

Review Article

Approaches for the Development of Rapid Serological Assays for Surveillance and Diagnosis of Infections Caused by Zoonotic Flaviviruses of the Japanese Encephalitis Virus Serocomplex

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Flaviviruses are responsible for a number of important mosquito-borne diseases of man and animals globally. The short viraemic period in infected hosts means that serological assays are often the diagnostic method of choice. This paper will focus on the traditional methods to diagnose flaviviral infections as well as describing the modern rapid platforms and approaches for diagnostic antigen preparation.

1. Introduction

Flaviviruses are responsible for a number of important mosquito-borne diseases of man and animals globally. The *Flavivirus* genus, consists of 50 species and 23 viral subtypes [1], which are further separated into 12 groups based on phylogenetic analysis of the NS5 gene [2], as well as antigenic and ecological similarities [1]. Members of the globally distributed Japanese encephalitis serocomplex, include viruses such as West Nile virus (WNV), Murray Valley encephalitis virus (MVEV), St. Louis encephalitis virus (SLEV), and Japanese encephalitis virus (JEV) [1]. Other serocomplexes include yellow fever virus (YFV), tick-borne encephalitis virus (TBEV), and Dengue virus (DENV) [3, 4]. Of recent concern is the rapid spread of a particularly virulent strain of WNV through North, Central, and South America [5]. This mosquito-borne virus can cause a fatal form of encephalitis in humans, birds, and horses [6, 7] and since its introduction to America in 1999, it has caused tens of thousands of clinical cases and thousands of deaths in humans and horses [8, 9]. More recently, a WNV outbreak occurred in Europe during 2010 and 2011, where it has been reported that there were 197 human cases of neuroinvasive disease caused by WNV in 2010 and a further 31 cases between July and August 2011 [10, 11]. In 2011 there was also an outbreak of encephalitis in

horses caused by the WNV subtype, Kunjin virus (KUNV), in the south-eastern areas of Australia [12].

The viruses of the JEV serocomplex are maintained in nature in a cycle involving mosquitoes and in most cases, birds as the vertebrate host. However, pigs usually serve as an amplifying host during outbreaks of JEV. While horses and humans can become infected, they are classified as “dead-end hosts” as the viraemia is usually insufficient to subsequently infect feeding mosquitoes [13]. Similarly, this low level of viraemia, particularly at the time of clinical presentation for both humans and horses limits the likelihood of detecting viral antigen or RNA. Thus, diagnosis of flaviviral infections is commonly achieved using serological assays such as plaque reduction neutralization tests (which are time consuming and require the handling of live virus) and ELISA. While assays such as ELISA are ideal for high-throughput screening, they are not readily adaptable to rapid, pen-side testing. The cross-reactive nature of the immune response to flaviviral infections also causes problems for the specific diagnosis of flaviviral infections and the development of rapid immunoassays [14].

There have been numerous approaches to improve the specificity of serological-based flavivirus diagnostic assays. These include preparing sub-unit antigens, identifying immunogenic peptides and competitive blocking assays.

Although this review will focus predominantly on the approaches used to improve diagnostic assays for WNV, these approaches are applicable to the other viruses of the JEV serocomplex, since they have similar ecology, epidemiology and pathology.

2. Considerations for the Development of Flavivirus Diagnostic Assays

2.1. Viraemia and Antibody Development during Flavivirus Infection. In humans, clinical symptoms of WNV disease, such as a mild febrile illness with fever, headache, and fatigue [15], generally appear two to 14 days postinfection [16–18]. However, in some infections acquired through blood transfusion, symptoms were not observed for up to 22 days [18]. After a laboratory-acquired infection of the Australian WNV subtype, KUNV, the infected individual developed symptoms 9 days after the presumed infection date [19]. Less than 1% of WNV infections result in neurological disease, which can be fatal [20–23]. Although viraemia can be present at symptom onset during flaviviral infections, the delay in most patients presenting to a medical practitioner [22, 24] means that detection of blood-borne virus or viral RNA can be difficult. While the exact time from the bite of a WNV-infected mosquito to detectable levels of circulating viral RNA in the patient's blood is not known [25], an early study involving the inoculation of terminally ill cancer patients with Egyptian strains of WNV, indicated that virus could be detected in the blood one to two days postinfection [26]. Following the laboratory-acquired infection of the individual with KUNV, the virus was isolated 8–10 days postinfection [19]. Screening of viraemic blood donors in the USA by Busch et al. (2008), revealed persistence of viral RNA for about 19 days, as determined by nucleic acid amplification testing [25]. In this study, IgM seroconversion occurred at about four days after the detection of RNA. However, in two laboratory-acquired infections, IgM was not detected until 13–17 days postinfection [16]. Interestingly, in one study, anti-WNV IgM was shown to persist for an average of 156 days [25] and was present for well over a year [27], rendering IgM serological assays unable to differentiate past and recent infections. IgG seroconversion occurs one to four days after the appearance of IgM [25, 28]. In a study exploring the IgM and IgG responses to JEV infection, similar kinetics to those reported for WNV were observed [29]. In this study, of the 32 patients that were admitted to hospital with acute encephalitis, 53% of the JEV seropositive patients had IgM present at admission, and all had IgM by 7 days postadmission. By day 180, 39% of the patients still had detectable IgM. The IgG response peaked 30 days after admission and was still detectable in half of the cases after 180 days.

WNV infection of horses in the USA has had a significant effect on the equine industry [30]. Approximately 10% of infected horses show signs of disease [13]. The symptoms are generally neurological and include ataxia, paralysis, and altered behaviour [31]. Experimental infections have induced a virus neutralising immune response by seven to

12 days postinfection [13, 32, 33] and clear IgM seroconversion around day seven postinfection [13]. The detection of virus in the blood of the experimentally infected horses in the study by Bunning et al. (2002) [13] occurred from days 1 to 3 postinfection and persisted until day 5 or 6 for all of the horses, except for one which only had detectable viraemia on days 3 and 4. Only one of the horses developed clinical signs of disease, which occurred on day 8 postinfection, and thus outside the window for virus detection, highlighting the requirement for antibody-based diagnostic assays for horses as well. Infection of horses with KUNV or MVEV can also cause a neurological disease [12, 34–36], although experimental infection of a limited number of horses with MVEV failed to induce clinical symptoms [37]. In this study, five of the 11 experimentally infected animals had detectable levels of the virus in their blood from day 1 to day 5 postinfection. All of the horses developed an antibody response to MVEV seven to fourteen days postinfection.

2.2. Geographical Distribution of the Pathogenic Flaviviruses and Considerations for Serological Diagnostic Assay Specificity. The presence of flaviviruses of the JEV serocomplex within the same geographic regions poses problems for the diagnostic specificity of serological assays due to the development of cross-reactive antibodies during flaviviral infections [4]. In North America, the presence of WNV and SLEV complicates serological specificity when diagnosing infections in humans and horses [38, 39]. In South America, in addition to WNV and SLEV, flaviviruses that should also be considered when testing equine sera include Ilheus virus and Bussuquara virus, although recent serosurveys have not found any equines with Bussuquara virus-neutralising antibodies [40–42]. In Europe, the African flavivirus, Usutu virus, was detected for the first time in sentinel horses, chickens, and birds during surveillance for WNV in 2008 and 2009 [43] and has since caused neurological disorders in patients in Italy [44, 45]. WNV caused a major outbreak of encephalitis in Greece in 2010 and cross-reactivity between WNV and DENV was observed when testing patient sera from this outbreak [46].

Australia has the largest number of different JEV serogroup species in the world. There have been incursions of JEV in the northern areas of the continent, while in recent years there have been outbreaks of dengue virus in northern Queensland. Kunjin virus is endemic in Northern Australia and in 2011 there was an outbreak of equine encephalitis with a 10–15% case fatality rate, caused by a virulent KUNV strain in South Eastern Australia [12]. In addition to KUNV and JEV, humans and horses also develop infections to MVEV and Kokobera, which can also complicate serological diagnosis [34–37] (May et al., manuscript under revision). Indeed, sequential exposure to different flaviviruses enhances the production of flavivirus cross-reactive antibodies, leading to false positives in some serological assays [47].

Prior vaccination against one or more flaviviruses must also be considered when interpreting assay specificity. Human vaccines are available for tick-borne encephalitis virus, JEV and YFV and a recent study showed cross-neutralisation

of DENV, WNV, and/or louping ill virus (LIV, which is a member of the TBEV serocomplex) by human serum samples taken following vaccination against TBEV and JEV [48]. The likelihood of samples cross-neutralising DENV and WNV was increased if the individuals were also vaccinated against YFV, in addition to JEV and TBEV. False positives in WNV assays have also been documented when assessing serum from JEV-vaccinated horses [49].

2.3. The Immunogenic Flavivirus Proteins. The flavivirus virion is spherical, enveloped, and approximately 500 Å in diameter [50]. It consists of a host-derived lipid bilayer containing 180 copies of the envelope (E) and membrane (M) proteins, which are arranged in a herringbone pattern [50, 51]. This envelope surrounds a nucleocapsid core which contains the single stranded, positive sense RNA genome of approximately 11 kb [52, 53]. The genome contains a single open reading frame (ORF), bounded by 5' and 3' untranslated regions [54]. The ORF is translated as a single polyprotein of three structural and seven nonstructural proteins, which are cleaved by viral and cellular proteases [55].

The immunodominant antigens during WNV infection are the E, prM, and NS1 proteins [56–62], although antibodies to the nonstructural proteins NS3 and NS5 have also been detected in humans [63, 64].

2.3.1. The Envelope Protein. The E protein is the dominant protein present on the surface of the flavivirus virion [50, 51]. It is a major target for neutralising antibodies [65–67]. The E protein monomer is divided into three domains. Domain I (DI) features an eight-stranded β barrel [51, 68, 69] and in many of the recent virulent WNV strains isolated, it contains the sole E protein N-linked glycan moiety [53]. Domain II (DII) contains the highly conserved fusion peptide [70] and Domain III (DIII) is an immunoglobulin-like structure [71] which is likely to participate in receptor binding [68, 72].

WNV E protein neutralising epitopes have been well-defined and map to all three domains [71, 73–75]. The majority of potent neutralising monoclonal antibodies (mAb) bind DIII, in particular residues 302–309 and 330–333 of the lateral ridge [71, 75–77]. Importantly, these epitopes are more likely to be type specific, but are not the target of most antibodies generated during WNV infection of humans and horses [59, 74]. Recent studies have revealed that most antibodies are directed to DII epitopes [59], which tend to be highly cross-reactive [78, 79].

2.3.2. prM/M. prM is a 20–25 kDa precursor to M protein and is present on immature, noninfectious particles [80, 81] and to a lesser extent on secreted infectious particles [82, 83]. During the virus maturation process, cleavage of prM by the cellular protease, furin, at a highly conserved cleavage site [84], results in the release of the singly glycosylated pr protein [84–86]. Furin cleavage of prM occurs only after exposure of the protein to low pH in the late endosomes [84].

While the crystal structure for WNV prM has not yet been determined, DENV prM was recently crystallised and the structure elucidated [87]. This study revealed that the pr

protein consisted of seven beta strands and confirmed that the protein structure was stabilised by the presence of three disulphide bonds. The pr peptide covers the E protein fusion peptide loop (DII) in the immature virus, thus pre-venting premature fusion of the virus particle with the host cell membrane during transport through the *trans*-Golgi network [80, 88].

Antibodies to prM are generated during flaviviral infection [59, 62, 89]. Western blot analysis using whole viral antigen and WNV-immune horse serum clearly demonstrates that the majority of antibodies generated during WNV infection are directed to the E and prM proteins [62]. Generally, the anti-prM antibodies do not elicit potent virus neutralisation *in vitro*. However, they can be protective *in vivo* [90–93]. A study by Cardosa et al. (2002) suggested that antibodies to prM may be virus specific, due to the ability of this antigen to differentiate DENV and JEV infections in humans [89]. However, in another study, immune serum from SLEV- and WNV-infected humans showed cross-reactivity between WNV and SLEV prM [94]. Nevertheless, prM may be a potential candidate antigen for incorporation into species-specific assays.

2.3.3. NS1. NS1 is a glycosylated, nonstructural protein, of about 48 kDa which has a highly conserved structure that is stabilised by six disulfide bridges [95, 96]. To date, there has been no success in crystallising NS1. However, electron microscopy has recently provided some insight into the structure of the hexameric secreted form of NS1 [97, 98]. NS1 is thought to have an important role in RNA replication [99–102] and has been colocalised with the double-stranded RNA replicative form [100]. NS1 also appears to have a role in immune evasion, as it has been shown to attenuate complement activation [103]. While a large amount of NS1 remains in the infected cell [104], it is also actively secreted at relatively high levels [104–107]. This secreted protein stimulates a strong [108–110], protective, but non-neutralising antibody response [111–113] and it has been targeted by serological assays [56, 58, 114–116]. There have also been numerous mAbs generated to this protein [111, 117–120], some of which have been incorporated into antigen capture assays for the early detection of virus infection [104, 121].

3. Serological Diagnosis of Flaviviral Infections

3.1. Traditional Approaches

3.1.1. Plaque Reduction Neutralisation Tests. The Plaque Reduction Neutralisation Test (PRNT) is the gold standard for the serological diagnosis of flaviviral infections. PRNT and virus neutralisation tests (VNT) assess for serum antibodies that bind to the viral envelope protein and prevent virus entry into the cell (normally Vero cells for flavivirus assessment) *in vitro* [122, 123]. In PRNT, neutralisation of the virus by antibodies in the infected patient's serum is evidenced by a reduction of plaques relative to the serum dilution. Virus neutralisation in VNTs results in the absence,

or, decrease in visible cytopathic effect in the cell monolayer. PRNT can be highly specific, although the accuracy of interpretation of the results depends upon simultaneous assessment against flaviviruses endemic to a given area, to allow for comparison of end-point titres. Other disadvantages of these tests are that they are labour intensive, require skilled personnel, a minimum of five days to perform, and the handling of live virus, which requires a BSL-3 (Biosafety level 3) facility (Table 1).

3.1.2. Haemagglutination Inhibition Assay. Haemagglutination and Haemagglutination Inhibition assays (HI) have been widely used for the detection of arthropod-borne viruses and the antibodies developed to these viruses, respectively [14, 124]. These assays exploit the ability of the envelope glycoprotein to bind and agglutinate avian erythrocytes so that they form a visible lattice in a U-bottom microtitre plate. In the HI assay, antibodies from infected individuals prevent the agglutination of the erythrocytes, which subsequently form a pellet. While the HI assay was used extensively in the past for flavivirus serology, this technique has now been largely superseded, in favour of assays with better sensitivity and specificity, but is still used in some instances for surveillance [125, 126]. The advantages of HI assays are that providing avian red blood cells are available, the assays can be performed with minimal training and equipment and the antigen used can be inactivated by a simple extraction process. However, as for PRNT, there is a requirement for the simultaneous assessment for flaviviruses endemic for the area and multiple different pH buffers are required for each different antigen. A constant supply of fresh avian red blood cells is also necessary and there is a high level of cross-reactivity amongst the flaviviruses [127].

3.1.3. Immunofluorescence Assay. The Immunofluorescence assay (IFA) can be used to differentiate the IgM and IgG responses to flaviviral infection. It involves incubating patient serum with glass slides, upon which are fixed flavivirus-infected cells. The patient's virus-specific antibodies are then detected with a fluorophore-conjugated antispecies IgM or IgG immunoglobulin. The benefit of this assay is that prefixed slides can be stored at 4°C and a BSL-3 facility is not required to perform the assay and results can be obtained quickly, particularly due to the commercialisation of IFA kits (e.g., Focus Diagnostics Arbovirus IFA). However, cross-reactivity of immune antibodies with closely related flaviviruses can impair the accuracy of the diagnosis and there is a requirement for a fluorescent microscope to evaluate the results.

3.1.4. ELISA. ELISA is routinely used for the diagnosis of WNV infection [140]. IgG- and IgM-capture (MAC-ELISA) ELISAs for WNV were originally developed by the Centers for Disease Control (CDC) and applied to human and equine WNV infection diagnosis [32, 141–143]. Prior to this, IgG and IgM capture ELISAs were developed in the 1980s for the detection of antibodies to JEV [29]. The MAC-ELISA

was developed to diagnose recent infections and involves the capturing of test-serum IgM with immobilised anti-species IgM, followed by the addition of WNV antigen and detection with a flavivirus-specific, horseradish peroxidase-conjugated monoclonal antibody. The first Food and Drug Administration- (FDA-) cleared assay of this nature was developed by PanBio, who have since released improved versions with increased accuracy [144]. The persistence of IgM from WNV infections [25, 27, 28] led to the development of avidity testing, in order to improve the usability of ELISA for differentiating past and recent infections [145]. Specificity problems associated with flavivirus cross-reactivity, have been reduced through the application of algorithms [146]. The sensitivity and specificity of commercially available ELISAs has been reviewed by Zhang et al. (2009) [147]. A survey of public health and commercial diagnostic reference laboratories in 2008 revealed that ELISA or microsphere immunoassay-based IgM and IgG assays were most commonly used for the diagnosis of WNV infections, with significantly fewer laboratories using PCR, PRNT, and culture isolation [148].

Defined epitope blocking ELISAs have also been used to increase the specificity of WNV serodiagnosis and have been useful for differentiating flaviviral infections through targeting epitopes on NS1 [56, 58, 116] or E protein [149]. A recent study in horses has shown exquisite specificity of the blocking ELISA originally published by Hall et al. (1995) [56] when assessing serum from horses that have been sequentially infected with SLEV or DENV followed by WNV [47]. The WNV blocking ELISA was positive only when testing serum from the horses following exposure to WNV, despite previous injection with SLEV or DENV. In contrast, depending on the day, postinjection with WNV, PRNT and IgM ELISA could not always be used to accurately diagnose WNV as the most recent injected virus. Despite these data, current blocking ELISAs are unable to differentiate infections caused by different subtypes of WNV [36] and for diagnosing WNV infection in patients who have received flavivirus vaccinations or have had previous flaviviral infections [150]. Blocking ELISAs have been used extensively for surveillance for WNV in North America [41, 151–154] and for detection of KUNV and MVEV in Australia [36].

3.1.5. Immunoblot. Western blot using lysates of flavivirus-infected cell monolayers can potentially differentiate flaviviral infections [89, 94]. In a study by Ocegüera et al. (2007) [94], where serum antibody reactivity to E, NS1, and prM proteins of WNV and SLEV was analysed, NS1 was most useful in differentiating WNV and SLEV infections in humans, whereas prM was the most specific antigen for differentiating JEV and DENV virus infections in a study by Cardoso et al. (2002) [89]. Western blot using crude lysates was also used to assess for the seroprevalence of WNV in horses in sub-Saharan Africa [155]. A drawback to using Western blot to analyse sera is that frequently serum antibodies will also recognise other proteins within the cell lysates and this can make accurate interpretation of the results difficult.

TABLE 1: Comparison of commonly used serological assays for the diagnosis of JEV serocomplex flaviviral infections.

Assay	Differentiation between IgM and IgG	Time to run assay	Handling of live virus by the end user	Species specific:#	Sensitivity*	Specificity*	High throughput	Target flaviviral protein for serum antibodies	Commercialised or available from government Agencies?
PRNT	No, but an increased titre using paired sera is indicative of a recent infection	Days	Yes	No	High. It is the gold standard assay	High. It is the gold standard assay, but requires the simultaneous testing of endemic flaviviruses	No	Neutralising epitopes of the E protein	No
HI	No, but an increased titre using paired sera is indicative of a recent infection	Hours	No once the antigen has been prepared	No	High	Low. Requires the simultaneous testing of endemic flaviviruses	No	E protein	No
IFA	Yes	Hours	No once the slides have been prepared	Yes	Moderate, for example, IgM IFA not as sensitive as ELISA [128, 129].	IgM: Moderate, for example, cross-reactivity 17% [130]; 14% [128] IgG: Low, for example, cross-reactivity: 63% [130]; 45% [128]	No	All	Yes, for example, Focus Diagnostics and Scimedx
ELISA IgG/IgM	Yes	Hours	No	Yes	High, for example, Focus Diagnostics WNV DxSelect IgM: 100% [131], but cross-reactivity with some cases of other flaviviral infections [132]	IgM: Moderate to High, for example, Focus Diagnostics WNV DxSelect IgM: 100% [131], but cross-reactivity with some cases of other flaviviral infections [132]	Yes	E protein	Yes, for example, Focus Diagnostics, Panbio, CDC
Blocking/Competitive	No	Hours	No	No	High, for example, [58]	High, for example, [47]	Yes	E or NSI	No, but mAbs can be purchased from Millipore.
Immunoblot using whole viral antigen	Yes	Hours	No	Yes	Moderate, for example, [94]	Depends on the protein targeted, for example, [94]	No	E, prM and NSI	No
Lateral Flow	IgM only	Minutes	No	Yes	High 98.8% [133]	High 95.3% [133]	No	E protein	Yes, for example, RapidWNV [133]
MIA	Yes	Hours	No	Yes	High	Moderate, for example, high specificity for known negative samples, but some cross-reactivity with other flaviviruses [134] High specificity for NS5 MIA [63]	Yes	E protein [135] NS5 [63]	Yes

Is the assay normally set up to be specific for the species being tested, for example, horse, human, and so forth?

* The sensitivity and specificity of the assays depends on the standard to which they were compared.

3.2. Recent Platform and Serological Assay Developments

3.2.1. High-Throughput, Rapid Microneutralisation Assays.

An automated colorimetric microneutralization assay has recently been described for the detection of, and differentiation between, WNV and SLEV infections in humans [156]. Of the 152 PRNT-confirmed negative, WNV-positive or SLEV-positive sera, there was concordance between PRNT and the rapid microneutralisation assay for all samples except one. The advantages of this assay over traditional PRNT are that the testing duration is significantly reduced and the assay can be performed in a 96-well format, enabling 8 dilutions of each sample to be analysed simultaneously. Like PRNT, neutral red is used to stain live cells; however, in this automated assay, the stained live cells are solubilised and the optical density quantified using a plate reader. The obvious drawback to this assay is that the handling of live virus is still a necessity.

3.2.2. Lateral Flow. The FDA has recently approved a lateral flow device for the diagnosis of WNV infection in humans [133]. This assay has significant advantages over ELISA (which can take several hours to perform) in that a result is obtained within 15 minutes [157]. Lateral flow assays routinely consist of antigens or antibodies immobilised on nitrocellulose strips and utilise gold particles or coloured latex as reporter molecules. The assay is commonly housed in a plastic or cardboard cassette. In the case of the WNV lateral flow assay, anti-WNV IgM antibodies in patient serum form a tertiary complex with biotinylated anti-human IgM, recombinant WNV E protein, and an anti-E mAb which is coupled to colloidal gold particles. This complex is then captured by immobilised streptavidin on the nitrocellulose strip to form a pink line. This assay displayed 98.8% sensitivity and 95.3% specificity, as compared to other predicate assays [133]. However, such devices are most suitable for situations where only a small number of tests are to be performed and where qualitative results are acceptable.

3.2.3. Microsphere Immunoassay (MIA). Microsphere immunoassay (MIA) is a bead-based microfluidic system in which an antigen is attached to encoded microbeads that can be identified using a fluorescence-activated cell sorting system (FACS). Target analytes that bind the beads during the assay procedure are detected using a fluorescent molecule. MIA offers two advantages over other serological assays such as ELISA: firstly, it is possible to multiplex the assay such that one serum can be assessed for reactivity to many antigens in a single run. Secondly, MIA offers higher sensitivity through the use of fluorescence [158]. In one study using MIA, WNV NS3 and NS5 antigens were assessed for the specific detection of recent WNV infections and to differentiate those patients that had been vaccinated against flaviviral infection [63]. The NS5 MIA showed 92% sensitivity ($n = 61$) for PRNT-confirmed WNV positive samples that were collected 7 to 77 days postsymptom onset, while none of the samples from JEV-vaccinated individuals were positive and only 5% of the samples from YFV vaccinated individuals were

positive, indicating good diagnostic specificity of the assay. Further, when assessed for cross-reactivity with DENV- and SLEV-positive samples, there was 9% and 5% false positives observed, respectively, for the NS5 MIA. At the time of this study's publication, it was thought that NS5 could be used to detect antibodies from patients with recent WNV infections; however, a subsequent comprehensive study using the same platform showed that 77% of sera tested showed the presence of anti-NS5 antibodies after 90 days and 13% of patients retained anti-NS5 antibodies after one year [64].

The MIA platform also enabled the simultaneous assessment and validation of various WNV antigens for the detection of WNV antibodies in horse sera [159] and has also been validated for the detection of anti-WNV and anti-SLEV IgM antibodies in human serum [134]. Rather than NS3 or NS5 antigen, this assay is based on the reactivity of serum antibodies to antigen captured by the anti-E monoclonal antibody, 6B6C-1 [134]. Many laboratories in the USA have adopted this assay for WNV diagnosis [134, 148]. One obvious drawback of the MIA platform is the requirement for expensive, specialised equipment.

3.2.4. Biosensors and Microfluidic Systems. The design of biosensors and microfluidic devices is targeted to field use and point of care. Some are based on dipstick and lateral flow formats, while other newer concepts use microcapillaries to direct the flow of biological samples and assay reagents through various chambers in the device. However, these are just two examples of platforms which fall into this category. In terms of advances in flaviviral research in this area, Teles (2011) provides a comprehensive critical review of biosensor-based assays for the diagnosis of Dengue infection [160]. Similarly a microfluidic system based on virus-coated magnetic beads for the detection of IgM and IgG antibodies against DENV within 30 minutes has been described by Lee et al., (2009) [161].

In terms of advances in WNV diagnosis using biosensors and microfluidic systems, a linear, 15 amino acid fragment of domain III of WNV was successfully used as an antigen on an amperometric immunosensor [162]. In a second study, a surface enhanced Raman scattering immunoassay was shown to be highly sensitive for the detection of anti-WNV immunoglobulin [163]. Using gold particles coated with WNV E protein, this assay was capable of detecting 50 pg/mL anti-WNV E immunoglobulin in rabbit serum, which is 400 times more sensitive than a standard direct sandwich ELISA. However, this assay still requires validation with clinical or field sera.

3.2.5. Autologous Red Blood Cell Agglutination Assays. While not a new technology, the autologous red blood cell (RBC) agglutination assay platform has only recently been assessed in a pilot study for the detection of antibodies to WNV. This technology was patented in the early 1990's by AGEN Biomedical Ltd. and had considerable advantages over traditional haemagglutination assays, ELISA and radioimmunoassays due to its ease of use and speed in which specific and sensitive diagnosis could be made [164, 165]. Originally

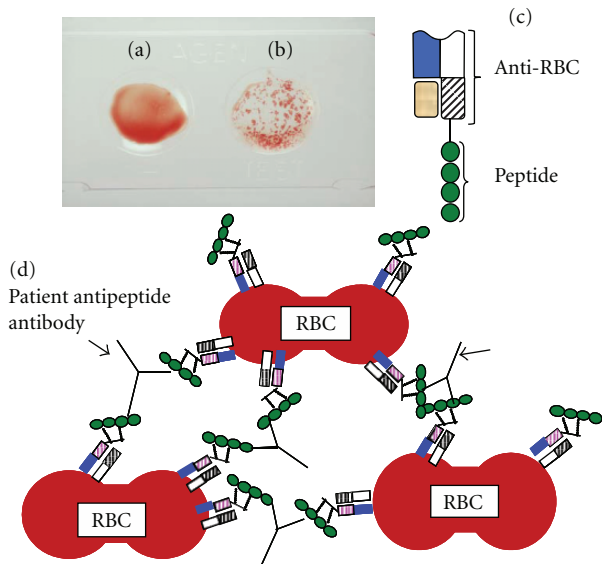


FIGURE 1: Autologous agglutination assay. Agglutination of RBC's (b, d) by the crosslinking of the assay reagent (c) and blood-borne anti-peptide antibodies. Agglutination does not occur in non-immune samples (a).

developed for the detection of anti-HIV antibodies in patient serum, the RBC agglutination assay reagent comprised a mAb with high affinity to human RBCs, chemically coupled to a specific viral antigen [164, 166, 167]. When a drop of infected patient blood was mixed with the mAb-peptide fusion protein, within two minutes, visible agglutination occurred [164, 166, 167] (Figure 1).

The advantages of this agglutination assay technology are many. It is rapid, portable and minimises sample handling. Because the read-out system is the patient's own erythrocytes, there is no need for secondary reagents such as fluorophore- or gold-labelled antibodies, reagent-coated latex beads or fixed heterologous erythrocytes. Further, this assay requires minimal training, specialised equipment, electricity or running water, making it useful for emergency testing, field surveillance testing, mass screening and use in developing countries [164, 167–169]. A functional RBC agglutination assay requires the manufacture of only one component comprised of two biologicals—a peptide and an antibody fragment. In contrast, lateral-flow diagnostics require the optimisation and validation of gold-conjugation technologies and the striping of biologicals onto membranes. These processes are more technically—and time—demanding.

Several groups have explored the production of autologous RBC agglutination reagents by recombinant methods [168, 170–174]. The feasibility of this approach was initially established in a bacterial expression system that used a single chain Fv fragment (scFv) derived from the original anti-erythrocyte mAb recombinantly fused to the HIV peptide [170, 171]. Although functional reagents were produced, the recovery of the reagent required extensive extraction and purification procedures. We recently described the secreted expression of the human-based recombinant autologous

agglutination reagent in mammalian cells, as both single chain (scFv) and intact mAbs [175]. The anti-RBC antibody was fused to a WNV diagnostic tag (WN19) and was shown to be functional in agglutination assays and suitable for diagnosis of WNV infection in horses. However, the reagent was not expressed at high enough levels within the cell culture medium for the true potential of this assay system to be realised. In particular, we aimed to develop a system based on generic vectors into which antigenic peptides could rapidly be incorporated and one in which the culture supernatant could be used for agglutination reactions without further processing. Recombinant expression of these agglutination reagents would enable rapid, on-demand production for minimal cost. This is particularly important for developing countries where outbreaks of new pathogens are frequent and there is a requirement for inexpensive, “point-of-care” assays.

4. Antigen Formulations to Improve Assay Specificity

The specificity of serological assays for the diagnosis of flaviviral infections predominantly relies on the antigen used in the assay. A number of alternative antigens to the complete E protein or total viral antigen have been assessed in recent years, particularly for improving the specificity of WNV seroassays. Some of these studies are listed in Table 2.

4.1. E Protein. The flaviviral E protein is frequently used in serological diagnostic assays [176]. However, some E protein epitopes induce flavivirus cross-reactive antibodies [66, 78] and recent attempts to improve the specificity of assays using E protein-based antigens have focused on using peptides or individual domains of the E protein [136–138]. By eliminating the cross-reactive epitopes in the E protein DII, Roberson et al. (2007) reported a WNV diagnostic antigen that conferred considerably higher specificity to diagnostic assays than its wild-type counterpart [136], reducing the number of false positives by 21–22%. In a different approach, Beasley et al. (2004) developed a recombinant protein based on the E protein DIII (EDIII). When the EDIII subunit protein was used in ELISA, there was clear discrimination of WNV-immune mouse ascitic fluid from mouse immune ascitic fluids generated against JEV, MVEV, SLEV, DENV and YFV [137]. While the EDIII ELISA displayed strong correlation with HI and PRNT for the detection of anti-WNV IgG in field trials with monkey, horse, and human sera, the specificity of the ELISA was not challenged with sera obtained from natural infections with other flaviviruses of the JEV serocomplex. In a more defined analysis of DIII antigens, a linear, 15-amino-acid fragment of this domain was successfully used in ELISA [138] for human WNV infection diagnosis and has also been assessed as an antigen on an amperometric immunosensor [162]. The comparative specificity of these E protein subunit antigens has not been fully investigated. However, in the ELISA, 100% specificity and 67% sensitivity was achieved when compared with a commercial WNV IgG ELISA kit.

TABLE 2: Antigen formulations to improve serological assay specificity.

Antigen	Reference	Assay Format	Serum	Sensitivity	Specificity
E protein: removal of DII cross-reactive epitopes from virus-like particles. One antigen for WNV and one antigen for SLEV	Roberson et al., 2007 [136]	IgM ELISA	Human. 134 sera including those with disease states classified as SLEV, WNV, JEV or DENV	Mutant WNV antigen: 100%* Mutant SLEV antigen: 100%#	Mutant WNV antigen: 88.6%* versus 70.9% for WT\$ Mutant SLEV antigen: 88.7%# versus 69.1% for WT\$
WNV E protein subunit (r-EIII)	Beasley et al., 2004 [137]	WNV ELISA	Polyclonal mouse immune ascitic fluid- anti-WNV, -JEV, -SLEV, -MVEV, -DENV, -YFV Human: PRNT/HI confirmed WNV pos ($n = 11$); WNV neg ($n = 4$, plus 1 SLEV pos) Horse: 57 PRNT/HI confirmed	Strong reactivity of anti-WNV ascitic fluid with r-EIII at 1 : 64 dilution 100%	Negligible reactivity of anti-JEV, -SLEV, -MVEV, -DENV and -YFV ascitic fluid with r-EDIII at 1 : 64 dilution. 100%
WNV E protein peptide DIII (Ep15)	Herrmann et al., 2007 [138]	IgG ELISA	Human. 66 sera including 7 WNV pos, 3 WN neg, 2 DENV pos, 54 unknown.	89% [†] ($n = 35$) 67% [‡]	87.5% [‡] ($n = 16$) 100% [‡]
WNV E protein peptide DI (WN19)	Hobson-Peters et al., 2008, 2011 [60, 61]	Western Blot	Horse. VNT confirmed	69% ($n = 11$ WNV pos)[60] [†] 80% WNV PRNT confirmed field samples ($n = 5$) [61]	100% [†] ($n = 4$ WNV/MVEV neg) Cannot differentiate MVEV-positive sera. 100% MVEV positive sera ($n = 4$) bound WN19.
JEV and DENV prM-native viral antigen	Cardosa et al., 2002 [89]	Western Blot	Human. 16 JEV pos, 22 DENV pos. Fig. 31 JEV pos	DENV assay: 95% JEV assay: 100% JEV assay: 93.5%	DENV assay: 100% JEV assay: 73% DENV assay: 100%
WNV prM-native viral antigen	Setoh et al., 2011 [62]	Western Blot	Horse. VNT confirmed. 16 WNV pos, 6 WNV negative (2 of these Kokobera positive)	87.5%	100%
JEV prM/M peptide	Hua et al., 2010 [139]	ELISA	Rabbit	Peptide bound by anti-JEV serum	Peptide not bound by anti-WNV and -DENV sera.

* Ability to distinguish WNV infections from other arbovirus infections.

\$WT = Wild Type.

Ability to distinguish SLEV infections from other arbovirus infections.

[†] After removal of 6 strong IgM positives and classifying equivocal results as false negative (for sensitivity) or false positive (for specificity).

[‡] Upon comparison with commercial IgG kit.

[‡] Percentages based on those serum samples that did not cross-react with the scFv carrier protein.

Our laboratory has identified a peptide (WN19) in Domain I of the E protein which has been successfully trialled in a small field study for the detection of anti-WNV antibodies in horse sera using Western blot [60, 61]. The peptide WN19 sequence encompasses the WNV envelope protein glycosylation site at position 154 and it was shown that the carbohydrate moiety was required for recognition of peptide WN19 by most WNV-immune horse sera assessed. The disadvantage of compartmentalising any target antigen is that there is often a reduction in sensitivity. This is evidenced by our data where a number of samples with VNT titres ≥ 160 reacted only weakly with peptide WN19, or not at all [60]. In Western blot, peptide WN19 was also detected by horse sera containing MVEV-neutralising antibodies.

4.2. prM. The prM antigen has been successfully used to differentiate DENV and JEV infections in humans using Western blot [89]. Western blot analysis has also shown that prM is consistently recognised by WNV-immune horse serum [62]. An obstacle to the use of the prM antigen for differentiating flaviviral infections is a difficulty in expressing a correctly folded protein. High-level expression of membrane proteins is inherently difficult and compartmentalising the prM protein so that only the soluble pr peptide is expressed, results in the elimination of the epitope(s) recognised by WNV-immune serum [62]. A detailed analysis on the use of prM to differentiate infections caused by viruses of the JEV serogroup is yet to be published.

A continuous JEV prM/M peptide that is recognised by anti-JEV rabbit serum and not by anti-WNV or -DENV serum has also been identified. However, the efficacy of this peptide in assays with clinical sera is yet to be determined [139].

5. Conclusions

The continued spread of flaviviruses worldwide warrants the need for rapid serological assays of increased specificity. In countries such as Australia, where multiple arboviruses infecting horses and humans can cocirculate, as well as the increased prevalence of other encephalitic-disease causing viruses such as Hendra, there is a need for the development of rapid, pen-side immunoassays. While ELISA is ideal for high-throughput testing, this assay format is not suitable for rapid point of care and veterinary pen-side testing. The development of rapid, portable flavivirus immunoassays has been impaired by the cross-reactive immune response generated against these pathogens in vertebrates. The difficulty lies in identifying a single antigen that confers both high sensitivity and specificity to the immunoassay. Multiplex assays such as MIA are ideal for giving an accurate profile of the immune response against various flaviviral antigens simultaneously. It is feasible that this platform could be used to test serum samples against NS3, NS5, E domain subunits such as DIII and the DI WN19 peptide, NS1 and prM in a single assay. However, application of flaviviral antigens to a multiplexed rapid, point-of-care device such as lateral flow has not yet been reported. Devices for multiplexed ABO blood typing

agglutination assays such as the patented EldonCard system or microfluidic device [177] may be readily adaptable for use with the autologous agglutination system, particularly if larger flaviviral antigens such as E DIII or NS1 can be recombinantly fused to the RBC-binding antibody.

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