin, 1947). Furthermore, rapid generation of neutralizing antibodies in humans can further decrease virus titers (Solomon et al., 1998). It was reported that dengue virus can be detected in plasma, leukocytes, and tissue obtained from liver, spleen, lymph nodes, lung and thymus autopsies (Scott et al., 1980; Rosen et al., 1999). Isolation of viruses from clinical samples can be performed by culturing the clinical samples on mosquito cell lines such as AP-61, Tra-284, C6/36, AP64, and CLA-1 or mammalian cell lines such as LLCMK2, Vero, and BHK21 (Guzmán and Kouri, 1996). These cell-culture methods for virus isolation were gradually replaced by reverse-transcriptase polymerase chain reaction (RT-PCR) for rapid diagnosis. Virus isolation has met with minimal success due to lower assay sensitivity and longer detection times being inherent limitations of the indirect immunoassays used to identify the virus serotype using serotype-specific monoclonal antibodies (Gentry et al., 1982; Henchal et al., 1983). In contrast, the use of RT-PCR in conjunction with cell-culture-based virus isolation can significantly improve detection sensitivities while allowing rapid identification of the virus isolate (De Paula et al., 2003).

5.2. Molecular diagnosis

Molecular methods such as RT-PCR and nucleic-acid hybridization are employed for differential diagnosis of flavivirus infections. RT-PCR is a PCR-based method used to amplify and detect viral RNA from the onset of illness. Lanciotti et al., initially adopted a two step nested RT-PCR protocol which was reported to be highly sensitive for the detection of dengue infections (Lanciotti et al., 1992). This method was later modified by Harris et al., to a single step multiplex RT-PCR assay that has been adopted internationally (Harris et al., 1998). For example, in DENV infection, RT-PCR is capable of detecting and distinguishing four dengue virus serotypes. The major advantage of PCR-based methods is that it allows the detection of viral RNA from the onset of illness and the assay is specific. This method however, is more complex, expensive and has to be conducted in a laboratory with specialized equipment and trained personnel. Hence, this is not always feasible especially in remote settings.

5.3. Serological diagnosis

Numerous serological assays are available to diagnose flavivirus infections such as plaque reduction neutralization test (PRNT), dot-blot assay, Western-blotting, indirect immunofluorescent antibody test and IgM/IgG antibody-capture ELISA. In plaque reduction neutralization test (PRNT), the patient's serum is mixed with the virus. The presence of any virus-specific neutralizing antibodies in the serum allows binding to the virus to form a complex. This antibody-virus complex is then overlayed onto a monolayer of cells. Virus infection of the cell is prevented due to neutralization of the virus by specific antibodies. The last antibody dilution with more than 50% neutralization potency is quantified as the neutralizing antibody titer. This method requires trained personnel in virus plaque titration and a biosafety level 3 containment is necessary to grow the virus which may not be readily available in many public health diagnostic laboratories (Johnson et al., 2009). It is a quantitative detection assay for measuring the neutralizing antibody titer and is reported to be more sensitive than ELISA (WHO, 2007). Although PRNT is sensitive and specific, it requires a procured list of flaviviruses and their respective antigens for analysis. It is more time-consuming, labour intensive and is recommended to be performed in reference laboratories particularly for samples that are not distinguishable by ELISA (WHO, 2007).

In contrast, IgM ELISA offers a more rapid and easier alternative to diagnose flavivirus infections as specific IgM antibodies can be readily detected in the CSF or serum samples of infected patients. As with all antibody-based serodiagnosis, the early acute phase of the disease usually presents a negative window period of detection as the antibody response has not mounted. IgM can only been detected on days 3 to day 5. Furthermore, IgG is undetectable during the acute phase of flavivirus infection or primary flavivirus infection. However, during secondary infection, rapid anamnestic IgG response can be detected as early as 3 days after the onset of illness in response to infection as IgG recognizes shared epitopes on multiple viral proteins of the different serotypes (Lam et al., 2000). The major limitation of IgM/IgG immunoassay is the cross-reactivity of the antibodies to conserved regions of the flavivirus E protein, thus confounding the diagnosis in areas where multiple flaviviruses circulate. For instance, this immunoassay was inefficient at differentiating ZIKV and DENV infections during the recent ZIKV epidemic in Brazil which occurred in a DENV endemic region. As flaviviruses share high degree of structural and sequence homology, antibodies directed against these flaviviruses can result in extensive cross-reactivities on serological assays leading to false-positive results. To circumvent this, IgM and IgG serological assays should be performed concurrently with NS1 antigen capture immunoassays. This is reviewed in the next section. A typical IgG capture ELISA is shown in Fig. 4.

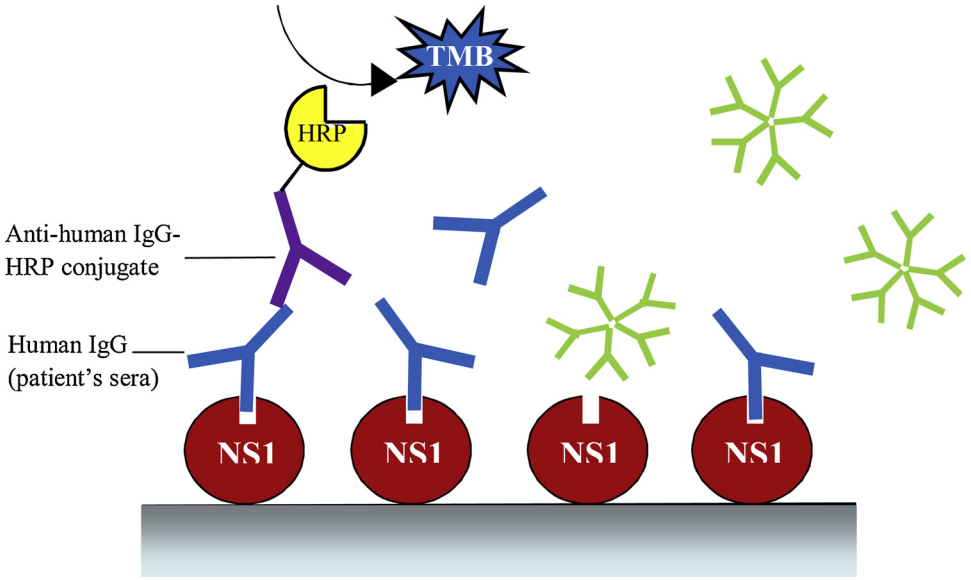

Fig. 4. Schematic showing IgG capture ELISA in detecting the presence of IgG antibody in infected individuals. After antibody response mounted, IgG antibody in the sera of infected individuals binds to the NS1 antigen. This is followed by the incubation of anti-human IgG –HRP conjugate as detecting antibody for color visualization. HRP catalyzes the conversion of a substrate to a color signal that is spectrophotometrically measurable.

5.4. NS1 antigen capture

NS1 protein is an ideal biomarker for diagnostic assays. NS1 is secreted from the infected cell and can be detected at high concentrations in the blood even before the antibody response is mounted. This implies that NS1 can be detected at the same time as viral RNA, typically from the onset of symptoms to 9 days or longer after the onset of disease (Muller et al., 2017). This assay detects the presence of the NS1 antigen in blood and acts as a marker for viremia whereby the NS1 concentration is directly correlated with the viral load (Young et al., 2000). This correlation has been reflected in quantitative-capture ELISA which showed that NS1 is secreted at high concentrations in the range of low nanogram per milliliter to micrograms per milliliter in some dengue-infected individuals. In subsequent kinetic studies of NS1 in secondary infections, it was found that high concentrations of NS1 of more than 600 ng/ml in the first 72 h of infection indicates the potential development of a more severe form of disease such as DHF or DSS (Libraty et al., 2002). Hence, NS1 antigen capture ELISA (Fig. 5) was developed as a simpler, rapid immunoassay with high sensitivity and specificity for diagnosing flavivirus infection in both urban and remote settings. It allows early diagnosis for a timely treatment to be given to the patient before virus pathogenesis spreads throughout the CNS.

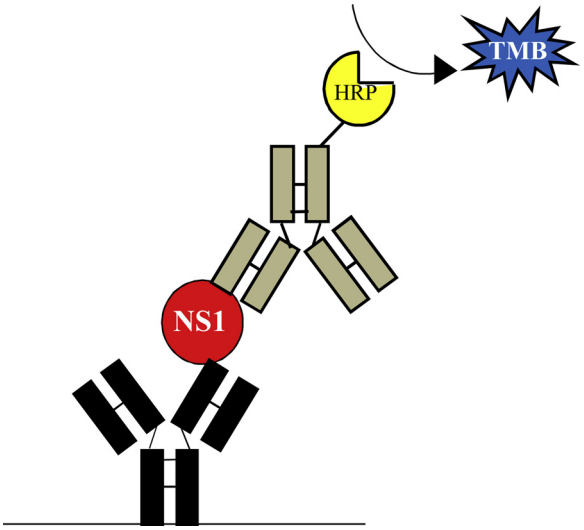

Fig. 5. Schematic representation of NS1 antigen capture sandwich immunoassay for detecting the presence of NS1 antigen in sera of infected patients. The presence of NS1 antigen in sera allows the binding of NS1 antigen to the pre-absorbed anti-NS1 antibody. This is followed by incubation with another anti-NS1 antibody HRP-conjugate which recognizes another epitope of NS1. HRP catalyzes the conversion of a substrate to a color signal that is spectrophotometrically measurable.

6. Flavivirus vaccines

Although the major flaviviral diseases are widespread in various geographical locations, no antiflaviviral treatments are available to date. As such, the development of flaviviral vaccines has been prioritized with grants being funded for better surveillance, diagnostics, vaccine development and intergration of vaccines in routine immunization regimens (Hombach et al., 2005). In 1951, Max Theiler was awarded the Nobel prize in Medicine for his finding in attenuating the wild-type virus through serial passaging in mouse and chicken tissue (Monath, 2005). This together with the development of YFV 17D live-attenuated vaccine has since become the basis for flaviviral vaccine development.

To control JE, few vaccines are currently in use including mouse-brain inactivated vaccine, inactivated vaccine cultivated on primary hamster kidney cells and live attenuated vaccine. Mouse brain-derived formalin inactivated whole virus vaccine based on the Nakayama or Beijing-1 strain were first produced in Japan (Hoke et al., 1988). It was approved for use internationally by WHO and licensed under the tradename of JE-Vax®. This vaccine has been routinely used for immunization in Asia. As the vaccine is cultivated in mice brains, safety remains a concern (WHO, 2006; Solomon, 2006; Sugawara et al., 2002). Local adverse reactions such as tenderness, redness and swelling have been reported in 20% of the vaccinees. Systemic side effects such as fever, rashes, chills, myalgia, nausea, vomiting, abdominal pain and headache are present in 10% of the vaccinees (Control, C.f.D. and Prevention, 1993). Few cases of hypersensitivity reactions such as allergic oedema and anaphylaxis were reported following vaccination due to the presence of porcine gelatin stabilizers that were included in the vaccine formulation (WHO, 2006; Sakaguchi et al., 2001).

Owing to the side effects generated, production of this vaccine ceased in 2005 and WHO has placed high priority on the generation of new JE vaccines. In 1988, a live-attenuated JE vaccine was developed in China. This vaccine was produced based on the SA-14-14-2 strain cultivated on primary hamster kidney cells. The virus was passaged through weanling mice followed by culturing on primary baby hamster

kidney cells. This vaccine has been administered to over 100 million children in China (Solomon et al., 2000). According to WHO, this live-attenuated SA-14-14-2 vaccine accounts for more than 50% of the global production of JE vaccines in 2005. As it is a live-attenuated vaccine, it poses a risk of mutation from vaccine strain into pathogenic strain (Tsai, 2000). However, the production and control standards have been upgraded to comply with WHO production standards. Although it was not WHO-prequalified, this vaccine was proven to be safe and effective in children since 2006 and it was licensed for use in Nepal, Sri Lanka, India and South Korea (Solomon, 2006; Paulke-Korinek and Kollaritsch, 2008).

Another candidate of live-attenuated JE vaccine that is being developed is ChimeriVax™-JE. This chimeric and genetically-modified vaccine uses the backbone of yellow-fever virus 17D (YFV-17D) as the antigenic target. The genes encoding the structural protein; prM and E protein of JEV SA14-14-2 strain were replaced into the non-structural gene of YFV-17D (Monath et al., 2003). The new vaccine strain was then cultivated on Vero cells (Monath et al., 2003). As the prM and E-gene sequence of this newly established vaccine strain was derived from JE virus, neutralizing antibodies against JEV-specific epitopes could be elicited. This chimeric vaccine induces subclinical infection in the host which resembles the YFV-17D vaccine's infection, however, it was shown to have antigenic specificity against JEV (Guirakhoo et al., 1999; Monath et al., 2000, 2002; Chambers et al., 1999). Preexisting immunity against the YFV virus did not negatively affect antibody responses exerted against the JE antigen (Monath et al., 2003, 2002). Preclinical studies have been performed to assess the safety, immunogenicity, and protective efficacy of the vaccine (Monath et al., 2002). Clinical trials suggest that the vaccine is well-tolerated and highly immunogenic in humans when administered subcutaneously (Monath et al., 2003).

Another type of vaccine is a Vero cell-derived inactivated vaccine which was developed in Austria and licensed in 2009 under the tradename of IXIARO in US and Europe. IXIARO is a purified, formalin-inactivated whole-virus JE vaccine based on SA-14-14-2 strain and cultivated in Vero cells. The attenuated JE vaccine SA14-14-2 strain was first passaged in primary dog kidney cells (PDK) for eight times followed by passaging in Vero cells for five times to be used as the master seed for inactivated vaccine (Srivastava et al., 2001). One dose of IXIARO contains 6 μg of inactivated virus adsorbed to 0.1% aluminium hydroxide. IXIARO was reported to provide seroprotection in clinical trials with long-term seroprotection of up to 12 months (Duggan and Plosker, 2009). Furthermore, IXIARO did not show a decrease in immunogenicity in vacinees with pre-existing immunity against flaviviruses of the same family, tick-borne virus or on vacinees with concomitant administration of Hepatitis A vaccine (Duggan and Plosker, 2009).

As dengue is the most important mosquitoes-borne flavivirus disease, research is ongoing to develop various dengue vaccines and therapeutic candidates. On 1st May 2019, FDA has recently approved Dengvaxia® (CYD-TDV) as the first dengue vaccine which licensed for use in several countries including European Union, Latin America and Asia. It is catered for people of 9–45 years old who were previously infected with dengue. Dengvaxia® is a live-attenuated, recombinant tetravalent dengue vaccine which uses the backbone of YFV 17D where the prM and E structural genes had been replaced with wild type dengue virus (Guy et al., 2011). The recombinant tetravalent vaccine was prepared by combining all the four serotypes of the dengue viruses into a single vaccine with no adjuvants or preservatives added (Guy et al., 2011). Although it is currently the only available dengue vaccine, significant controversy was raised when it posed an increased risk of severe dengue infection for dengue-naïve individuals during their primary dengue infection after vaccination (Aguiar et al., 2016) due to ADE. The main disadvantage of Dengvaxia® is that the vaccine is only restricted to targeted populations (9–45 years old with previous exposure to dengue fever). Hence, the development of safe, effective and affordable dengue vaccines remains a high priority.

West Nile virus vaccine, the ChimeriVax-WN vaccine has been developed and is undergoing phase I clinical trials. By utilizing the ChimeriVax technology, ChimeriVax-WN vaccine was shown to be less neurovirulent than the YFV 17D vaccine in mice model (Arroyo et al., 2004). Other West Nile vaccine candidates include a live-attenuated chimeric West Nile/dengue vaccine. Developed by NIH, this candidate vaccine comprises prM and E structural genes of WN on the backbone of dengue type 4 virus (DEN4) and is further attenuated by deletion of the 5′ non-coding regions of DEN4. This vaccine candidate was shown to be immunogenic, less neurovirulent and neuroinvasive in mice models compared to its parental WNV strain (Pletnev et al., 2006).

For Zika infection, the currently available vaccine candidates include a DNA-based vaccine, purified formalin-inactivated Zika vaccine (PIZV) and live attenuated Zika vaccine. The DNA-based vaccine was produced based on expression of prM and E proteins of ZIKV. This prM-E DNA vaccine was reported to confer complete protection to animal models against ZIKV challenge (Larocca et al., 2016). This vaccine is reported to be undergoing phase II clinical trials to further assess the safety and immunogenicity of the vaccine as well as the optimal dose for administration. The PIZV vaccine was developed based on Zika virus strain, PRVABC59 isolated during the Zika outbreak in 2015 (Lanciotti et al., 2016). Phase I clinical trials of PIZV showed that it conferred complete protection to mice and non-human primates upon viral challenge with high neutralizing antibody titers observed (Modjarrad et al., 2018). This vaccine is currently undergoing a fourth Phase I trial to address safety and immunogenicity issues in vaccine development (Modjarrad et al., 2018). Another candidate vaccine which is in phase I clinical trials is the live-attenuated Zika virus vaccine known as rZIKV/D4Δ30-713 developed by the NIH (Whitehead et al., 2017). This vaccine candidate is a chimeric virus with dengue virus type 4 backbone that expresses the Zika virus surface protein (Li et al., 2018). This attenuated ZIKV chimera was also aimed to be produced as a pentavalent vaccine that is immunogenic against both dengue virus and Zika virus (Whitehead et al., 2017). With extensive research and various ZIKV vaccine candidates marching into clinical trials phase, a licensed ZIKV vaccine would be expected very soon.

7. Monoclonal antibodies for flavivirus infection

While flaviviral infections remain as important infectious diseases, antiviral agent are urgently needed. Treatment against flavivirus infections is only limited to management of complications and effective care. Although licensed vaccines are available as a preventive measure for some of the flaviviral diseases such as JEV, TBEV, YFV, none has been successfully developed and approved for DENV. Development of vaccines for dengue against all four antigenically distinct serotypes has been a huge challenge for decades. Besides, the sporadic outbreak of flavivirus infections in a previously unaffected geographical region is another challenge for vaccine development as complete prevention of the diseases is only possible through universal immunization across huge geographical regions. In this context, antiviral therapy plays a significant role against the flaviviruses. Antiviral agents are urgently needed to block viral replication in the brain for flaviviral encephalitis or modulate the host immune response to fight against the infection. There are many antiviral candidates for therapeutic purposes, however, in this review we will focus primarily on therapeutic monoclonal antibodies against flavivirus infections.

During flavivirus infection, monoclonal antibody (mAb) can protect the host by blocking virus attachment to the cell surface, disrupting viral membrane fusion, mediating viral clearance via Fc-dependent effector functions or direct neutralization of the virus (Levine et al., 1991; Pierson and Diamond, 2008). E glycoprotein is the principal antigen that elicits neutralizing antibodies against flavivirus infection (Pierson et al., 2008; Beltramello et al., 2010). Structural studies revealed that E protein of several flaviviruses share common features whereby the E

protein is folded into three antigenic domains (Paulke-Korinek and Kollaritsch, 2008; Modis et al., 2004; Nybakken et al., 2006; Dai et al., 2016). Domain I has a central β barrel and is a hinge to link domain II and domain III. Domain II is a dimerization domain and is responsible for membrane fusion during viral entry whereas domain III is a C-terminal like immunoglobulin-like module which acts as a receptor binding site where virions attach to the host cell receptor. Most neutralizing antibodies isolated against flaviviruses localize to domain III (DIII) of E protein (Sukupolvi-Petty et al., 2007; Rey et al., 1995; Chambers et al., 1990). Isolated antibodies against DIII have reported to be virus or serotype specific, possibly due to lower sequence similarity among the flaviviruses.

For WNV infection, mAb E16 has been identified as the most potent neutralizing antibody targeting DIII of E protein. E16 was generated from mice immunized with recombinant WNV E protein by hybridoma techniques (Oliphant et al., 2005). E16 was shown to neutralize all WNV strains tested, blocked infectivity of strain 956 and showed no cross-reactivity or neutralization against other flaviviruses such as DENV, YFV, JEV and SLEV (Oliphant et al., 2005). In mice model challenged with WNV, administration of a single dose of 100 μg of E16 at day 2 of infection protected more than 90% of the mice from lethal infection and single dose of 2 mg of E16 at day 5 resulted in 90% survivability. The Fc region was reported to enhance the potency of E16 in mice, indicating that E16 controls WNV infection through ADCC and C1q-related effector functions. The inhibitory effect of E16 improves clinical and virologic outcome, suggesting that E16 can mediate viral clearance from infected neuron. Humanized E16 also demonstrated protective efficacy against mice challenged with WNV. Structural studies with the FAb fragment of E16 suggested that E16 mAb neutralizes WNV by inhibiting viral entry, possibly through disrupting the conformational rearrangement of E protein before membrane fusion (Kaufmann et al., 2006).

The potent neutralizing mAbs that have been successfully isolated against DENV include mAb 1A1-D2 and 4E11 (Roehrig et al., 1998; Megret et al., 1992). MAb 1A1-D2 was reported to strongly neutralize DENV1, DENV2 and DENV3. Structural studies revealed that when the Fab fragment of 1A1-D2 complexed with domain III of DENV2E protein, epitope would be partially occluded on the virus but interestingly, the binding of the antibody to the virus at 37 °C was not affected (Roehrig et al., 1998). This suggested that the virus was in dynamic motion, thereby making hidden epitopes briefly available (Lok et al., 2008). Cryo-EM image of Fab 1A1D-2 complexed with DENV showed a significant reorganization of the E protein, disrupting the mature virion structure and preventing its binding to the cell-surface receptor (Lok et al., 2008; Cockburn et al., 2012). 4E11 is another potent neutralizing antibody isolated against E protein of DENV. In contrast to mAb 1A1-D2, 4E11 was shown to neutralized all four dengue serotypes (Thullier et al., 1999). 4E11 was reported to binds to DIII A strand, disrupting the architecture of mature virion and inactivate the virus particle (Cockburn et al., 2012).

For JEV infection, two potent therapeutic mAbs have been successfully isolated and characterized, 2F2 and 2H4 (Qiu et al., 2018). In 1989, these two mAbs were isolated and reported to enhance survival of JEV-infected animal models such as mice, goats, and monkeys (Zhang et al., 1989). Recently, these two mAbs were further characterized for its neutralization mechanism in response to JEV infection (Qiu et al., 2018). 2F2 and 2H4 bound to the E protein of JEV with no cross- reactivity observed against DENV1, DENV2, YFV and ZIKV, indicating that both isolated mAbs were JEV-specific. Neutralizing antibodies 2F2 and 2H4 proved to be highly potent and conferred therapeutic protection in mice models. Administration of 20 μg of 2H2 and 2F4 respectively have shown to exhibit 100% survival and complete viral clearance in the brain of the infected mice by day 9 and day 7 respectively. Structural studies showed that both mAbs occupy most of the surface of JEV, and prevent the rearrangement of E protein thereby blocking the viral attachment and viral fusion to its receptor. In another study performed by E Fernandez et al., it was claimed that a panel of neutralizing monoclonal antibodies have been generated against four different JEV genotypes (Fernandez et al., 2018). These mAbs primarily recognized the lateral ridge on the DIII of E protein and inhibit JEV infection by blocking viral fusion. Administration of a single dose 250 μg of JEV-31 and JEV-169 were shown to confer protection to mice infected with JEV GIII whereas limited therapeutic activity was recorded upon administration of the mAbs to JEV GI-infected mice. In the same study, human monoclonal antibodies were isolated from vaccinated individuals against JEV-SA14-14-2. Of the human neutralizing antibodies isolated, hJEV75 provided significant protection to mice infected with JEV GIII and mice infected with JEV GI strains respectively.

With the recent ZIKV outbreaks, extensive research has been performed for the development of effective therapeutic agents. Several mAbs have been characterized and were found to recognize the lateral ridge of E protein DIII. ZV54 and ZV67 are mAbs isolated form mice immunized with Zika virus (Zhao et al., 2016). These antibodies were reported to be ZIKV-specific and capable of neutralizing African, Asian and American Zika virus strains. *in vivo* passive transfer of these mAbs was shown to protect IFN-γ deficient mice against lethal ZIKV challenge. ZV54 and ZV67 are two structurally similar mAbs that differ by two contact residues. Crystallographic analysis of ZV67-ZIKV DIII complex suggested that, structurally similar ZV54 and ZV67 bound to the ZIKV DIII lateral ridge in a similar manner as WNV E16 (Zhao et al., 2016). This indicated that lateral ridge DIII epitopes are highly conserved among flaviviruses.

8. Flavivirus NS1: the crucial and potent biomarker for immunodiagnostic and immunotherapeutics

Flavivirus NS1 is a highly conserved, non-structural glycosylated protein which is held by six intramolecular disulfide bonds. It plays a crucial role in modulating host cell machinery for effective virus propagation. After viral genome being inserted into the cytoplasm of the host cell, the signal peptide in the viral mRNA leads the mRNA to the ER lumen for translation of the structural and non-structural proteins. The polypeptides are then cleaved by signalase, furin and viral serine protease. The presence of signal peptide at the C-terminal of E protein cleaves and translocates the hydrophilic monomeric NS1 from translated polypeptide into the ER lumen (Muller and Young, 2013). NS1 monomers of ZIKV, WNV and DENV contains three domains; the β- roll, wing and β-ladder domains. Homodimer NS1 is formed by extending the β-ladder domain and connecting at the β- roll domain, forming a cross-shaped protein. The hydrophobic surface of the β- roll and wing domains could mediate the interaction with the cell membrane whereas the loops connecting the surface β- ladder is the region for potential host protein interaction due to its hydrophilicity. Monomeric NS1 is cleaved at the highly conserved octapeptide sequence located in the C-terminus of NS1. It is then modified by addition of carbohydrate moieties at multiple sites to form a dimeric NS1, leading to acquisition of hydrophobic character and resulting in membrane association. Following dimerization, NS1 is trafficked to three sites; site for viral replication within the cell, the cell surface and being secreted into the extracellular space (Muller and Young, 2013). The cell-associated NS1 along with other non-structural proteins and viral RNA formed a replication complex which resides in the membranous vesicle packets (VP) induced within the infected cells. The replication complex which comprises of all seven non-structural protein and selected host factor are responsible in facilitating viral RNA replication in the cytoplasm (Mackenzie et al., 1996). A small portion of cell-associated NS1 is found at the surface of infected cell. In mammalian cell, expressed NS1 is trafficked through the Golgi apparatus where the exposed carbohydrate moieties of the dimeric form of NS1 were then trimmed by glyosidases and glycotransferases to form a soluble hexameric lipoprotein that eventually secretes out of the infected cell. NS1 hexamer is a high

density lipoprotein that is held together by weak hydrophobic interaction and it could dissociate into dimers in the presence of non-ionic detergents (Flamand et al., 1999). Secreted NS1 is often used as a biomarker for early diagnosis of flavivirus infection as it can be detected in the blood at early stages as evidenced by high level of NS1 secretion of up to 50 μg/ml in the sera of DENV-infected patient (Young et al., 2000; Li et al., 2012). Secreted NS1 is also reported to be highly immunogenic where both the proteins and the antibodies it elicits play an important role in disease pathogenesis (Falgout et al., 1990).

In primary dengue infection, relatively low concentration of anti-NS1 IgM and IgG responses elicited (Falkler et al., 1973). As NS1 is not a component of the virion particle, hence, anti-NS1 antibodies generated are not the neutralizing antibodies. Interestingly, multiple studies shown that administration of anti-NS1 antibodies were able to protect the mice against lethal viral challenge (Schlesinger et al., 1985; Gould et al., 1986). This indicates that the protective immunity conferred by anti-NS1 antibodies were likely due to complement-mediated lysis of infected cells following antibody recognition of cell-surface associated NS1. Beside complement-mediated lysis of infected cell, studies using complement and specific Fc-γ receptor knockout mice shown that administration of anti-NS1 WNV antibodies of varying isotypes gave protection to the mice through phagocytosis and clearance of the infected cell through Fc-γ receptor I or IV recognition of cell-surface NS1-bound antibodies (Chung et al., 2007, 2006; Diamond et al., 2008). These implied that infected mice could also be protected by complement-independent activation.

Although live attenuated chimeric viruses are the vaccine candidates against flaviviral infections, there is a growing interest in making NS1 as the potential candidate for the generation of subunit vaccines. NS1 was delivered as vaccine candidates via different strategies including immunization with recombinant or native protein subunit, live recombinant vaccinia virus, defective recombinant adenovirus, naked DNA and peptide-based approach. Hence, NS1 is the target of cell-mediated immunity besides being capable of eliciting complement dependent and independent antibody-mediated protection.

The correct diagnosis of a flavivirus infection is rather challenging especially in geographical regions where multiple flaviviruses co-exist. The conventional serological assays suffer from high antigenic cross-reactivity mainly from the E protein where the antibody response is targeted. Furthermore, the serological assay of IgM capture ELISA not only suffer from cross-reactivity issue, early detection of the disease is not possible as antibody responses have yet mounted. On this basis, NS1 emerged as suitable biomarker for diagnosing flaviviral infection. NS1 antigen capture ELISA for DENV revealed that NS1 is secreted at high level from the onset of symptoms in infected individuals (Young et al., 2000). This makes early diagnosis possible and obviates the need to use complex instruments for more accurate diagnosis. The development of diagnostic tools based on NS1 detection can indeed revolutionized flavivirus infections diagnosis in view of its simplicity, low-cost, high sensitivity and specificity of the assays.

9. Conclusion

The sporadic emergence and continual expansion of the geographical regions for flaviviruses necessitate effective antiviral therapy against flavivirus infections. While flaviviral disease is the main form of infectious disease with high morbidity and mortality rate recorded, no approved antiviral treatment has yet been found. The isolation and characterization of therapeutic antibodies, structural studies for identification of neutralizing epitopes, mechanism of neutralization, pathogenesis, viral inhibitors and others remain in the forefront of flaviviral research. Despite extensive research associated with flavivirus infections, various anti-flaviviral antibodies generated so far have failed in clinical trials. The factors associated with the failures of therapeutic antibodies in clinical trials should be investigated extensively. For instance, flaviviruses are heterogenous and dynamic. The degree of maturation and dynamic state of the virions affect the availability of the epitopes at a given time. Besides, flaviviruses undergo structural transition from smooth surface at room temperature to adopting a 'bumpy' surface at human body temperature (Austin et al., 2012). This implies that the structure of virions produced by different cell types, exposed neutralizing epitopes and immunoreactivity with the neutralizing antibodies could be different *in vitro* and *in vivo*. This explains why mAbs that can neutralize virus in infected mice provide no neutralizing ability or protective efficacy in human clinical trials. Antibody-based therapy is a promising future for tackling flavivirus infections. Although generation of anti-flavivirus antibodies/compound poses great challenges, the goal of finding a cure for flaviviral disease could come into realization.

Authors' contributions

All authors made contribution to this study and approved the final manuscript.

Declaration of Competing Interest

The authors declare that they have no competing interests.

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