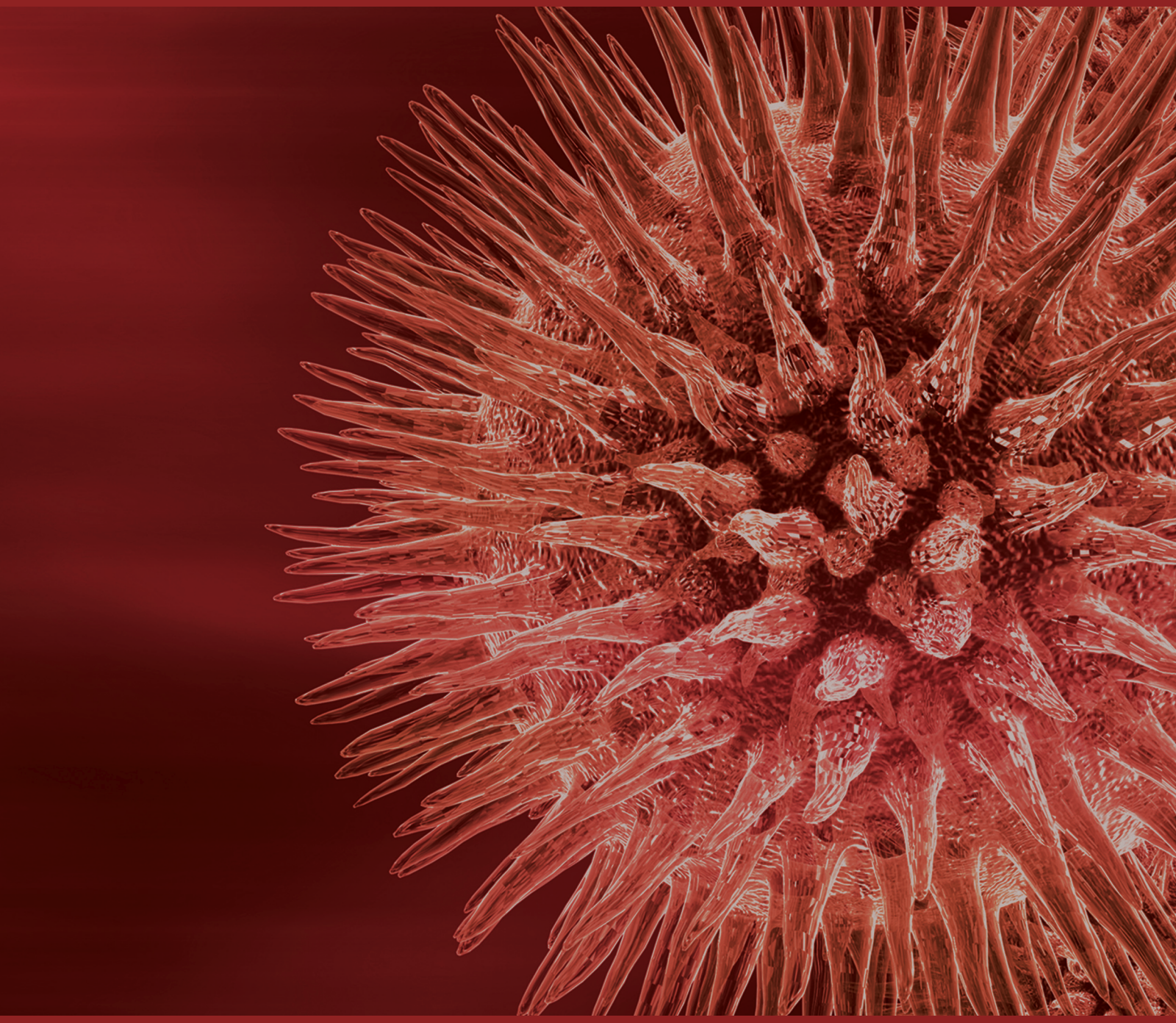
Journal of Biomedicine and Biotechnology

Advances in Arbovirus Surveillance, Detection and Diagnosis

Guest Editors: Roy A. Hall, Bradley J. Blitvich, Cheryl A. Johansen, and Stuart D. Blacksell



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Editorial

Advances in Arbovirus Surveillance, Detection and Diagnosis

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Arthropod-borne viruses (arboviruses) are responsible for many important vector-borne diseases of man and animals including dengue, yellow fever, Japanese encephalitis, tick-borne encephalitis, Rift Valley fever, West Nile fever, chikungunya, Ross River disease, and bluetongue. Detection or accurate prediction of virus activity in vector populations and specific diagnosis of infection in the human or animal host are crucial components of effective control and treatment strategies and facilitate early warning of potential or existing outbreaks and initiation of vector management and/or vaccination programs.

One of the key elements of the control of arbovirus transmission is early detection of virus activity or increased virus activity in vector populations. Surveillance programs designed to monitor these parameters provide an early warning system of increased risk of transmission and disease outbreak. One approach routinely used by groups to monitor virus activity is sentinel animal surveillance. This is particularly useful for veterinary or zoonotic arboviruses where domestic animals (e.g., cattle, pigs or chickens) can be effectively employed. While serological monitoring of strategically positioned flocks or herds can provide early warning of virus transmission in a specific region, there are many difficulties with this system: the costs of maintaining the animals (particularly in remote areas), lack of specificity of serological assays, and the ability to only target viruses that infect the selected sentinel animal or those that are transmitted by vectors that feed on the sentinel host. To address these problems, a new approach (reviewed by A. F. van den Hurk and colleagues in this issue) has recently been applied to the specific detection of virus activity in mosquito populations, which is particularly useful for remote locations. The specific detection of viral RNA expectorated by infected mosquitoes feeding on a sugar-coated matrix has enabled these investigators to collect samples over prolonged periods in remote locations and specifically identify viruses carried by any mosquito feeding on the sugar bait. The potential application of this system to a wide variety of arbovirus surveillance scenarios is very promising.

Another important component of arbovirus surveillance is the detection of virus in arthropod vector populations. Traditional methods include trapping vectors such as mosquitoes, ticks, and midges, identifying them to species level and analysing vector pools for known viruses of interest. Virus isolation by inoculating mice, embryonated eggs, or colonized vector species has largely been replaced by *in vitro* methods (e.g., inoculation of arthropod and vertebrate cell lines), but is still the most effective means for monitoring virus activity for some viruses. However, the enhanced technologies for the specific detection of viral nucleic acid, such as multiplexed real-time PCR protocols, provide more rapid, sensitive, and specific approaches for detecting virus activity in vector populations. Furthermore, the recent application of next-generation sequencing technologies to rapidly analyze

nucleic acid of unidentified viral isolates provides a revolutionary approach for the discovery and genetic characterization of new vector-borne viruses.

Monitoring arthropod vector populations is also an important component of arbovirus surveillance, particularly for detecting an increase in known vectors or the introduction of a new species into an area (e.g., recent incursions of *Aedes albopictus* into Europe and Australia). In addition to the labor-intensive methods of trapping and identifying vectors by morphology or genetic analysis, new strategies such as satellite-based remote sensing of vector breeding sites and assessment of the risk of virus transmission based on proximity to human or animal habitation (see the paper by Susan N. Rossmann and colleagues in this issue) can rapidly provide highly useful data on a very large scale.

Accurate and timely diagnosis of arbovirus infections is also crucial to ensure appropriate patient management, for the reporting of virus activity in a region and to allow instigation of control strategies such as vector management, vaccination, and public awareness campaigns. Serological assays are predominantly used for this purpose; however, many problems exist with traditional methods including the use of live virus for antigen production and plaque reduction neutralization tests, lack of specificity due to cross-reactivity between related viruses, and the costs of running individual assays for each viral antigen used. However the use of recombinant viral antigens (whole proteins, domains or peptides) in multiplexed formats such as microsphere immunoassays provides rapid, sensitive, and specific analyses that can be coupled to large-scale antigen production methods (see the paper by J. He and colleagues in this issue) and high-throughput robotic systems in the diagnostic laboratory. For some viral infections, enhanced real-time PCR protocols provide a more specific alternative with the detection of viral nucleic acid in human or animal samples (see the review by N. Johnson and colleagues in this issue). The availability of several commercial point-of-care assays, particularly for dengue and West Nile viruses, also provides a useful tool for the clinician or veterinarian treating the patient on presentation at the clinic (see the reviews by S. D. Blacksell and J. M. Hobson-Peters in this issue).

The new technologies and novel approaches referred to above and elaborated on in the papers in this special issue provide an excellent platform for the advancement of arbovirus surveillance and diagnosis. Thorough evaluation of their effectiveness against traditional methods in the field and clinic and their application to different arboviral diseases will allow their routine implementation and unleash the potential to vastly improve our ability to manage these diseases in the future.

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Review Article

Commercial Dengue Rapid Diagnostic Tests for Point-of-Care Application: Recent Evaluations and Future Needs?

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Dengue fever, dengue haemorrhagic fever, and dengue shock syndrome (DF/DHF/DSS) are tropical diseases that cause significant humanitarian and economic hardship. It is estimated that more than 2.5 billion people are at risk of infection and more than 100 countries have endemic dengue virus transmission. Laboratory tests are essential to provide an accurate diagnosis of dengue virus infection so that appropriate treatment and patient management may be administered. In many dengue endemic settings, laboratory diagnostic resources are limited and simple rapid diagnostic tests (RDTs) provide opportunities for point-of-care diagnosis. This paper addresses current issues relating to the application of commercial dengue RDTs for the diagnosis of acute dengue virus infection, recent diagnostic evaluations, and identifies future needs.

1. Introduction

1.1. The Burden of Dengue. Dengue fever, dengue haemorrhagic fever and dengue shock syndrome (DF/DHF/DSS) are a group of tropical disease states that cause significant humanitarian and economic hardship. DF/DHF/DSS are caused by the dengue virus, which belongs to the flavivirus genus of the family *Flaviviridae*. The flavivirus genus includes approximately 70 viruses of which there are 3 antigenic complexes; the Japanese encephalitis virus, tick-borne encephalitis, and the dengue virus complexes [1]. There are four distinct serotypes of dengue virus (i.e., dengue virus serotypes 1–4) which all cause clinical disease. It is estimated that more than 2.5 billion people are at risk of infection and more than 100 countries have endemic dengue virus transmission. While exact numbers of dengue virus cases are not available, for the period 2000–2004, the annual average was 925,896 cases, which was almost double when compared to the 479,848 cases that were reported for the period 1990–1999 [2]. About 250,000 to 500,000 cases of DHF are reported annually although the true incidence is not really known [3]. In dengue endemic regions which include countries in Asia and the Americas, the burden of dengue is approximately 1,300 disability-adjusted life years (DALYs) per million population, which is similar to the disease burden of other childhood and tropical diseases, including tuberculosis, in these regions [2].

1.2. Why Do We Need Rapid Diagnostic Tests (RDTs) and Who Controls the Quality? Laboratory tests are essential to provide an accurate diagnosis of acute dengue virus infection at patient presentation to a clinical setting so that appropriate treatment and patient management may be administered. In many dengue endemic settings, laboratory diagnostic resources are limited and simple rapid diagnostic tests (RDTs) provide opportunities for point-of-care diagnosis. The characteristics of the ideal diagnostic test are said to be defined by the ASSURED criteria: (1) Affordable by those at risk of infection; (2) Sensitive (few false-negatives); (3) Specific (few false-positives); (4) User-friendly (simple to perform and requiring minimal training); (5) Rapid (to enable treatment at first visit) and Robust (does not require refrigerated storage); (6) Equipment-free; (7) Delivered to those who need it [4].

The need for simple point-of-care diagnostic tests has led to the proliferation of antibody-based RDTs for tropical infections such as dengue, leptospirosis, melioidosis, and malaria using the immunochromatographic test (ICT) format. Unfortunately, many dengue antibody-based RDTs had substandard performance for the diagnosis of acute dengue at patient presentation which leads to the large-scale evaluations funded by independent international organisations such as World Health Organization (WHO) [5, 6] to determine which are the best of the commercial assays. Until these large-scale evaluations were performed, many "backyard" manufacturers marketed their products via the internet with little or no independent verification of the manufacturer's performance claims. Results from these evaluations have provided independent performance details to consumers, and poor results challenged manufacturers to improve RDT performance. The RDT market still remains largely unregulated with the exception of the USA where in vitro devices require approval by the Food and Drug Administration (FDA) however, in the absence of national regulations, high-quality, independent assessments in peer-reviewed journals provide the best guide to quality.

1.3. Rapid Test Formats. Immunochromatographic tests for the detection of dengue virus nonstructural protein 1 (NS1) antigen, IgM, IgG, and IgA antibodies have been developed by a number of commercial companies and have found wide application because of their ease of use and rapidity of results. These dengue RDTs are presented in the form of a lateral flow cassette that allows the flow of sample in a horizontal plane or a wick-style test that is performed in a tube and draws sample vertically by capillary action. Dengue virus RDTs use a cocktail of dried antigens and colloidal gold-labelled monoclonal antibodies (specific for dengue NS1 antigen, IgM, IgG, or IgA antibodies) on a pad at the head of a nitrocellulose strip which is impregnated with either antidengue NS1 antigen, IgM, IgG, or IgA antibody lines. Test sample and running buffer are added to the pad which releases the colloidal gold from the pad and facilitates the mixing of the patient sample with the gold complex and facilitates the migration of the reagents and sample by capillary action along the nitrocellulose strip towards the anti-human IgM, IgG, or IgA antibody lines. The presence of dengue virus NS1 antigen or IgM, IgG, or IgA antibodies is signified by the development of maroon lines in the location of the antibody lines. The dengue RDTs have the advantage that they can be performed in approximately 10–15 minutes and requires no specialized equipment or training, making them ideal for low-technology environments; however, this format has the weakness of subjective reading by the operator.

1.4. Rapid Test Evaluation Methodologies. Diagnostic assays are usually evaluated in terms of sensitivity and specificity that is calculated using a 2×2 cross-tabulation where a "gold standard" result (the peer-acknowledged, most accurate test) or reference standard result (normally, the test most widely used) is compared with the rapid test to determine diagnostic accuracy. A test that is 100% sensitive and specific is deemed to be a perfect test. The choice of gold standard assay, final patient result, or comparison with nonreference assay as the reference comparator can have a large influence on the final diagnostic accuracy results. Unfortunately, there is a lack of conformity in the evaluation methodologies and choice of reference assays for dengue RDT diagnostic assessments; however, it should be noted that this issue is not confined only to dengue diagnostics. Guidelines for the evaluations of dengue diagnostic assays have recently been published [7] which is hoped will provide a framework for a uniform approach to diagnostic assessments.

1.5. Dynamics of Dengue Virus Infection: Implications for Diagnosis. The dynamics of dengue virus infection have a potentially large influence on the interpretation of RDTs (Figures 1 and 2). Following the initial infection, the dengue virus replicates to high titers in the blood before patients are unwell enough to present to a physician, with viraemia peaking at the time or shortly after the onset of symptoms. Virus remains detectable in the blood for up to 2 to 12 days after the onset of symptoms and may reach titers of up to 1×10^8 50% infectious doses (ID_{50}) per mL or $10^{8.5}$ 50% mosquito infectious doses (MID_{50}) [8, 9]. During the viraemic phase of dengue infection, NS1 antigen is produced concomitantly during the virus replication process. NS1 antigen is a 46- to 50-kilodalton glycoprotein highly conserved by all dengue serotypes and is expressed in either membrane-associated or secreted forms [10, 11]. Soluble NS1 circulates in the serum of patients during the viraemic phase of infection of dengue virus infections and hence is an excellent diagnostic target for acute dengue diagnosis. Difference in the persistence of soluble NS1 antigen in serum between primary (5-6 days post-onset of illness) and secondary dengue infections (6–12 days) has been, noted and it is hypothesised that the presence of anti-NS1 antibodies, that are more frequently detected in dengue secondary infection [12], modulates the formation of antibody-antigen complexes which impede the ability of the test to detect free NS1 antigen [13, 14].

An understanding of the features of the host humoral immune response to dengue virus infection also is important for the interpretation of dengue RDTs. Dengue IgM antibodies are a reliable marker of recent infection but not necessarily acute infection. In primary dengue virus infections, IgM antibodies develop following the decline of viraemia between days 3–5 after the onset of infection using very sensitive detection methods [15, 16] and reache peak levels approximately 2 weeks later [17]. Persistence of IgM antibodies following primary infection using linear regression method has been estimated at 179 days (95% confidence interval, 155 to 215 days) [18]. In dengue endemic settings where in secondary infections dominate, IgM antibodies may be detectable by RDTs as soon as after 2-3 days of infection [19–21] and peak IgM antibody levels are usually lower than in primary infections [8, 22]. Persistence of IgM antibodies following secondary infection is estimated to be shorter than that of primary infections at 139 days (95% confidence interval, 119 to 167 days) [18], and other published estimates of IgM antibody persistence range from 2 months to 6

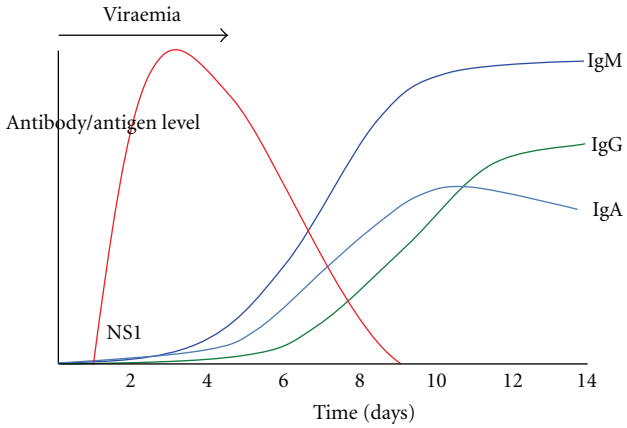

Figure 1: Graphical representation of the kinetics of dengue NS1 antigen and IgM, IgG, and IgA antibodies during a primary dengue infection.

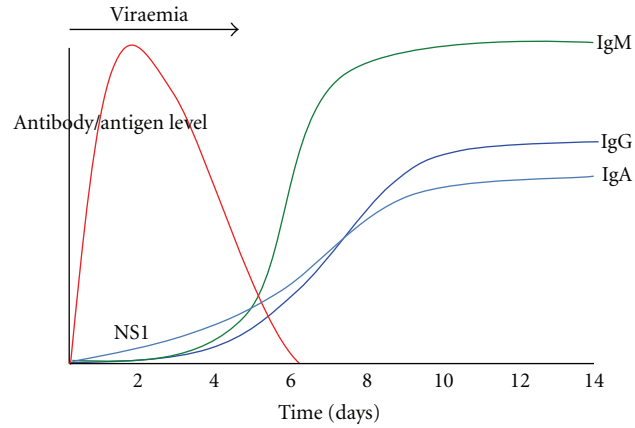

Figure 2: Graphical representation of the kinetics of dengue NS1 antigen and IgM, IgG, and IgA antibodies during a secondary dengue infection.

months [8, 23]. The IgG antibody response develops a few days after the onset of the IgM antibody response and is serotype specific and may persist for many years following a single infection. Secondary dengue virus infections generate an anamnestic IgG antibody response that is characterised by a rapid rise in IgG antibodies detectable at days 4-5 of illness [16] which is much sooner than the normal IgG antibody response of a primary infection. Dengue IgA antibodies have been reported in serum of dengue fever patients only between days 8 and 11 after onset of fever [17]. However, in the more severe forms of the disease, IgA antibodies were reportedly undetectable in DHF patients in the acute phase of illness (days 2 to 4) but increased in the following early convalescent phase (days 5 to 14) and, in DSS patients, increased to the highest levels on days 8 to 11 and slightly decreased 15 days after onset of fever [17]. In primary dengue infection, the onset of detectable levels of IgA antibodies has been reported on average at 5.5 days after onset of fever, and, in secondary infection, IgA antibodies increased slowly during the first days of the study [22]. The rates of positivity for IgA antibodies in serum were reportedly significantly higher in secondary infections than in primary infections (100 versus 84.6%) [24].

2. Diagnostic Evaluations

2.1. Performance of Antibody-Based Diagnostics. Dengue IgM and IgG antibody-based RDTs have been in existence for approximately 15 years in various forms by different manufacturers (see Table 1 for description of contemporary commercial dengue IgM and IgG-based RDTs). Multiple diagnostic evaluations were performed from the late 1990s to the mid-2000s [25–29]; however, significant heterogeneity in evaluation methodologies makes direct comparison of diagnostic accuracy problematic [30]. In 2005, WHO Western Pacific Regional Office (WPRO) commissioned an independent evaluation of dengue IgM RDT performance for acute diagnosis as well as an evaluation of storage conditions using stored samples from Thailand [6] and prospectively recruited patient samples from Lao PDR [31]. The results for the majority of the evaluated dengue IgM antibody RDTs demonstrated a lack of sensitivity for acute dengue infection diagnosis that ranged from 6.4% to 65.3% and specificities ranged from 69.1% to 100% (selected results are presented in Table 2). Subsequently, WHO sponsored a multicentre evaluation of dengue IgM antibody RDTs where test sensitivities ranged from 21% to 99% and specificities ranged from 77% to 98% when compared with reference ELISAs [5]. Subsequent evaluations of the Panbio Duo IgM RDT reported sensitivities ranging from 65.3 to 81.8% and specificities ranging from 75.0 to 97.6% (Table 2). Recent assessments of the Standard Diagnostics (SD) IgM RDT demonstrated improved sensitivity compared to the very poor 1st generation device results from the WHO study [6] (21.8%), with 2nd generation device having reported sensitivities of 53.5% [21] and 79.2% [19]. The improvement in the 2nd generation SD IgM RDT is evidence of the positive feedback of diagnostic evaluations to the manufacturers.

2.2. Performance of NS1 Antigen-Based Diagnostics. The most important development in dengue diagnostics in recent years is the advent of the specific detection of dengue virus NS1 antigen (see Table 1 for description of contemporary commercial dengue NS1 antigen RDTs). Dengue RDTs that detect NS1 antigen employ a number of serotype-specific anti-NS1 monoclonal antibodies to capture and detect soluble NS1 antigen in serum, plasma, or blood. The first commercial assays for dengue NS1 antigen detection used the ELISA format [14, 36] and demonstrated excellent sensitivity and specificity in the early phase of infection that diminished with falling viraemia levels. The major commercial diagnostics manufacturers, Panbio, Biorad, and SD, have all developed RDT-based NS1 antigen tests, and all have equivalent ELISA-based assays. The diagnostic performance of NS1-based RDTs from the abovementioned manufacturers has been evaluated in numerous geographical locations with the results from 21 diagnostic evaluations presented in Table 3. Twelve studies evaluated the Biorad STRIP RDT

Table 1: Characteristics of dengue rapid diagnostic tests mentioned in this paper.

Manufacturer	Product name	Analytes	Storage temperature (°C)	Quoted accuracy (Sn/Sp[a])	Sample[b]	$1^0/2^{0c}$	Format[d]	Sample volume (ul)	Maximum time (min)[e]
Merlin	Dengue Fever IgG and IgM Combo Device	IgM/IgG	2–30°C	IgM 96/98 IgG 97/98	S/P/WB	Yes	LF	1	30
Standard Diagnostics	BIOLINE Dengue Duo NS1 antigen and IgG and IgM Combo Device	NS1 Ag IgM/IgG	1–30°C	NS1-Ag 92.8/98.4 IgM/IgG 99.4/93.0	S/P/WB	Yes	LF	NS1-Ag 100 IgM/IgG 10	20
Biosynex	Immunoquick Dengue Fever IgG and IgM	IgM/IgG	2–30°C	IgM 97.6/98.3 IgG 95.2/96.6	S/P/WB	Yes	W	1	20
Biorad	STRIP	NS1 Ag	2–8°C	NS1-Ag 92.3/98.8	S/P	No	W	50	15
Alere	Panbio Dengue Early Rapid Kit	NS1 Ag	2–8°C	Not stated	S	No	LF	50	15
Alere	Panbio Dengue Duo Cassette	IgM/IgG	2–8°C	S[b] convalescent—1^{0c}−85.1/91.6; 2^0−98.8/91.6 P acute—1^0−58.3/45.0; 2^0−100/45.0 WB acute—1^0−71.4/91.2; 2^0−77.4/91.2 WB convalescent—1^0−78.6/85.3; 2^0−100/85.3	S/P/WB	Yes	LF	10	15
MP Diagnostics	ASSURE	IgA	2–28°C	Not stated	S/P/WB	No	LF	25	15–20

Sn/sp[a]: sensitivity/specificity.
[b]S–serum; P: plasma; WB: whole blood.
[c]Primary and secondary infections: manufacturer claims RDT can differentiate
[d]W: wickstyle; LF: lateral flow.
[e]Maximum time in minutes to confirm a negative result.

Table 2: Description of selected recent diagnostic assessments of dengue IgM, IgA, and IgG antibody RDTs.

Assay	Study	Year	Location	Sample timing	Reference comparator	Antibody target	Sensitivity (95% CI)	Specificity (95% CI)
SD Bioline Dengue IgM (1st Generation)	Blacksell et al. [6].	2006	Thailand	Admission	AFRIMS MAC and GAC-ELISA paired samples	IgM	21.8 (17.4–26.7)	98.8 (95.7–99.9)
	Nga et al. [32]	2007	Vietnam	3 weeks illness	Focus IgM/IgG ELISA	IgM	10.6 (6.0–18.0)	99.0 (94.3–99.8)
						IgG	90.4 (84.6–94.2)	88.9 (77.8–94.8)
SD Bioline Dengue Duo (2nd Generation)	Wang and Sekaran [21]	2010	Malaysia	1–15 days	Virus isolation, RT-PCR, rising titer in a paired samples using MAC ELISA	IgM	53.5	100
	Blacksell et al. [19]	2011	Sri Lanka	Median 5; IQR 2–7 days illness	AFRIMS MAC and GAC-ELISA paired samples	IgM	79.2 (70.5–87.2)	89.4 (83.5–93.7)
Panbio Dengue Duo cassette	Blacksell et al. [6]	2006	Thailand	Admission	AFRIMS MAC and GAC-ELISA paired samples	IgM	65.3 (59.9–70.5)	97.6 (93.9–99.3)
	Nga et al. [32]	2007	Vietnam	3 weeks illness	Focus IgM/IgG ELISA	IgM	67.3 (57.8–75.6)	91.7 (84.4–95.7)
						IgG	66.4 (58.4–73.6)	94.4 (84.9–98.1)
	Moorthy et al. [33]	2009	South India	Not stated	Panbio MAC and GAC-ELISA	IgM	81.8	75.0
						IgG	87.5	66.6
	Blacksell et al. [19]	2011	Sri Lanka	Median 5; IQR 2–7 days illness	AFRIMS MAC and GAC-ELISA paired samples	IgM	70.7 (60.7–79.4)	80.0 (73.0–85.9)
Merlin IgM	Blacksell et al. [19]	2011	Sri Lanka	Median 5; IQR 2–7 days illness	AFRIMS MAC and GAC-ELISA paired samples	IgM	72.7 (62.9–81.2)	73.8 (66.2–80.4)
Biosynex IgM	Blacksell et al. [19]	2011	Sri Lanka	Median 5; IQR 2–7 days illness	AFRIMS MAC and GAC-ELISA paired samples	IgM	79.8 (70.5–87.2)	46.3 (38.3–54.3)
MP Diagnostics ASSURE	Tan et al. [34]	2011	Singapore	Acute	NS1 Ag and MAC ELISAs	IgA	86.7	86.1
	Ahmed et al. [35]	2010	Bangladesh	Acute and Convalescent	NS1 Ag and MAC ELISAs	IgA	99.4	100

for the diagnosis of acute dengue infection using admission samples, and the results demonstrated considerable variation in sensitivity (49.8%–98.7%) but the specificities reported were more consistent with all being >90%. For 25% (3/12) of the studies, the sensitivity was >89%; however, all of these studies used a skewed comparator of either virus isolation, RT-PCR, or NS1-ELISA and did not examine the possibility of false-negative results by testing paired serum samples to examine for dynamic rise in serological assays such as IgM (MAC) or IgG (GAC) capture ELISAs. Studies that used a more representative combination of virus or antigen detection and serology as reference comparators gave sensitivities for the Biorad STRIP RDT of between 49.4% [37] and 78.9% [38]. The SD Bioline Dengue Duo RDT NS1 antigen detection strip was evaluated for acute dengue diagnosis in four studies (Table 3) with consistently high specificity estimates (96.7–100%) and sensitivities that ranged from 48.5% [19] to 65.4% [21] with the studies either using a combination of virus detection and serology [21, 39, 40] as comparators or serology alone [19]. The Panbio Early Rapid RDT NS1 antigen detection strip was evaluated in two studies using samples from three locations (Vietnam, Malaysia, and Sri Lanka) with high specificity estimates (92.5–96.7%) and sensitivities that ranged from 58.6% [19] to 69.2% [20] for admission samples. A few studies have compared the diagnostic accuracy of NS1 antigen RDTs in primary and secondary dengue infections. Generally, NS1-antigen RDTs demonstrated higher sensitivities in primary infections when compared to secondary infections [39, 41–43]; however, other studies have reported the opposite [37]. As mentioned earlier, it has been suggested that this phenomenon of lowered NS1-antigen detection in dengue secondary infections is caused by NS1 antigen complexing with anti-NS1 antibodies [12–14]. This observation results in an inability of the NS1-antigen RDT to detect complexed NS1 antigen and should not be interpreted insensitivity on the part of the diagnostic assay.

2.3. Combination of NS1 Antigen and IgM Antibody Results. To take advantage of the entire temporal spectrum of patient presentation during the acute phase of dengue infection (usually from 1 to 7 days after onset of fever), NS1 antigen and IgM antibody results have been combined in a Boolean manner using AND/OR operators. NS1 antigen is present in the serum in the early phase of infection; however, patients that present late in the course of infection may have undetectable levels of NS1 antigen. Dengue IgM antibodies are usually present following 2–5 days of infection, and, by combining the results of dengue NS1 antigen and IgM antibody testing, accurate diagnosis during acute presentation is afforded. This approach was initially described [48] by combining the results of the Panbio NS1 antigen and IgM antibody ELISAs in Lao PDR. Subsequently, studies [19–21] have combined NS1 antigen and antibody results to exploit the temporal diagnostic characteristics of each analyte (Table 4). Combining the SD Bioline Dengue Duo RDT NS1 antigen and IgM antibody results for acute diagnosis, the sensitivity ranged from 75.5% [39] to 92.9% [19] and the specificity from 88.8% [19] to 100% [39]. Combining the Panbio Early Rapid RDT NS1 antigen and IgM antibody results, the sensitivity ranged from 89.0% to 89.9%; the only specificity reported was 75.0% [19].

3. Future Needs for Dengue Rapid Tests

Despite recent improvements in the RDTs, there are a number of issues that require further investigation.

3.1. Standardisation of Diagnostic Assessments. The aforementioned lack of conformity in the evaluation of dengue RDTs remains a problem and a standardised approach must be considered when performing diagnostic assessments so that there is comparability between studies. The recently published guidelines for the evaluations of dengue diagnostic assays [7] should be followed whenever possible.

3.2. Determining Geographical Variation and Practical Aspects of Test Use. To further strengthen the current diagnostic accuracy estimates, prospective recruitment studies are required in different dengue-endemic locations where there are variations in dengue infection status (primary versus secondary), days of illness, and prior to presentation. Further studies are also required to examine some of the more practical aspects of dengue RDT performance that includes the influence of operator training, interoperator variation, and ease of use of the assays. Where case-control studies are to be performed using characterised archived samples, consideration should also be given to the appropriateness of the composition of dengue patient (serotypes, days of illness) and non-dengue patient (other dengue-like fevers) cohorts.

3.3. Differentiation of Primary versus Secondary Dengue Infections. Patients with secondary or later dengue infections are considered to have an increased risk of the more severe forms of the disease, and therefore the accurate detection of primary and secondary at presentation to a clinical facility may become a promising patient management tool. Some manufacturers of antibody-based RDTs claim their assays are able to differentiate primary and secondary dengue virus infections using the following criteria: (1) acute primary dengue virus infection defined as an IgM-positive and IgG-negative (IgM+/IgG−) result and (2) acute secondary dengue virus infection defined as IgM-positive and IgG-positive (IgM+/IgG+) or IgM-negative and IgG-positive (IgM−/IgG+) results. Examination of the voracity of the manufacturer's claims is limited to a few studies [6, 19, 31] and is often conducted in dengue endemic settings where there a dominance of secondary dengue infections. Such studies have demonstrated that RDTs cannot reliably differentiate the different dengue infection states.

3.4. Sample Type and the Effect of Anticoagulants and Preservatives. Many manufacturers allow the use of serum, plasma, or whole blood (Table 1) for use in dengue RDTs in both antigen and antibody formats. Interestingly, the Panbio Duo antibody RDTs only permits the use of serum. Unfortunately, there is little quantitative evidence that all sample types

Table 3: Description of selected recent diagnostic assessments of dengue NS1 RDTs.

Assay	Study	Year	Location	Sample timing	Reference comparator	Sensitivity (95% CI)	Specificity (95% CI)
Biorad STRIP	Dussart et al. [44]	2008	French Guiana	82% <5 days illness	RT-PCR or paired MAC and GAC-ELISA	77.6 (72.1–82.4)	100 (92.6–100)
	Shu et al. [42]	2009	Taiwan	Median 2; 1–7 days illness	RT-PCR or paired MAC and GAC-ELISA	77.3 (0.54–0.92)	100
	Hang et al.[41]	2009	Vietnam	1–6 days illness	RT-PCR or paired MAC and GAC-ELISA	72.8 (64.1–80.3)	100 (91.6–100)
	Chaiyaratana et al. [43]	2009	Thailand	1–8 days illness	NS1 Ag ELISA	98.9 (96.8–100)	90.6 (85.6–95.7)
	Zainah et al. [45]	2009	Malaysia	Not stated	Viral culture, nested RT-PCR, NS1 Ag ELISA	90.4 (86.6–94.4)	99.5 (97.4–99.9)
	Ramierez et al. [46]	2009	Venezuela	2–6 days illness	RT-PCR or paired MAC-ELISA	67.8 (57.4–76.7)	94.4 (80.9–99.4)
	Lima et al. [47]	2009	Brazil	1–6 days illness	Combinations of viral culture, nested RT-PCR, NS1 Ag ELISA	89.6 (84.7–93.2)	99.1 (96.9–99.9)
	Pok et al. [38]	2010	Singapore	1-8 days illness	"Recife" classification (7)	78.9 (70–86.1)	99 (94.6–99.9)
	Tricou et al. [39]	2010	Vietnam	1–6 days illness	RT-PCR or paired MAC and GAC-ELISA	61.6 (55.2–67.8)	100 (93.8–100)
	Najioullah et al. [37]	2011	Martinique	Not stated	RT-PCR	49.4% (43.2–55.6)	100
	Osorio et al. [40]	2010	Colombia	Median 4; range 2–7 days illness	Viral culture, nested RT-PCR or paired MAC and GAC-ELISA	57.7 (47.6–67.3)	95.3 (84.2–99.4)
	Blacksell et al. [19]	2011	Sri Lanka	Median 5; IQR 2–7 days illness	AFRIMS MAC and GAC-ELISA paired samples	58.6 (48.2–68.4)	98.8 (95.6 −99.9)

Table 3: Continued.

Assay	Study	Year	Location	Sample timing	Reference comparator	Sensitivity (95% CI)	Specificity (95% CI)
SD Bioline Dengue Duo	Tricou et al. [39]	2010	Vietnam	1–6 days illness	RT-PCR or paired MAC and GAC-ELISA	62.4 (56.1–68.5)	100 (93.8–100)
	Wang and Sekaran [21]	2010	Malaysia	1–15 days	Virus isolation, RT-PCR, paired MAC ELISA	65.4 (58.5–72.3)	98.8 (96.2–100)
	Osorio et al. [40]	2010	Colombia	Median 4; range 2–7 days illness	Viral culture, nested RT-PCR or paired MAC and GAC-ELISA	51 (44.1–57.7)	96.7 (90.8–99.3)
	Blacksell et al. [19]	2011	Sri Lanka	Median 5; IQR 2–7 days illness	AFRIMS MAC and GAC-ELISA paired samples	48.5 (38.5–58.7)	99.4 (96.6–100)
Panbio Early Rapid NS1	Fry et al. [20]	2011	Vietnam	1-5 days; 84.5% <3 days illness	RT-PCR or paired MAC and GAC-ELISA	69.2 (62.8–75.6)	96% (92.2– 99.8)
	Fry et al. [20]	2011	Malaysia	1-15 days; 70% ≤5 days illness	RT-PCR or paired MAC and GAC-ELISA	68.9 (61.8–76.1)	96.7 (82.8– 99.9)
	Blacksell et al. [19]	2011	Sri Lanka	Median 5; IQR 2–7 days illness	AFRIMS MAC and GAC-ELISA paired samples	58.6 (48.2–68.4)	92.5 (87.3–96.1)

Table 4: Description of selected recent diagnostic assessments of dengue RDT combining NS1 antigen, IgM, and IgG antibody results.

Assay	Study	Year	Location	Sample timing	Reference comparator	Analyte combination	Sensitivity (95% CI)	Specificity (95% CI)
SD Dengue Duo Bioline	Tricou et al. [39]	2010	Vietnam	1–6 days illness	RT-PCR or paired MAC and GAC-ELISA	NS1/IgM	75.5 (69.6 – 80.8)	100 (93.8 – 100)
						NS1/IgM/IgG	83.7 (78.4 – 88.1)	97.9 (88.7 – 99.9)
	Wang and Sekaran [21]	2010	Malaysia	1–15 days	Virus isolation, RT-PCR, rising titer in a paired samples using MAC ELISA	NS1/IgM	88.7 (84.0 –93.3)	98.8 (96.3–100)
	Osorio et al. [40]	2010	Colombia	Median 4; range 2–7 days illness	Viral culture, nested RT-PCR or paired MAC and GAC-ELISA	NS1/IgM	78.4 (72.4 –83.7)	91.3 (83.6–96.2)
						NS1/IgM/IgG	80.7 (75–85.7)	89.1 (81–94.7)
	Blacksell et al. [19]	2011	Sri Lanka	Median 5; IQR 2-7 days illness	AFRIMS MAC and GAC-ELISA paired samples	NS1/IgM	92.9 (83.9 –97.1)	88.8 (82.8–93.2
Panbio Early Rapid NS1 and Duo assay	Fry et al. [20]	2011	Malaysia	1–15 days; 70% ≤5 days illness	RT-PCR or paired MAC and GAC-ELISA	NS1/IgM	89.0 (85.2 – 92.8)	Not reported
						NS1/IgM/IgG	93.0	Not reported
	Blacksell et al. [19]	2011	Sri Lanka	Median 5; IQR 2–7 days illness	AFRIMS MAC and GAC-ELISA paired samples	NS1/IgM	89.9 (82.2–95.0)	75.0 (67.6–81.5)

perform equally and it is incumbent of manufacturers to provide these performance details. The effect of sample anticoagulants and whole blood on RDT performance and ease of reading also require examination in a field setting.

3.5. Storage Considerations. Dengue endemic regions are normally located in tropical regions that have high temperature and high humidity climates. Many of the contemporary dengue rapid tests require refrigeration (i.e., 2–8°C) (see Table 1); however, some manufacturers specify storage at 2–30°C; however, ambient tropical temperatures often exceed 30°C. There is an urgent need to examine the effect of storage temperature on contemporary dengue RDTs as the only previous investigation concentrated on earlier-generation antibody-based tests [6].

3.6. Prognostic Markers of Disease Severity. While the acute diagnosis of dengue infection is clinically useful, in a dengue endemic setting where the majority of infections are seen in outpatient settings, there is a clear need to also have prognostic details of disease severity. The more severe forms of dengue infection (DHF and DSS) require patient admission to hospital and critical care facilities, and prognostic indicators of clinical severity would provide direction for patient management. Quantifying secreted NS1 antigen has been hypothesised as a marker of disease severity [14], and subsequent studies have shown that dengue NS1 antigen levels correlate with severity disease where plasma levels of secreted NS1 correlated with viraemia levels and were higher in patients with DHF than in those with the clinically less-severe dengue fever [13, 49].

4. Conclusions

Despite improvements in the accuracy of IgM-based RDTs, this format is not sufficiently sensitive for acute dengue diagnosis alone. Acute dengue diagnosis using IgG-based RDTs is not recommended due to the lifelong persistence of dengue IgG antibodies and hence the possibility of misdiagnosis by false-positive detection. NS1-antigen-based diagnostics are an important component of modern point of care diagnostics; however, they are only sensitive in the early phase of infection and therefore are not suitable for sole use in dengue-endemic settings where late clinical presentations may occur. To take advantage of the results of testing modalities across the entire temporal spectrum of patient presentation, dengue NS1 antigen, and IgM antibody, RDT results must be combined; however, there is a need to educate clinicians and scientists of this fact. The challenge for manufacturers and researchers is to address the gaps in the more practical aspects of dengue RDT performance including samples types, RDT storage, disease severity, and conduct of future diagnostic assessments.

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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 vireamic 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 humans 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 laboaratory-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 Kokoberra, 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 crystalised 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 Oceguera 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 Cardosa 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: 93–100% [131]	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 NS1	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 NS1	No
Lateral Flow	IgM only	Minutes	No	Yes	High 98.8% [133]	High 95.3% [133]	No	E protein	Yes, for example, RapidWN [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

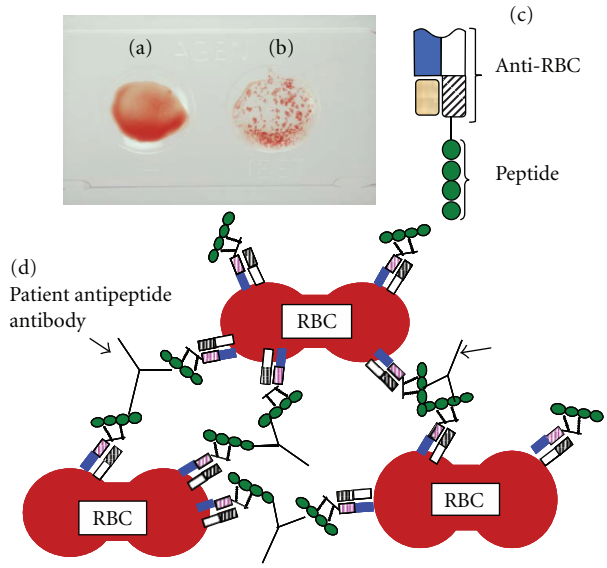

Figure 1: Autologous agglutination assay. Agglutination of RBC's (b, d) by the crosslinking of the assay reagent (c) and blood-borne antipeptide antibodies. Agglutination does not occur in nonimmune samples (a).

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 occured [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	Strong reactivity of anti-WNV ascitic fluid with r-EIII at 1 : 64 dilution	Negligible reactivity of anti-JEV, -SLEV, -MVEV, -DENV and -YFV ascitic fluid with r-EDIII at 1 : 64 dilution.
		IgG ELISA	Human: PRNT/HI confirmed WNV pos ($n = 11$); WNV neg ($n = 4$, plus 1 SLEV pos)	100%	100%
		IgG ELISA	Horse: 57 PRNT/HI confirmed	89%¶($n = 35$)	87.5%¶ ($n = 16$)
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.	67%‡	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]†	100%† ($n = 4$ WNV/MVEV neg)
				80% WNV PRNT confirmed field samples ($n = 5$) [61]	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.	DENV assay: 95% JEV assay: 100%	DENV assay: 100% JEV assay: 73%
			Pig. 31 JEV pos	JEV assay: 93.5%	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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Review Article

Evolution of Mosquito-Based Arbovirus Surveillance Systems in Australia

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Control of arboviral disease is dependent on the sensitive and timely detection of elevated virus activity or the identification of emergent or exotic viruses. The emergence of Japanese encephalitis virus (JEV) in northern Australia revealed numerous problems with performing arbovirus surveillance in remote locations. A sentinel pig programme detected JEV activity, although there were a number of financial, logistical, diagnostic and ethical limitations. A system was developed which detected viral RNA in mosquitoes collected by solar or propane powered CO_2-baited traps. However, this method was hampered by trap-component malfunction, microbial contamination and large mosquito numbers which overwhelmed diagnostic capabilities. A novel approach involves allowing mosquitoes within a box trap to probe a sugar-baited nucleic-acid preservation card that is processed for expectorated arboviruses. In a longitudinal field trial, both Ross River and Barmah Forest viruses were detected numerous times from multiple traps over different weeks. Further refinements, including the development of unpowered traps and use of yeast-generated CO_2, could enhance the applicability of this system to remote locations. New diagnostic technology, such as next generation sequencing and biosensors, will increase the capacity for recognizing emergent or exotic viruses, while cloud computing platforms will facilitate rapid dissemination of data.

1. Introduction

Over 75 different arboviruses have been isolated in Australia, with some being the aetiological agents of human disease [1]. Ross River virus (RRV) and Barmah Forest virus (BFV), both belonging to the genus Alphavirus, are responsible for the greatest number of annual disease notifications [2]. While Murray Valley encephalitis (MVEV) and Kunjin virus (KUNV; a subtype of West Nile virus (WNV)) are endemic in northern Australia, they can cause periodic outbreaks of acute encephalitis in southern and central regions. Dengue outbreaks occur regularly in northern Queensland, the only region in Australia where *Aedes aegypti*, the primary vector of dengue viruses (DENVs), occurs [3]. When Japanese encephalitis virus (JEV) emerged in the mid-1990s in the Torres Strait and Cape York Peninsula (Figure 1), it was feared that it would become a serious public health issue on the Australian mainland [4]. Finally, as competent vectors are present, there is always the potential for exotic arboviruses, such as the North American strain of WNV, chikungunya virus (CHIKV), and Rift Valley fever virus, to be introduced into Australia [5–7].

There is a need for informed decisions to be made regarding the implementation of control strategies for both endemic and exotic arboviruses. A comprehensive surveillance strategy is essential to ensure that elevated or emergent virus activity is detected before an outbreak occurs, as well as for establishing a baseline of arbovirus activity.

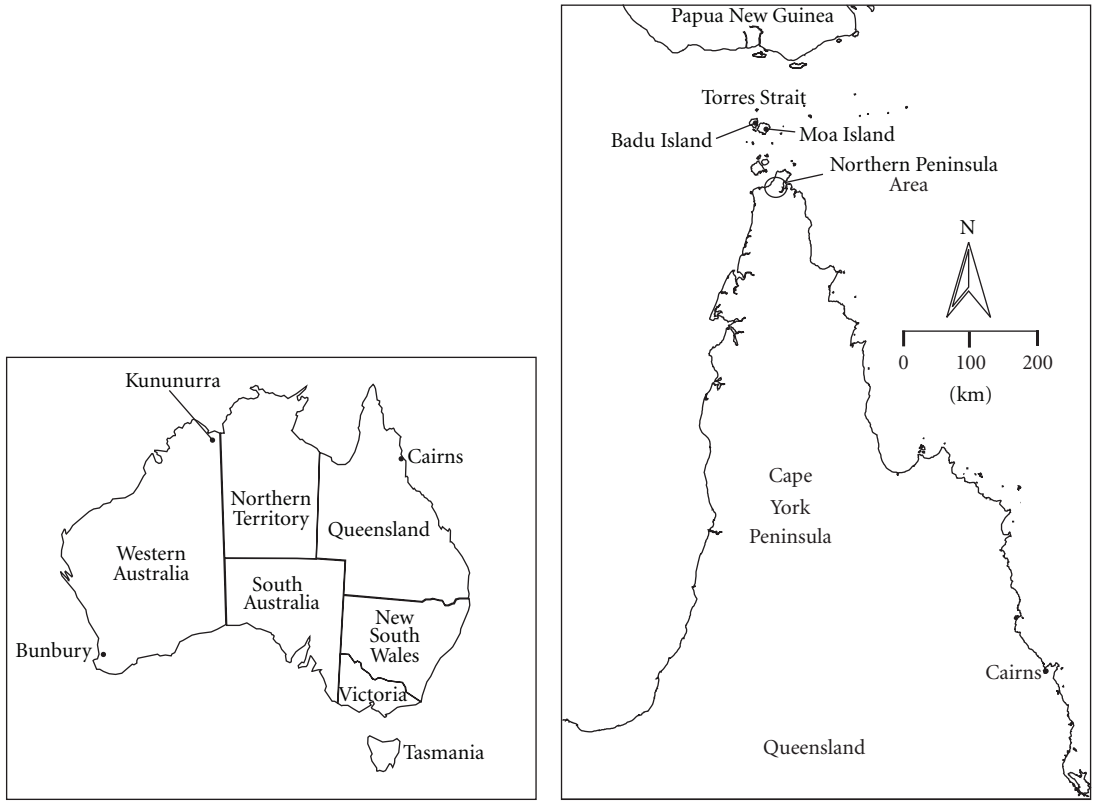

Figure 1: Map of (a) Australia and (b) northern Queensland showing locations mentioned in the text.

Together with meteorological and vertebrate host data, this may then facilitate accurate prediction of virus fluctuations and outbreaks. The relevant authorities can use the data obtained from such strategies to formulate control initiatives, including vaccination, mosquito control, and/or public education. The mainland Australian states maintain proactive mosquito and sentinel animal-based arbovirus surveillance programmes, which are primarily used to detect elevated RRV and MVEV activity. Outbreaks of other arboviruses are often only recognised when human or animal cases are diagnosed, a situation exemplified by the regular dengue outbreaks in northern Queensland and the original outbreak of JEV in northern Australia.

The emergence of JEV in northern Australia highlighted a number of unique problems which compromised the implementation of a sustainable surveillance programme to detect future incursions of the virus. This area of northern Australia, encompassing the Torres Strait and Cape York Peninsula is remote, located hundreds of kilometres from diagnostic laboratories. Many of the locations in this region are only accessible by aircraft or boat and wet season rainfall renders most mainland sites inaccessible to road transport between December and May, the period when JEV activity mainly occurs. We describe the history of JEV surveillance in Australia and the development of a novel surveillance system for JEV in remote locations, which also has application for other arboviruses, both within Australia and overseas.

2. Sentinel Animals for Arbovirus Surveillance

Sentinel animals have been utilized in Australia since the late 1960s to monitor arbovirus activity [8]. A sentinel animal programme involves placing immunologically naïve animals in a given location, where they are periodically bled and the serum samples submitted for the detection of virus-specific antibodies and/or virus. Sentinel chickens were initially deployed in response to an outbreak of MVEV in southeastern Australia in 1974 [9] and are still employed to detect virus activity in all mainland states [10, 11], except Queensland. While sentinel livestock (especially cattle) have been used to detect important veterinary arboviruses, such as bluetongue and bovine ephemeral fever viruses, they have also occasionally seroconverted to MVEV and KUNV [12]. Following the incipient outbreak of JEV on Badu Island in the Torres Strait in 1995, a sentinel pig surveillance system was established to detect further JEV activity in the Torres Strait and determine whether the virus had extended onto Cape York Peninsula on the Australian mainland [13]. In the Torres Strait, this programme was considered successful, detecting JEV in all years (except 1999) between 1996 and 2006.

Despite the ability to detect arbovirus activity, deployment of sentinel animals has a number of drawbacks which compromise their efficacy as a surveillance tool. There are ethical implications associated with using animals. Cross-reactions in serological assays make it difficult to distinguish

closely related viruses, such as JEV and MVEV. Larger animals, such as pigs, can be difficult to bleed, representing an occupational health and safety issue. Some sentinel animals are amplifying hosts of the virus they are deployed to detect (i.e., pigs and JEV), so they may actually contribute to virus transmission cycles. Clearly, an alternative method to sentinel animals needs to be developed for arbovirus surveillance in remote areas.

3. Mosquito-Based Surveillance of Arboviruses

In Australia, mosquito-based arbovirus surveillance currently involves collecting mosquitoes in CO_2-baited encephalitis vector surveillance (EVS; [14]) or Centers for Disease Control (CDC; [15]) light traps. Once collected, pools of 25–100 individual mosquitoes are processed and a filtered homogenate is inoculated into cell culture and viral antigen is detected with an enzyme-linked immunosorbent assay (ELISA; [16]) or immunofluorescence assay (IFA; [17]). Some Australian states conduct ongoing mosquito trapping for virus isolation, which either runs throughout the year [11] or between November and April [10]. Other states only undertake trapping in response to outbreaks or incursions of virus [18, 19]. Traps are deployed overnight and mosquitoes collected within 24 hours. Unfortunately, longer periods of deployment are hampered by relatively short battery life necessitating recharging or replacement of batteries, exhaustion of CO_2 when dry ice is used, and decreased survival/viability of mosquitoes for extended periods in collection containers. Traps need to be collected within 24 hours and more than 30 traps can be set in a night [11] highlighting the labour-intensive nature of this method of surveillance. This problem is compounded in remote locations, where logistical issues and high costs prevent collection of traps after 24 hours of deployment.

In an attempt to overcome these limitations, a unique system of surveillance based on detecting arboviruses in mosquitoes collected in traps over 7 days has been under development since 2000. This system involves (a) solar-, long life battery-, or propane gas-powered traps, which can run for extended periods; (b) CO_2 administered from large capacity compressed gas cylinders; and (c) molecular-based assays which are able to detect viral RNA in mosquitoes which have been held under field conditions for at least 7 days. One trap that showed considerable early potential was the propane-powered Mosquito Magnet (20; Woodstream Corporation, Lititz, PA, USA). This trap functions by combusting propane gas to produce CO_2, heat, and moisture as mosquito attractants, while a thermoelectric generator converts excess heat into electricity to power the trap fan [20]. Importantly, a single propane gas cylinder lasts 3 weeks, making this an ideal trap for remote areas. In efficacy trials conducted in northern Australia, the Mosquito Magnet collected at least as many *Culex sitiens* subgroup mosquitoes (the primary JEV vectors) as the "gold standard" CO_2-baited Centers for Disease Control light trap [21].

Since virus degradation after 7 days under tropical conditions was to be expected, molecular methods for viral RNA detection were tested for sensitivity. Preservation of infectious virus between remote trapping location and laboratory depends on a cold chain which is difficult to maintain. A number of laboratory-based experiments demonstrated that RT-PCR detected WNV, St. Louis encephalitis virus, western equine encephalitis virus, and DENV RNA in mosquitoes stored at room temperature or under simulated conditions of high temperature and humidity [22–24]. PCR-based detection methods were able to detect JEV RNA in a single mosquito in pools of up to 1,000 uninfected mosquitoes stored for at least 14 days under simulated tropical conditions [25]. Similarly, during a field trial in Cairns, northern Australia, single laboratory-infected mosquitoes were detected in pools of up to 1,000 mosquitoes stored for 14 days within a functioning Mosquito Magnet [26]. Furthermore, RT-PCR was used to detect DENV-2 RNA in laboratory-infected mosquitoes adhered to sticky traps set under natural conditions of high heat and humidity [27], while DENV-3 RNA was detected in 6 pools of *Ae. aegypti* removed from sticky ovitraps deployed during an outbreak in Cairns [28].

Between 2001 and 2005, a field trial was conducted in the Torres Strait and northern Cape York Peninsula with the objective of comparing the mosquito-based surveillance system with the sentinel pig programme [29]. Weekly mosquito collections from either the Mosquito Magnet and/or the Northern Australian Quarantine Strategy Mozzie Trap (NMT; 29), a trap developed for the purposes of long term deployment, were submitted for detection of JEV RNA. Sentinel pigs were bled weekly and serum samples were submitted for detection of JEV-specific antibodies by ELISA or viral RNA by real-time TaqMan RT-PCR [30]. The mosquito-based system successfully detected JEV and demonstrated the feasibility of the concept. However, it did not detect JEV before detection in the sentinel pigs on either Badu Island or St. Pauls community on Moa Island and did not detect virus on the mainland. There were a number of other logistical issues with the mosquito-based system. Firstly, the collection of large numbers of non-target *Aedes* spp. congested fans and necessitated presorting of collections. Indeed, a single weekly Mosquito Magnet collection yielded >178,000 mosquitoes of which <1% were *Cx. sitiens* subgroup mosquitoes. The number of non-target species was significantly reduced when 1-octen-3-ol (octenol), a chemical used to increase collections of some mosquito species, was removed from the traps [31]. A lack of ventilation, especially in the NMT, caused considerable bacterial and fungal contamination of collected mosquitoes, which may have led to degradation of viral RNA and therefore reduced the ability to detect viral RNA by TaqMan RT-PCR. The hot, humid conditions coupled with a lack of regular maintenance led to blocked gas lines and component malfunction, which decreased the efficiency of both types of traps. Finally, the system was determined to be insufficiently sensitive, as it was estimated that over 47,000 *Cx. sitiens* subgroup mosquitoes would have to be processed from the mainland for a single JEV detection, equating to 114

(a) (b)

Figure 2: CO_2-baited updraft box trap deployed near Bunbury, Western Australia. (a) The trap is powered by a battery and baited with CO_2 released from a compressed gas cylinder. (b) Close-up view of trap showing collected mosquitoes (photographs courtesy of Scott Dandridge).

trap nights [29]. Although the results from the field trial were promising, the issues identified during the trial prevented mosquito-based surveillance on a large scale as a replacement for sentinel animals.

4. Development of a Mosquito-Free Arbovirus Surveillance System

To circumvent some of the issues listed previously, particularly the need to process large numbers of mosquitoes, a strategy was developed whereby collected mosquitoes probe a substrate, and the substrate, not the mosquitoes, is submitted for virus detection. It was originally suggested by Doggett and others that virus could be transferred from infected to uninfected mosquitoes while feeding from the same sucrose soaked cotton pledget [32]. However, they were unable to detect virus on the sucrose pledget using a fixed cell culture ELISA. Based on these observations, a series of experiments utilising TaqMan RT-PCR was conducted to assess whether mosquitoes expectorate viruses when they sugar feed [33]. Not only were JEV, MVEV, and KUNV RNA detected in pledgets removed from batches of up to 50 infected mosquitoes, but also JEV RNA was detected in 73% of pledgets that had been exposed to individual infected mosquitoes [33].

The discovery that mosquitoes expectorate virus when they sugar feed formed the basis for a "mosquito-free" surveillance system. In this system, mosquitoes are attracted to a CO_2-baited trap, which possesses a holding container where mosquitoes are provided access to a sugar-baited substrate [34]. Several substrates were tested in the laboratory and it was found that Flinders Technology Associates (FTA; Whatman International Ltd, Maidstone, UK) cards could bind and preserve viral RNA for at least 28 days, inactivate virus on contact for safer handling, and resist bacterial and fungal contamination. Honey was chosen as the sugar source, because it remains moist on the FTA cards for at least a week and honey contains antibacterial properties which would also limit degradation of viral RNA [34–36]. Importantly, CHIKV, KUNV, and RRV RNA could be detected on >70% of honey-baited FTA cards that had been fed upon by infected mosquitoes.

In parallel with the laboratory-based experiments, a CO_2-baited updraft box trap which collected and housed mosquitoes was developed and tested. This trap utilized updraft technology [37] and incorporated a motorised fan which draws mosquitoes into a collection container where mosquitoes could access the honey-baited FTA cards. In preliminary field trials, the updraft box trap collected more mosquitoes than a CO_2-baited CDC trap and between 77–95% of collected mosquitoes fed on the honey-baited substrates [34].

The final step in the development of the "mosquito-free" surveillance system was to test the efficacy of the system for detection of arboviruses in a field setting [34]. Trials were undertaken during 2008 and 2009 at locations where RRV and BFV activity historically occurred near Bunbury, southwestern Western Australia, and Cairns (Figure 2). At each location, two updraft box traps were set, with each trap

containing 4–6 honey-soaked FTA cards. Traps were serviced weekly and the FTA cards and mosquitoes were sent to the laboratory for detection of viral RNA using TaqMan RT-PCR. During the trials, RRV and BFV RNA was detected in both FTA cards and mosquito pools [34].

Although the efficacy of the honey-baited system of arbovirus surveillance had been demonstrated under field conditions, its sensitivity compared to sentinel animals had yet to be evaluated. Consequently, a field trial was conducted during the 2009-2010 wet season with the objective to compare the honey-bait system and sentinel chickens for the detection of MVEV or KUNV activity at Kununurra in the remote north of Western Australia. The honey-soaked cards were submitted for virus detection using TaqMan RT-PCR and the chicken sera tested for virus-specific antibodies in a modified blocking ELISA [38]. Unfortunately, during the 14-week trial, there was no evidence of flavivirus activity in either the sentinel chickens or the honey-baited FTA cards, which was only the second time in over 20 years that MVEV or KUNV activity was not detected at the study location. A field comparison of the honey-bait system and a sentinel animal system is still to be conducted during a period of recognized arbovirus activity.

5. New Directions for Mosquito Collection

All of the mosquito traps described previously utilise various combinations of CO_2 and light to attract mosquitoes to the trap, and battery-powered fans to draw attracted mosquitoes into a collection bag or chamber. However, access to electricity for powering traps can be a significant hurdle for remote trapping and hot humid conditions can damage motorised components. Even though the Mosquito Magnet traps utilised combustion of propane to power the fan, as well as create heat and CO_2 to attract mosquitoes, the issues with dependability meant that it was not practical for use in remote locations.

Recently, we have developed a passive nonmechanical fanless trap for collection of mosquitoes in the honey-bait surveillance system. This trap extends work that was conducted over 40 years ago by Schreck and others who developed a passive trap consisting of a plexiglass box with screened cone entry points that collected large numbers of mosquitoes [39]. Our trap consists of a translucent plastic crate connected via a hose to an external CO_2 source. Mosquitoes enter the trap through the bottom of the crate, attracted by the CO_2. Once inside, the mosquitoes are attracted to the outdoor light transmitted through the sides of the translucent crate, helping to retain mosquitoes inside where they feed on honey-soaked FTA cards. In field trials in Cairns, Australia, and Florida, USA, passive traps baited with CO_2 from dry ice collected 185% and 50% of the number of mosquitoes, respectively, as a CO_2-baited CDC light trap (S. Ritchie, G. Cortis and D. Shroyer, unpublished data). As mosquitoes escaping from the passive trap were observed in the Florida trial (D. Shroyer, personal communication), further refinements are needed to maximize mosquito capture and retention.

Finding a suitable CO_2 source to attract mosquitoes in remote locations poses many problems. Dry ice is impractical for long-term trapping. When using compressed gas cylinders as a CO_2 source, issues such as expense, heavy weight, requirement for specialised regulators, and transport as dangerous goods need to be considered. A relatively simple and inexpensive system that offers great potential as a CO_2 source is the production of CO_2 during fermentation reactions involving yeast, sugar, and water [40, 41]. Such fermentation-derived CO_2 could be greatly improved by the development of yeast strains that produce high levels of CO_2 at lower temperatures, and storage systems that only release CO_2 when vectors are active, for example, at night.

The addition of semiochemicals that work either alone or synergistically with CO_2 could also be used to increase collections of hematophagous insects including mosquitoes [42, 43]. When used as an attractant in traps, these lures significantly increase the collection of mosquitoes such as *Ae. albopictus* and *Anopheles gambiae* [44, 45]. One chemical that has received much attention is octenol which, when added to CO_2 in light traps, could significantly increase collections of important vectors of RRV [46] and JEV [31], as well as other biting flies, including culicoides [47] and phlebotomines [48]. However, due to intraspecific differences in responses to octenol [31], preliminary trials should be conducted to determine the suitability for its use as an added attractant.

The CO_2-baited passive traps could be modified into simplified "killing traps" that not only facilitate detection of arboviruses but also kill attracted mosquitoes. The honey bait could be laced with nonrepellent, rapid-acting insecticides such as bendiocarb [49] or imidacloprid [50]. Because mosquitoes do not need to be retained in the trap, it can be more open, increasing mosquito access to the honey-soaked FTA cards and to insecticides, thus increasing killing power. Furthermore, nonrepellent insecticides can be used to treat the interior of the box trap or vaporized, nonrepellent insecticides such as dichlorvos and metofluthrin [51] can be placed inside the box to kill attracted mosquitoes. A perimeter line of surveillance and killing traps could be used to maximize surveillance while providing limited control. This trap line could consist of conduit connecting a large CO_2 source to several trap units, similar to the lure and kill method used to control *Ae. taeniorhynchus* at a resort near Naples, Florida [42].

Modern sensor technology and data transfer systems could be harnessed to create remote mosquito sensors and traps that notify users of not only the magnitude and identity of mosquitoes sensed but also possible infection with arboviruses on honey-soaked cards. Indeed, sensors that detect and identify mosquitoes by wing-beat frequency have been developed and teamed with lasers to track and kill detected mosquitoes (http://intellectualventureslab.com/?page_id=563). Acoustic signals could be used to estimate the number of mosquitoes within a trap, and biosensor systems [52] could be utilized to identify arboviruses expectorated onto honey-baited cards.

6. New Directions for Detection of Virus in Substrates or Mosquitoes

The sensitive detection of the viruses obtained by surveillance is a crucial aspect of any methodology. Furthermore, there has been a recent emergence and re-emergence of viral pathogens such as JEV, WNV, CHIKV, Usutu virus, and Alkhurma virus, so testing routinely for exotic and new viruses becomes an important component of surveillance to protect both human and animal health. However, many of the molecular methods for detection of arboviruses are limited to characterized viruses with specific primer and probe sets.

Testing for novel viruses without prior knowledge of the pathogen has become possible with new high throughput sequencing methods, also known as next generation sequencing (NGS). They enable the rapid and sensitive detection of large numbers of known and unknown viruses in a sample. Various chemistries for NGS are available and continually being developed, but most perform a reaction with a template clonally amplified on microscopic beads (reviewed in [53]). The combined outputs from hundreds of thousands or even millions of such beads generate enormous amounts of sequencing data. The generated data is independent of sequence, so that any nucleic acid in a sample is a potential template and therefore it by-passes the challenges associated with the detection of viruses that cannot be cultured in the laboratory. Using NGS, the potential exists for the discovery of exotic and new viruses. In addition, software tools being developed for the related field of microbial metagenomics [54], or the study of microbial communities using primarily NGS methods, will facilitate the analysis of the large amounts of sequence data generated from samples obtained in the honey-bait surveillance system.

Like any new technology, there are some drawbacks to NGS. It is currently relatively expensive in comparison with traditional detection methods, although the cost per base sequenced is decreasing rapidly [55, 56]. Some potential also exists for bias introduced by the nucleic acid amplification steps of the methods [57], and this should be a consideration during experimental design. Nonetheless, in support of their application to arbovirus surveillance, NGS and metagenomics have been successfully applied to the surveillance of viruses in bat communities [58, 59], in fermented food [60], and in human clinical samples [61–63]. There is considerable potential for this technology to identify new arboviral threats to human and animal health, to examine how arbovirus populations change with time, and to reveal how environmental factors affect the emergence of new strains and influence spread from animal reservoirs into human populations.

7. Conclusions

Effective surveillance forms a vital component of any programme aimed at reducing the impact of arboviruses on human and animal health. A multidisciplinary, holistic system is the best approach. It could incorporate the latest scientific advances such as NGS for virus detection, sophisticated surveillance tools such as biosensors to collect data on mosquitoes and viruses, meteorological data, and production of effective, low-cost, nonmechanical traps coupled with yeast-generated CO_2. Such uncomplicated traps have the greatest applicability for deployment in remote locations. The information obtained from such a system could then be uploaded for dissemination to end users employing data sharing technology, such as a "cloud computing" platform [64]. Arbovirus surveillance data in north-eastern Australia has been erratic in the past mainly due to its remoteness. The simplicity of using the described traps and the honey-baited FTA cards together with advancing information technology would ensure continuous collection of surveillance data.

Even though vector species identification and infection rates in mosquito populations cannot be determined using the honey-bait system, it can provide an early warning of impending virus activity, in much the same way as a sentinel animal programme. Disease control strategies, such as mosquito control, vaccination, or health promotion initiatives, can then be implemented. Focussed trapping can also be undertaken to incriminate vector species, calculate virus carriage rates, as well as determine important entomological characteristics that can influence transmission, including host feeding patterns and the genetic structure of the vector populations.

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Research Article

Proximity of Residence to Bodies of Water and Risk for West Nile Virus Infection: A Case-Control Study in Houston, Texas

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West Nile virus (WNV), a mosquito-borne virus, has clinically affected hundreds of residents in the Houston metropolitan area since its introduction in 2002. This study aimed to determine if living within close proximity to a water source increases one's odds of infection with WNV. We identified 356 eligible WNV-positive cases and 356 controls using a population proportionate to size model with US Census Bureau data. We found that living near slow moving water sources was statistically associated with increased odds for human infection, while living near moderate moving water systems was associated with decreased odds for human infection. Living near bayous lined with vegetation as opposed to concrete also showed increased risk of infection. The habitats of slow moving and vegetation lined water sources appear to favor the mosquito-human transmission cycle. These methods can be used by resource-limited health entities to identify high-risk areas for arboviral disease surveillance and efficient mosquito management initiatives.

1. Introduction

Houston, Texas, is a metropolis in the southeastern United States with around four million residents [1]. West Nile virus (WNV) human cases were first reported locally in 2002 [2] and have since become endemic with human cases reported annually [3]. WNV is an arboviral disease from the Flaviviridae family whose main transmission cycle occurs between birds and mosquitoes; humans serve as an incidental host. In southeastern United States, *Culex quinquefasciatus* mosquitoes have been demonstrated as important vectors of WNV disease transmission [2, 4].

In the United States, WNV transmission season traditionally occurs from spring to fall, with a peak in late summer [2]. In warm weather, mosquito larval development occurs within days [5, 6] allowing for rapid reproduction of new mosquito populations. Mosquito larval development occurs in water bodies with each species having their own preferential type. *Culex quinquefasciatus* mosquitoes have a diverse larval habitat range, with high larval counts near human habitation [7, 8]. Mosquito control efforts in Houston, Texas, target residential areas where either mosquito pools or dead birds are positive for WNV disease. Targeted areas are identified through random mosquito trapping and reporting of dead birds by residents. The ecological dynamic between vector, reservoir, and human habitats is critical to understand when examining risk for human WNV infection. While this vector's larval habitat preferences are known, no studies to date have examined direct associations between larval water habitats and WNV human disease transmission. This paper presents a novel method for examining disease clustering and its spatial association with water sources.

2. Methods

A case-control study design was used to determine the association between water sources and the risk of human infection with WNV.

2.1. Case Selection. Cases were defined as WNV-positive patients identified through local surveillance performed by the Houston Department of Health and Human Services (HDHHS), Harris County Public Health and Environmental Services (HCPHES), or the Gulf Coast Regional Blood Center (GCRBC). Local surveillance identified cases either by state mandatory reporting laws or by national blood donation testing guidelines that required laboratory confirmation of WNV human disease. Previous research has shown that the highest rates of WNV human seroprevalence were among those who reported a history of being outside during the hours of dusk and dawn [9]. These hours are concurrent with the peak activity time of *Culex quinquefasciatus* mosquitoes. Since most people are at home during dusk and dawn, it was resolved that cases are most likely exposed while at home. It was determined appropriate to use cases' home address at time of disease development as their location of mosquito exposure. Cases' home addresses were collected via case investigations performed by HDHHS, HCPHES, or GCRBC during 2002 and 2009. Exclusion criteria included evidence of nonlocally acquired disease as documented in the case investigation nonrecognition of address by MapMarker USA version 14 geocoding software, or home address falling outside the metropolitan's geographic area as determined by the geocoding software. After applying the exclusion criteria, we had 356 residential addresses from cases for final analysis.

2.2. Control Selection. Controls were defined as selected block centroids generated from the United States Census Bureau decennial data (http://www.census.gov/). Controls were selected using two methods: a population proportionate to size sampling method which takes into account varying population densities within the metropolitan city and a random sampling method. There were three selection frames that were used to identify the final control. In descending order the frames were census tract level, block group, and finally block. The population proportionate to size sampling methods was used to select the initial frame: census tract level. It was understood that population distribution was uniform throughout the census tracts selected; therefore, we used a random selection method for the two additional frames: block group and block. Since the smallest defined census level is a block, the centroid of the block level was used as a surrogate for control households. Based on sample size calculations, a 1 : 1 case-control ratio was determined appropriate to satisfy statistical significance using discipline standards; therefore, 356 control addresses were selected for final analysis.

2.3. Data Analysis. Spatial analysis of case and control residential distances' to local water body sources was performed using MapInfo v9.5.1 software. Shapefiles of water sources within the metropolitan's geographic parameters were provided in kind by Dr. Irina Cech, professor at the University of Texas Health Science Center at Houston. The shapefiles were based on United States Geological Survey water source definitions and data. Case and control residential coordinates were superimposed onto the water source shapefile. Water source labels were used to identify the particular water source, that is, Cedar Spring, Lou River, Brays Bayou, and so forth. The water source type was inferred from these labels. Using the software's measurement tool, we measured the distance from each case/control point, to the closest water source, excluding salt water sources since *Culex quinquefasciatus* mosquitoes do not utilize salt water sources as larval habitats [5]. For each case/control point we recorded the proximity to the closest water source, the type of the particular water source, and the name of the particular water source. We used STATA v11.0 (College Station, Texas) to run all statistical analyses. Chi-squared tables and logistic regression were used to analyze the significance of proximity to a water source between the two populations. Odds ratios, 95% confidence intervals (CIs), and P values were computed to analyze the significance of three factors: specified residential proximity to a water source; proximity to a particular water source type; proximity to a particular water source. Attack rates (number of WNV human cases over total number of households) were calculated for each census tract and mapped to spatially identify areas of high WNV human transmission. A Getis Ord hot spot analysis was performed using ESRI ArcGIS 10.0 to determine concentrations of high and low human disease clustering. The GetisOrd (Gi) hot spot analysis identifies clusters of higher and lower magnitude than would be randomly found and statistical output is in the form of a Z score known as a GiZ score. Areas of high clustering were indicated by a GiZ score of 1.96 or greater, and areas of low clustering were indicated by a GiZ score of −1.96 or less.

3. Results

On average, cases and controls resided the same proximity from water sources [x_0 (controls) = 892 meters, x_1 (cases) = 931 meters]. Using linear regression, we found no statistical association between residential proximity to water and odds for human WNV infection. However, when we binomially-coded at varying distances ranging from 50 to 750 meters, we found a significant protective trend from distances ranging from 50 to 200 meters (Table 1). Living less than or equal to 200 meters from a water source ($x^2 = 6.67$, $P < 0.01$) was found to be protective from infection by a factor of 0.54.

Water source types were analyzed for association with odds for human WNV infection using odds ratios and chi-squared tests, as seen in Table 2. We examined the six most common water source types. Two water source types were statistically associated with odds of human infection. Living near a creek increased one's odds of human infection by a factor of 1.37 ($P = 0.09$). Living near a spring decreased one's odds of human infection by a factor of 0.55 ($P = 0.06$). To further analyze these associations, we created two groupings

Table 1: Distance of case residence compared to US Census control centroids to water source in meters, evaluated by odds ratio (OR), 95% confidence intervals (CI), and significance (*P* value).

Distance (m)	OR	95% CI	*P* value
50	0.10	(0.01, 0.42)	<0.01
100	0.21	(0.07, 0.42)	<0.01
150	0.35	(0.18, 0.66)	<0.01
200	0.54	(0.32, 0.89)	0.01
250	0.70	(0.46, 1.05)	0.07
300	0.76	(0.52, 1.11)	0.14
350	0.78	(0.54, 1.12)	0.16
400	0.82	(0.58, 1.16)	0.24
450	0.82	(0.59, 1.14)	0.22
500	0.85	(0.62, 1.17)	0.31
550	0.87	(0.63, 1.19)	0.35
600	0.78	(0.57, 1.07)	0.11
650	0.84	(0.62, 1.15)	0.26
700	0.89	(0.66, 1.21)	0.45
750	0.92	(0.68, 1.25)	0.60

based on slow moving and moderate moving water source types. A grouping of slow moving water bodies (creeks and gullies) was found to increase one's odds of human infection by a factor of 1.45 ($P = 0.03$). A grouping of narrow moderate moving water bodies (streams and rivers) was found to be protective against human infection by a factor of 0.50 ($P = 0.02$).

Particular water sources were evaluated for association with odds for human WNV infection by odds ratios and chi-squared tests, as seen in Table 3. The eleven most common specific water sources were analyzed. Two water body sources were significantly associated with increased odds for human infection. Living close to White Oak Bayou ($P = 0.01$) increased one's odds of human infection by a factor of 2.25. Additionally, living near Cypress Creek ($P = 0.02$) was also associated with increased odds of human infection by a factor of 2.54. Since Cypress Creek has several tributaries, an additional category was made that included all feeders for Cypress Creek. This group had the strongest significance of all water bodies ($P < 0.01$) with increased odds of human infection by a factor of 1.93. We also found that living close to Buffalo Bayou had increased odds of human infection by a factor of 1.59, which neared significance ($P = 0.07$).

Spatial distribution of WNV attack rates per 10,000 population by census tract illustrates that the highest risk area of transmission is in Northwest Houston as seen in Figure 1. Hot spot analysis confirmed that there were significant clusters of cases in Houston as seen in Figure 2. The areas of highest valued clusters were along the Northwest corner of Harris County, which overlaps Cypress Creek and its feeders. Figure 3 demonstrates the spatial relevance of the Houston area inlaid within Harris County, in relation to the state of Texas, and the United States of America.

Table 2: Proximity of residence to water source types in cases versus controls, evaluated by odds ratio (OR), 95% confidence intervals (CI), and significance (*P* value).

Water source type*	OR	95% CI	*P* value
Bayou	1.15	(0.84, 1.56)	0.36
Creek	1.37	(0.93, 2.02)	0.09
Ditch	0.49	(0.13, 1.60)	0.19
Gully	1.50	(0.73, 3.16)	0.23
Lake	1.50	(0.73, 3.16)	0.23
Stream	0.55	(0.27, 1.08)	0.06
Creek and gully	1.45	(1.02, 2.07)	0.03
Stream and river	0.50	(0.25, 0.95)	0.02

* As defined by the United States Geological Survey.

Table 3: Proximity of residence to particular water sources in cases versus controls, evaluated by odds ratio (OR), 95% confidence intervals (CI), and significance (*P* value).

Particular water source	OR	95% CI	*P* value
Bering Ditch	0.66	(0.14, 2.82)	0.52
Berry Bayou	1.00	(0.26, 3.78)	1.00
Brays Bayou	0.73	(0.43, 1.23)	0.21
Buffalo Bayou	1.59	(0.93, 2.75)	0.07
Cypress Creek	2.54	(1.10, 6.35)	0.02
Cypress Creek and tributaries	1.93	(1.14, 3.33)	0.01
Greens Bayou	0.66	(0.26, 1.59)	0.31
Halls Bayou	1.00	(0.40, 2.47)	1.00
Hunting Bayou	1.89	(0.69, 5.66)	0.17
Little White Oak Bayou	1.81	(0.74, 4.72)	0.15
Sims Bayou	0.57	(0.19, 1.61)	0.25
White Oak Bayou	2.25	(1.15, 4.55)	0.01

4. Discussion

This is the first known case-control study to perform a spatial analysis of human WNV infection risk with regard to proximity of residences to water sources serving as surrogates for potential aquatic larval habitats. Overall, we found no direct association between proximity of residences to water sources and odds of WNV human infection in Houston, Texas. However, we found a significant trend of decreased risk of infection among people living within 200 meters of a water source. It is conjectured that areas closest to water sources are the primary target of mosquito control programs, therefore decreasing the risk of transmission at closer distances. We did find a pattern of increasing odds ratios as distance increased by 50-meter intervals, suggesting that mosquitoes in Houston have an expansive flight range that is important in the ecology of disease transmission. *Culex quinquefasciatus* mosquitoes are known to have an expansive flight range with recapture documented up to 1000 meters outside of their release site [10]. One speculation could be that the use of adulticides along water bodies could temporarily suspend adult mosquito activity allowing for higher mosquito activity occurring at greater distances. Although adulticides are the primary mosquito

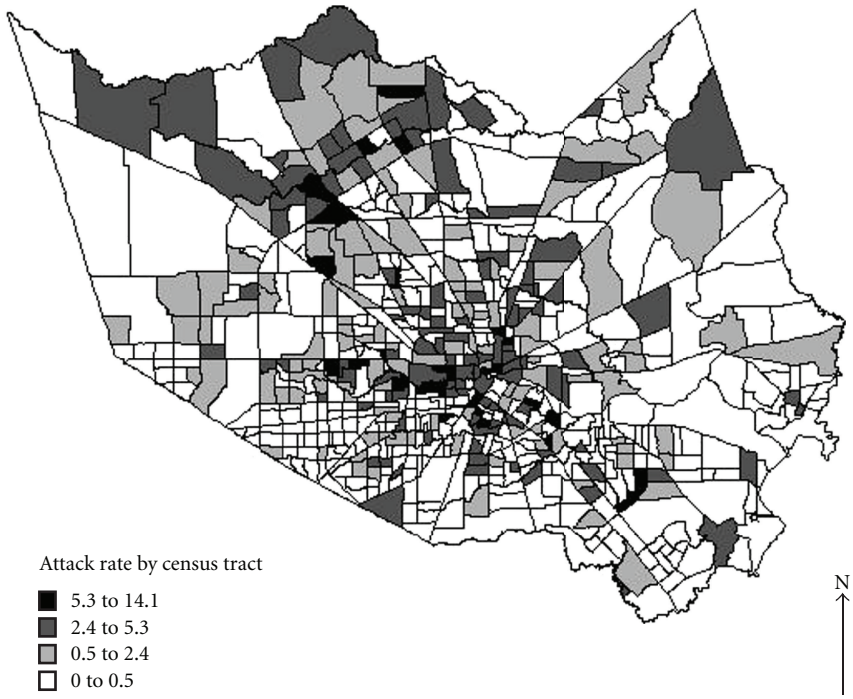

Figure 1: Attack rate: number of reported West Nile virus cases per 10,000 population using 2000 US census tract data in the Houston metropolitan area, Texas.

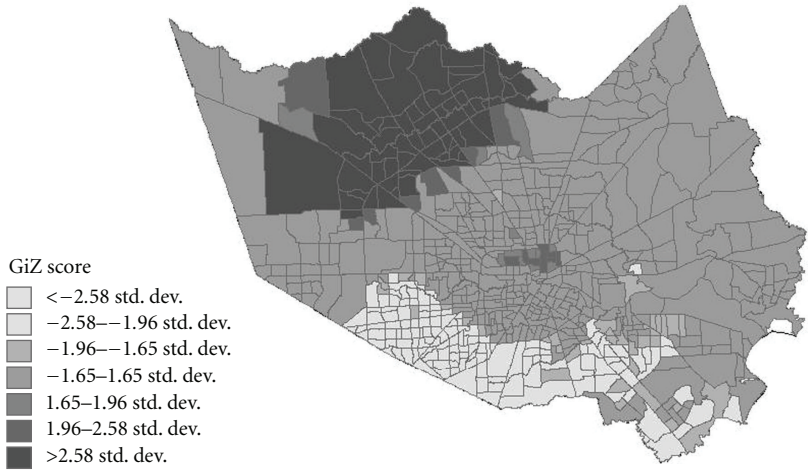

Figure 2: Hot spot cluster analysis of West Nile virus cases in the Houston metropolitan area, Texas.

control method used in this area, it is known that the use of adulticides is random and not associated with specific water bodies. Another speculation is that alternate breeding sites, specifically storm sewers, also play a role in disease transmission. In Houston, *Culex quinquefasciatus* are the dominate mosquito species collected from storm sewers, and storm sewers have been demonstrated as a preferential site for breeding, larval development, and daytime resting [11]. Unfortunately, we did not have access to sewer blueprints of the metropolitan area to further investigate this theory.

When analyzing residential proximity to water source types, we did find a strongly significant association for risk of human infection among residences near creeks and gullies, specifically Cypress Creek. It is believed that the slower movement of water and dense vegetation is preferential for the local transmitting *Culex* vector species. Due to low numbers of cases per creek, no additional specific creek sources were included in the final analysis. Cypress Creek is a large water source that flows throughout the northwest corner of the metropolitan Houston area. Figure 1 demonstrates that attack rates of human infection are strongest in the area where Cypress Creek flows. This finding is further substantiated by Figure 2, which shows the highest clusters of human WNV cases are in the area

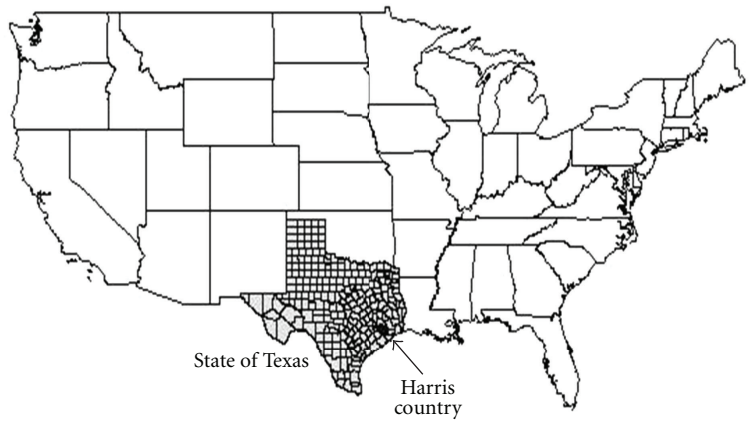

Figure 3: Geographic location of metropolitan Houston area inlaid within Harris County in relation to the State of Texas and the United States of America.

where Cypress Creek flows. We feel the true association of infection is with the particular water source Cypress Creek. Additional studies should perform mosquito pool testing around Cypress Creek and additional creeks and gullies throughout the metropolitan area to examine WNV field infection rates of mosquitoes in efforts to further validate our findings.

When analyzing residential proximity to water source types, we did find a strong protective association of residences closest to streams; however, no particular stream water sources were identified as being associated with infection. To further investigate these findings, we created a grouping of moderate moving water sources which included streams and rivers. This grouping had the strongest significance of protection from human WNV infection. Additionally, no particular river water sources were identified as being associated with infection. These findings are evidence that residences in closest proximity to moderate moving water sources are significantly protected against WNV human infection.

Houston is prone to flooding, and as part of the flood mitigation program, the city has an extensive network of bayous, which are man-made canals [2]. The surrounding habitats of bayous in Houston are varied with some being cast with concrete walls and others edged with grass, shrubs, and other vegetation. Overall, we did not find an association between the living near bayous and increased odds of infection. However, we did find that White Oak Bayou and Buffalo Bayou were significantly associated with increased odds of infection. These specific bayous are lined with extensive vegetation preferential to mosquito habitats. This is in sharp contradiction to the bayous lined with concrete, such as Brays Bayou, where the data suggested decreased odds of infection. We cogitate that the type of bayou lining and habitat dictates WNV transmission. Future research should incorporate bayou linings and their individual risk for local human habitants.

There are a few limitations of this study that are worth noting. One limitation was the potential for selection bias due to the inability to verify disease status of controls by serum antibody testing. Since WNV is a mandatory-reportable disease in the state of Texas, anyone who tested positive should have been reported to the local health department. The risk of misclassification of controls is possible if a resident at the address never developed symptoms or had mild disease that went undiagnosed as WNV. However, this risk is presumed minimal since current estimates of seroprevalence in Houston are relatively low [12]. Due to financial constraints, we were unable to obtain a serum sample from controls to verify disease status. Lastly, we were unable to test for potential confounders related to human-mosquito transmission, such as socioeconomic status, gender, rainfall, or other seasonal environmental factors. Complete records for these potential confounders were unavailable. Despite the inability to control for these potential confounds, we believe the results are sound considering people do not choose their residence location based on human-mosquito transmission hotspots.

The main strength of the study is the ability to determine high risk areas of WNV transmission around the Houston metropolitan area using minimal resources. The methods we used are simple to perform and could be of benefit to health authorities in other jurisdictions to identify areas with increased risk for WNV transmission. In resource-scarce public health departments, this inexpensive method could greatly increase the effectiveness of mosquito control programs. Our case-control selection methods would be simple to replicate. Since WNV is a reportable disease nationally, case investigations are performed for all patients that test positive. From these case investigations, health departments should have the addresses of the cases in their jurisdiction. Control selection would be easy to execute as census data is readily available from the US Census Bureau website that is updated both annually and decennially.

In conclusion, we found that living near slow moving water bodies, such as creeks and gullies, or bayous with heavy vegetation increased one's odds of infection with WNV. Most importantly, we identified Cypress Creek as an area of high WNV human infection that should be targeted by future mosquito control efforts. With the recent literature suggestive of increased ranges of arboviral vectors and areas of transmission, this method of spatial analysis could benefit other health authorities in areas experiencing active WNV transmission who need predictive models of exposure risk for targeted education and control efforts for disease prevention.

Conflict of Interests

The authors have no conflicts of interests to report.

Acknowledgments

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Review Article

Rapid Molecular Detection Methods for Arboviruses of Livestock of Importance to Northern Europe

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Arthropod-borne viruses (arboviruses) have been responsible for some of the most explosive epidemics of emerging infectious diseases over the past decade. Their impact on both human and livestock populations has been dramatic. The early detection either through surveillance or diagnosis of virus will be a critical feature in responding and resolving the emergence of such epidemics in the future. Although some of the most important emerging arboviruses are human pathogens, this paper aims to highlight those diseases that primarily affect livestock, although many are zoonotic and some occasionally cause human mortality. This paper also highlights the molecular detection methods specific to each virus and identifies those emerging diseases for which a rapid detection methods are not yet developed.

1. Introduction

In 1983, Odend' Hal [1] published a short book listing the worldwide distribution of animal viruses. It reported the classification as well as host, historical movements, and diagnostic techniques available. Of the 110 viruses cited, 35 were arboviruses, those viruses that are transmitted primarily by an arthropod vector. This first attempt at mapping animal viruses provides a useful baseline for reviewing the current state of arboviruses of livestock. Arboviruses are mainly classified to the virus families *Bunyaviridae*, *Togaviridae*, *Reoviridae,* and *Flaviviridae* [2]. The majority of these virus families have a ribonucleic acid genome, with the clear exception of the *Asfarviridae* to which African swine fever virus belongs (Table 1).

In Table 2 an updated list of arthropod-borne virus pathogens of humans, livestock, and wildlife is provided. During recent years several pathogenic arboviruses have apparently dispersed to new locations. The most well-known cases have been the movement of West Nile virus from the Old world to the New World [3] and the introduction of bluetongue virus into northern Europe [4]. This has prompted a wide range of authors to review the potential viruses that could emerge in the UK and Europe and assess the risk of such emergence events in the future [5–9].

The cooler climate experienced in northern latitudes (above 50°) means that there are fewer species and less diversity among particular arthropod species and the viruses they harbour than found in subtropical and tropical regions. Currently, there are few arthropod-borne diseases of livestock in Europe and as a result, livestock in the UK and many areas of northern Europe may be highly susceptible to many arthropod-borne viruses listed in Table 2. The health impact of an emergence of one could likely be severe. An exception to this is the presence of louping ill virus, which is considered to be the only arbovirus of veterinary importance that is endemic within the UK. The virus has been present for hundreds of years and is restricted to moorland locations, particularly in Devon, Cumbria, Wales, and Scotland [10]. This tick-borne virus causes fatal encephalitic disease in sheep, although it has been reported in a range of other species. A number of reports have suggested that West Nile virus and two other mosquito-borne viruses had been introduced into the UK, although cases of disease in horses have not been reported [11]. This is in clear contrast to the situation in Italy where WNV has repeatedly emerged

Table 1: Details of selected arbovirus families.

Virus family	Enveloped	Genome (sense)	Segmentation (number)	Example virus
Bunyaviridae	Yes	RNA SS[1] (−)	3	Rift Valley fever
Flaviviridae	Yes	RNA SS (+)	Nonsegmented	Dengue virus
Reoviridae	No	RNA DS[2]	10–12	Bluetongue virus
Rhabdoviridae	Yes	RNA SS (−)	Nonsegmented	Vesicular stomatitis virus
Togaviridae	Yes	RNA SS (+)	Nonsegmented	Chikungunya virus
Asfarviridae	Yes	DNA DS	Nonsegmented	African swine fever virus
Orthomyxoviridae	Yes	RNA SS (−)	8	Thogoto virus

[1] Single-stranded.
[2] Double-stranded.

to cause neurologic disease in horses from the Tuscany Region [12]. Recurring outbreaks of disease have occurred in both livestock and humans suggesting the permanent establishment of this virus in the mosquito population [13]. This has made assessment of future climate change trends essential to understanding the impact on both the ecology of the UK and risk of vector-borne disease introduction and establishment [9]. Such changes might enhance the establishment of invasive arthropod species such as the Asian tiger mosquito (*Aedes albopictus*) that in turn could directly import an exotic virus. It could also boost the population of indigenous vectors that could in turn increase the numbers of biting events, enhancing the likelihood of virus transmission. Furthermore, increases in temperatures can shorten the extrinsic incubation period, the time between the vector taking a blood meal and becoming infectious to a new host, thus enhancing virus persistence in a new area.

A wide range of routes would enable arthropod-borne viruses to translocate into a disease-free area. These can be divided into those that are part of the normal ecology and are influenced by the environment and climate that are presumably occurring all the time. Avian migration is an example of this pathway. Avian species are known to harbour many pathogens [14] and certain viruses such as avian influenza and Newcastle disease virus are transmitted around the globe through bird migration. For arthropod-borne viruses, virus movement can occur via transportation of the vector [15] or through infection of the host, particularly a viraemic animal that is subsequently fed on by an arthropod in a disease-free destination. The range of Crimean-Congo haemorrhagic fever appears to be increasing slowly in south-east Europe associated with spread by its tick vector (*Hyalomma* spp.). Bluetongue virus transmission into the UK is believed to have resulted from direct introduction of its midge vector (*Culicoides* spp.) assisted by wind movements in 2007 [16]. It is likely that movements by these routes, if they happen, are occurring continuously and cannot realistically be controlled. Therefore, effort needs to be directed towards reducing the impact of introduction. The alternative to natural introduction is often mediated by the actions of man. Again, this could occur through passive introduction of the vector through the movement of humans, livestock, or trade goods between endemic and disease-free areas or by the movement of infected livestock between countries. Theoretically, these mechanisms of entry can be regulated and effort should be directed to prevention programs.

A key aspect in preparing for the emergence of arthropod-borne diseases is the establishment of tests capable of detecting them. Development of such tests needs to address a number of fundamental issues. These include key features such as sensitivity of the assay and its specificity for the target virus. The assay must also be validated to provide assurance of its reliability, or at least give an indication of what might be missed. The assay under development needs to compete with existing technologies in terms of cost and speed to deliver desirable benefits to encourage adoption. The application of a particular test needs to be considered. Some tests may be applied to surveillance for virus, in which case the test needs to be amenable to cost-effective delivery of high volumes of samples. This in turn can complement serosurveys for particular viruses or be applied to sampling arthropod vectors in order to provide early warning of potential disease incursion.

For some technologies, the cost of individual tests is prohibitive for application to large numbers of samples or in resource-poor areas such as Africa. In each of these areas, molecular detection techniques have been very competitive as evidenced by the numerous tests developed in recent decades. Many of the assays reported in this paper provide a result considerably faster than more traditional detection methods such as virus isolation and plaque-reduction neutralization tests. Genetic variability of viruses is an inherent weakness in the use of molecular detection techniques with primer-mismatch being a constant problem. This has to some extent been overcome by the wealth of sequence data now available on many of the viruses that affect livestock.

Here we provide an overview of those arthropod-borne viruses that cause clinical disease in livestock and lead to economic losses. It will not consider important arboviruses that cause significant human disease with no livestock involvement such as yellow fever, dengue, Toscana virus, and chikungunya virus. Background information on the disease caused by particular viruses is described, the main arthropod vector and the reported current geographical distribution is provided for the viruses selected. This is followed by a brief review of reported rapid molecular tests that detect specific viruses or those tests that detect virus groups that contain numbers of animal pathogens.

Table 2: Pathogenic arboviruses (viruses in bold are dealt with in greater detail later in the paper).

Virus	Classification: family genus	Vector	Animals affected	Disease (SFI[1], HF[2], E[3])	Endemic presence	OIE* listed
African horse sickness virus	***Asfarviridae Asfivirus***	**Midge (*Culicoides* spp.)**	**Horses**	**SFI/HF**	**Africa**	**Yes**
African swine fever virus	***Reoviridae Orbivirus***	**Ticks (*Argasid* spp.)**	**Pigs**	**HF**	**Africa**	**Yes**
Akabane virus	***Bunyaviridae Orthobunyavirus***	**Mosquito (*Aedes* spp.) and midges**	**Cattle, sheep, horse,**	**Congenital abnormalities**	**East Asia, Turkey, South Africa**	**No**
Bluetongue virus	***Reoviridae Orbivirus***	**Midge (*Culicoides* spp.)**	**Cattle, sheep, goat**	**HF**	**Americas, Africa, Asia**	**Yes**
Bovine ephemeral fever virus	***Rhabdoviridae Ephemerovirus***	**Midge/mosquito**	**Cattle**	**SFI/respiratory**	**Africa, Asia, Australia**	**No**
California encephalitis virus	*Bunyaviridae Orthobunyavirus*	Mosquito	Humans, small mammals	E	Americas	No
Chikungunya virus	*Togaviridae Alphavirus*	Mosquito (*Aedes* spp.)	Humans	SFI	Africa, Asia, Europe	No
Colorado tick fever virus	*Reoviridae Coltivirus*	Tick (*Dermocentor andersonii*)	Humans, small mammals	SFI	North America	No
Crimean-Congo haemorrhagic fever virus	***Bunyaviridae Nairovirus***	**Ticks (*Hyalomma* spp.)**	**Humans**	**SFI/HF**	**Africa, Asia, Europe**	**No**
Dengue virus	*Flaviviridae Flavivirus*	Mosquitoes (*Aedes* spp.)	Humans	SFI/HF	Africa, Asia, Americas, Europe	No
Dugbe virus	*Bunyaviridae Nairovirus*	Ticks	Humans, cattle	SFI	Africa	No
Eastern equine encephalitis virus	***Togaviridae Alphavirus***	**Mosquitoes (*Culex* spp.)**	**Humans, equine**	**E**	**Americas**	**Yes**
Epizootic haemorrhagic disease virus	***Reoviridae Orbivirus***	**Midge (*Culicoides* spp.)**	**Cattle, deer**	**HF**	**Americas, Africa, Asia**	**Yes**
Equine encephalosis virus	***Reoviridae Orbivirus***	**Midge (*Culicoides* spp.)**	**Equine**	**E**	**Southern Africa, Israel**	**No**
Getah virus	*Togaviridae, Alphavirus*	Mosquito (*Culex* spp.)	Equine	SFI/E	Asia	No
Inkoo virus	*Bunyaviridae Orthobunyavirus*	Mosquito (*Aedes* spp.)	Cattle	SFI	Finland	No
Japanese encephalitis virus	***Flaviviridae Flavivirus***	**Mosquito (*Culex* spp.)**	**Humans, pigs, horses**	**E/abortion**	**Asia**	**Yes**
Kemerovo virus	*Reoviridae Orbivirus*	Tick (*Ixodes* spp.)	Humans, rodents, birds	SFI	Asia	No
Kyasanur Forest virus	*Flaviviridae Flavivirus*	Ticks (*Haemaphysalis* spp.)	Humans, small mammals	E	India	No
La Crosse virus	*Bunyaviridae Orthobunyavirus*	Mosquito (*Aedes* spp.)	Humans, Small mammals	E	North America	No
Louping ill virus	***Flaviviridae Flavivirus***	**Ticks (*Ixodes ricinus*)**	**Sheep, cattle**	**E**	**British Isles**	**No**
Murray Valley encephalitis virus	*Flaviviridae Flavivirus*	Mosquito (*Culex annulirostris*)	Humans, horse, cattle	E	Australia, Indonesia	No
Nairobi sheep disease virus	***Bunyaviridae Nairovirus***	**Tick (*Ripicephalus appendiculatus*)**	**Sheep, goats**	**HF/gastroenteritis**	**East Africa**	**Yes**
Omsk Haemorrhagic fever virus	*Flaviviridae Flavivirus*	Tick (*Dermacentor reticulatus*)	Humans	HF	Asia	No
Palyam virus	*Reoviridae Orbivirus*	Mosquitoes, midges	Cattle	Abortion	Africa, Asia and Australia	No
Peruvian horse sickness virus	*Reoviridae Orbivirus*	Mosquitoes	Horses	E	South America	No
Powassan virus	*Flaviviridae Flavivirus*	Mosquito (*Aedes*/*Anopheles* spp.)	Small/medium sized mammals		North America, Russia	No

Table 2: Continued.

Virus	Classification: family genus	Vector	Animals affected	Disease (SFI[1], HF[2], E[3])	Endemic presence	OIE* listed
Rift Valley fever virus	***Bunyaviridae Phlebovirus***	**Mosquito (*Aedes* spp.)**	**Humans, sheep, goats, camels**	**SFI/HF/abortion**	**Africa**	**Yes**
Russian spring-summer encephalitis virus	*Flaviviridae Flavivirus*	Tick (*Ixodes persulcatus*)	Humans, cattle, goats	E	Eurasia	No
Sandfly fever virus	*Bunyaviridae Phlebovirus*	Sandfly (*Phlebotomus perniciosus*)	Humans	SFI	Europe	No
Sindbis virus	*Togaviridae, Alphavirus*	Mosquito	Wildlife, avian	SFI	Africa, Europe, Asia	No
St. Louis encephalitis virus	*Flaviviridae Flavivirus*	Mosquito (*Culex* spp.)	Humans, avian	E	Americas	No
Semliki Forest virus	*Togaviridae Alphavirus*	Mosquito (*Aedes* spp.)	Equine	E	Africa	No
Tahyna virus	*Bunyaviridea Orthobunyavirus*	Mosquito (*Aedes* spp.)	Humans, pigs	SFI	Africa, Europe	No
Tick-borne encephalitis virus	*Flaviviridae Flavivirus*	Tick (*Ixodes* spp.)	Humans, wildlife	E	Europe, Asia	No
Thogoto virus	*Orthomyxoviridae Thogotovirus*	Tick (various spp.)	sheep	Abortion	Africa	No
Usutu virus	*Flaviviridae Flavivirus*	Mosquito	Avian, humans	E	Africa, Europe	No
Venezuelan equine encephalitis virus	***Togaviridae Alphavirus***	**Mosquito**	**Humans, horse**	**E**	**Americas**	**Yes**
Vesicular stomatitis virus	***Rhabdoviridae Vesiculovirus***	**Mosquito/sandfly/ midges/blackfly**	**Humans, cattle, horse, pigs**	**Mucosal vesicles**	**Americas**	**Yes**
Wesselsbron virus	***Flaviviridae Flavivirus***	**Mosquito (*Aedes* spp.)**	**Humans, sheep, cattle**	**HF**	**Africa, Asia**	**No**
West Nile virus	***Flaviviridae Flavivirus***	**Mosquito (*Culex* spp.)**	**humans, cattle, horse, avian**	**E**	**Africa, Eurasia, Americas**	**Yes**
Western equine encephalitis virus	***Togaviridae Alphavirus***	**Mosquito**	**Humans, cattle, horse**	**E**	**Americas**	**Yes**
Yellow fever virus	*Flaviviridae Flavivirus*	Mosquito (*Aedes* and *Haemagogus* spp.)	Humans, primates	HF	Africa, South America, Caribbean	No

[1] SFI: systemic Febrile Illness; [2]HF: haemorrhagic fever; [3]E: encephalitis.
*Office International des Epizooties (World Organisation for Animal Health).

2. Viruses Associated with Transmission by Midges

2.1. Bluetongue Virus

2.1.1. Clinical Disease. Bluetongue (BT) is a disease of ruminants with sheep being the most susceptible and affected species and cattle being affected to a lesser extent. Asymptomatic infections have been recorded in various other species such as deer, alpaca, llamas, and goats [17–19]. Since the emergence of BTV serotype 8 in northern Europe, significant clinical disease in cattle herds and goats has been reported [20]. The incubation period from exposure to disease is between two and fifteen days, averaging around 6 days [21]. In sheep, disease is first characterised by a fever and salivation, which develops into a more severe form causing mucopurulent discharge from the nasal passages and oedema of face and lips. The tongue may become cyanotic hence the name bluetongue and haemorrhages can form on the coronary band. Oral lesions can become haemorrhagic and ulcerative [21]. In affected cattle, crusts and erosions are visible on the nasal and oral mucosa, in or around the nostrils and lips. Other signs include salivation, fever, conjunctivitis, muscle necrosis, and stiffness in limbs.

2.1.2. Geographical Distribution. BTV is endemic throughout the world with the total of 24 serotypes circulating across the globe. In 2007, a new *Orbivirus*, Toggenburg virus, was isolated from goats in Switzerland and is now characterised as a tentative 25th serotype of bluetongue virus [22]. A further serotype has been detected in the Middle East [23].

Bluetongue virus was first reported in Africa when the disease was described in European Merino sheep that had been introduced into the Cape Colony [21]. Historically, the disease has predominantly been found between latitudes 40°N and 35°S until 1998 when the virus entered Europe and caused repeated outbreaks involving mainly five serotypes (BTV-1, BTV-2, BTV-4, BTV-9, and BTV-16) around the Mediterranean Basin affecting 12 European countries, three North African countries, and Israel [24]. In 2006, BTV-8

emerged in northern Europe with outbreaks in most western European countries and as far north as Denmark and southern Sweden [25]. BTV-8 emerged in south-east England in 2007 and spread to locations throughout the UK. However, the UK government introduced a voluntary vaccination programme in 2008, which controlled the disease and no further outbreaks have been reported within the UK [26]. In 2008, small localised outbreaks of BTV serotype-6 were reported in cattle herds in the Netherlands and Germany [27]. This was the second serotype to bypass southern Europe and emerge directly to northern Europe.

2.1.3. Vector. The only known biological vectors of bluetongue virus are the biting midges of the *Culicoides* genus. The most widespread vector is Afro-Asiatic *C. imicola*, which is distributed in Asia, the Middle East, most of Africa, and in southern and eastern Europe [28]. The absence and scarce distribution of *C. imicola* during the bluetongue outbreaks in Italy in 2000 onwards led to a search for novel vectors for virus transmission. Light-trapping and RT-PCR experiments identified the *C. pulicaris* and *C. obsoletus* complex (*C. obsoletus ss.*, *C. chiopterus*, *C. dewulfi*, *C. scoticus*) as potential vectors [29]. Later, field studies during the 2006 outbreak in Northern Europe also found no *C. imicola* in traps, but identified *C. dewulfi*, *C. obsoletus,* and *C. scoticus* as possible vectors [30]. In addition, these species have a palearctic distribution and they are known to be widespread throughout Europe. The high parity rates of *C. dewulfi* and *C. obsoletus* complex observed in Netherlands further supports the possibility of these species playing a role in bluetongue transmission [31]. These observations suggest that more than one species of midge are responsible for bluetongue virus distribution.

2.1.4. Molecular Diagnosis. Since the early 1990's, a range of RT-PCR assays targeting different BTV genome segments have been developed with a common drawback that they were only able to detect a limited number of serotypes [32–37]. Furthermore, most of these methods required the use of agarose gel electrophoresis for nucleic acid detection, which made them laborious and unpractical for diagnostic purposes. However, the spread of BTV in Northern Europe has seen the reporting of a number of conventional [38] and real-time RT-PCR assays detecting all 24 serotypes simultaneously [39–41]. These assays have been designed for diagnostic requirements; they are all rapid, reliable, and sensitive, enabling high throughput testing, which can be applied directly to clinical samples. The genome of bluetongue virus consists of ten double-stranded RNA segments coding for seven structural and three nonstructural (NS) proteins [42]. Several of these genome segments are highly conserved within serotypes, which make them potential targets for molecular detection. All recently published real-time RT-PCR and traditional RT-PCR assays use different BTV genome segments as a target. Orrù and coworkers [37] designed an assay targeting genome segment 10 (encodes NS3) using a stem-loop Molecular Beacon (MB) fluorescent probe. The probe can be used for both real-time RT-PCR detection and quantification purposes. Two groups have published duplex assays using two primer sets, where one assay targets BTV genome segment 7 (encoding the main BTV-specific antigen, VP7) and the other targets the segment 1 (encoding the viral polymerase, VP1) [39]. Using genome segment 1 as a target not only allows detection of all 24 serotypes, but will also detect geographic variants within individual serotypes by differentiating the samples to eastern (Middle-East, Asia, Australasia) and western (Africa, The Americas) genotypes [39]. Considering the high genetic variability through reassortment and mutations of RNA viruses, it is possible that current real-time RT-PCR assays might fail to detect some strains of BTV in the future. To address the problem of the genetic variability of BTV, the most recent real-time RT-PCR assay is based on the primer-probe energy transfer (PriProET) which is characterised by its tolerance towards mutations in probe region [42]. This assay is designed to detect all 24 serotypes.

2.2. African Horse Sickness Virus

2.2.1. Clinical Disease. African horse sickness virus (AHSV) is the causative agent of African horse sickness, a disease of *Equidae* with a high level of mortality. Horses are the most affected species whereas mules and donkeys show mild clinical signs or no signs at all, and zebras are considered a natural host and reservoir for AHSV [43]. Based on clinical and pathological findings, African horse sickness can manifest in four forms ranging from mild symptoms with no mortality to a severe disease with 95% mortality rate [43]. The mildest form, horse sickness fever, is characterised by mild to moderate fever lasting up to 5 days and affecting most commonly the African donkey. A cardiac (subacute) form is recognised by long-lasting fever, oedema of the head, neck, chest, or supraorbital fossae and petechial haemorrhages in the eyes and tongue with mortality rates around 50%. The most severe manifestation with highest mortality rates is a pulmonary (peracute) form, which is characterised by a rapid onset of disease. Death can occur without previous indication of illness or an animal can show signs of fever, depression and respiratory distress. The most commonly seen clinical presentation is a mixed (cardiac-pulmonary) form which can reach mortality rates as high as 70% 3–6 days after onset of fever.

2.2.2. Geographical Distribution. The virus is currently endemic in subtropical and tropical areas of Africa below the Saharan desert, which seems to provide a natural barrier against spread northwards [43]. Nine serotypes have been recognised which all have been reported in southern and eastern Africa. Serotypes 4 and 9 are found in western Africa and are the only serotypes that have caused outbreaks outside of Africa. The major outbreaks outside Africa have so far occurred in the Middle East, Spain, and Portugal. Spain has experienced five outbreaks of African horse sickness since 1966 [44]. The first outbreak in 1966 started from Gibraltar and was caused by serotype 9, which resulted in 637 animals dying or being slaughtered. In 1987, a number of subclinically infected zebras were imported into a Safari park near Madrid, which caused four further outbreaks between

1987 and 1990. However, these outbreaks were due to serotype 4 of AHSV. This was the first time that a serotype other than 9 had been recorded north of the Sahara desert. Before the eradication of the virus in Spain at the end of 1991, the outbreaks resulted in over 1300 horses dying or being destroyed and the virus spreading into Portugal, Tunisia, and Morocco [45].

2.2.3. Vector. Like bluetongue virus, African horse sickness virus is spread by biting midges of the *Culicoides* genus. The major vector for transmission is *C. imicola*, but also *C. bolitinis* has been shown to play a role in virus spread [46]. In 1998, over 100 horses died in an isolated population in Clarens Valley in South Africa and AHSV was isolated from the most locally abundant midge species, *C. bolitinis*, collected during light trap studies. It has been suggested that other *Culicoides* species might be involved, especially where low-grade cycling of virus is occurring.

2.2.4. Molecular Diagnosis. The first RT-PCR assays for AHSV detection and serogroup identification were time consuming and labour intensive procedures which involved either restriction fragment length polymorphism (RFLP) or dot-blot hybridisation assays [47–49]. These were followed by several conventional RT-PCR assays, which still required gel-based visualisation and took between four and six hours to complete [50–52]. However, a huge improvement in molecular diagnosis has occurred during recent years, resulting in numerous real-time RT-PCR assays that are able to detect, quantify, and discriminate the serotypes of AHSV in a short period of time [53–57]. Currently AHSV is only endemic in Africa, where laboratory conditions vary and real-time RT-PCR equipment can be too costly. Therefore, some studies have aimed to develop both real-time RT-PCR and conventional RT-PCR in parallel or solely improve conventional RT-PCR to provide better and faster diagnostic tools that are available in all circumstances [58]. These studies have shown high sensitivity and specificity of both conventional and real-time RT-PCR assays for all nine serotypes. Detection limit for both conventional and TaqMan real-time RT-PCR has been reported to be 1.2 $TCID_{50}$/mL [55]. More importantly new, improved RT-PCR assays can provide results within three hours of sample receipt.

2.3. Epizootic Haemorrhagic Fever Virus

2.3.1. Clinical Disease. Epizootic haemorrhagic fever virus (EHDV) causes a haemorrhagic disease in ruminants, especially in white-tailed deer in America and in cattle elsewhere. The clinical signs are often similar to those caused by bluetongue virus which complicates diagnosis. The clinical signs reported in cattle include reduction in milk production, fever, loss of appetite, weakness, excessive nasal and ocular discharge, oral ulcerations, discolouration of the udder, and oedema of hooves [59].

2.3.2. Geographical Distribution. There are currently ten serotypes of EHDV circulating throughout the world. EHDV-1 was first isolated in white-tailed deer in New Jersey in 1955 and it is still the most important infectious disease in deer in North America. All serotypes have caused clinical disease in cattle across the globe including North America, Africa (north and south), Australia [60], the island of Réunion [61], and Japan. Recent outbreaks have been reported around the Mediterranean including Morocco, Algeria, Israel in 2006, and Turkey in 2007 [62].

2.3.3. Vector. Culicoides spp. transmit the disease between ruminant hosts.

2.3.4. Molecular Diagnosis. The earliest EHDV RT-PCR assays have been based on American isolates, mainly on serotypes 1 and 2 targeting different genomic segments [63–65]. Since the spread of EHDV into new territories, the RT-PCR assays reported have been type-specific rather than serotype specific or even multiplex RT-PCR assays that can simultaneously detect both bluetongue and EHDV [66, 67]. A real-time RT-PCR has been reported that detects eight serotypes of EHDV [68].

2.4. Bovine Ephemeral Fever Virus

2.4.1. Clinical Disease. Bovine ephemeral fever virus (BEFV) causes disease in domestic cattle and water buffalo [69]. The disease is also known by the names 3-day sickness, stiff sickness, bovine epizootic fever, lazy man's disease, or dengue of cattle. The clinical outcome can vary from inapparent infection to death, but generally disease has four main phases. After an incubation period of between one and ten days, disease begins with a sudden fever that can be bi-, tri-, or polyphasic with peaks 12 to 18 hours apart. Fever lasts around half a day before the infected animal may become depressed and reluctant to move. Mucous discharge from the nose and profuse salivation can be observed and milk production is reduced or ceased altogether. This period of disability usually lasts between one and two days after which most animals start to recover. Although mortality rates as high as 30% have been observed, in most uncomplicated cases it is less than 2%. Sequelae include reduced milk production and other complications include pneumonia, mastitis, abortion in late pregnancy and temporary infertility of bulls [70].

2.4.2. Geographical Distribution. The bovine ephemeral fever was first recognised in Zimbabwe in 1906. The current distribution of BEFV includes all of Africa, the Middle East, Asia, and Australasia [71]. The outbreaks in the Middle East have occurred in Saudi Arabia and Israel [72, 73]. The disease has not been reported in Europe or the Americas.

2.4.3. Vector. Epidemiological studies indicate that BEFV is transmitted through flying insects. No arthropod vector has been shown to transmit the virus, but it has been isolated from *Culicoides* midges in Kenya and mosquitoes (*Culicine* mosquito species and *Anopheles bancroftii*) in Australia [74, 75].

2.4.4. Molecular Diagnosis. Currently, virus isolation seems to be the standard method for bovine ephemeral fever diagnosis and only two molecular diagnostic assays detecting BEFV have been reported. Real-time RT-PCR has been developed by Stram and coworkers [76], whereas Zheng and coworkers [77] reported the development of reverse transcription loop-mediated isothermal amplification (RT-LAMP) method. Both assays target the G gene and are highly sensitive assays, real-time RT-PCR being able to detect 10 BEFV genome copies in a sample [76]. The advantage of RT-LAMP is that no specialist equipment is required as there is no requirement for thermal cycling. However, a range of specialist apparatus is now available specifically for application with RT-LAMP such as lateral flow devices and turbidometers.

2.5. Akabane Virus

2.5.1. Disease. Akabane is a disease of ruminants. In adults the disease is generally asymptomatic with a transient viraemia occurring between one and six days after infection, which lasts for about six days. Occasional cases of encephalomyelitis have been observed in some infected animals [78]. The main economic impact of Akabane virus results from abortions, stillbirths, and congenital abnormalities that affect pregnant animals. Abnormalities vary depending on the trimester when infection occurs, although most are severely affected and euthanized shortly after birth.

2.5.2. Geographical Distribution. The disease occurs between latitudes 35°N and 35°S [79]. Serological evidence indicates that the virus is present throughout Africa, Asia, and the northern half of Australia. Disease has been observed in South Africa, Cyprus, the Middle East, and Japan.

2.5.3. Vector. Akabane virus has been isolated from *Culicoides* spp. in Australia (*C. brevitarsis* and *C. wadei)*, Africa (*C. milnei* and *C. imicola)*, Japan (*C. oxystoma*), and a number of mosquito species including *Aedes vexans*, *Culex tritaeniorhynchus* and *Anopheles funestus*.

2.5.4. Molecular Diagnosis. The genome of Akabane virus, like other orthobunyaviruses, is segmented, consisting of a small (s), medium (m), and large (l) segments. Standard RT-PCR assays have been described for detection of Akabane virus that target the S segment of the virus [78, 80]. A further development has been to incorporate detection of Akabane virus with that of Aino virus, a causative agent of congenital defects in cattle. This combined assay takes the format of a real-time multiplex RT-PCR that also targets the S segment [81]. The limit of detection is reported to be between 3 and 30 genome copies.

2.6. Equine Encephalosis Virus

2.6.1. Disease. Equine encephalosis virus (EEV) causes an acute disease in horses with a high fever and depressed appetite. A characteristic of the disease is the swelling of the lips and eyelids. Neurological disease is common and abortion can result from infection. Clinical features such as oedema are similar to those observed for AHSV and this should be considered in the diagnosis. However, fatalities are rare, particularly when supportive treatment is provided.

2.6.2. Geographical Distribution. The virus was originally isolated in South Africa and seven serotypes have been reported within the country [82]. A recent report has suggested the emergence of EEV in Israel [83].

2.6.3. Vector. Culicoides spp. are implicated in the transmission of this virus [84].

2.6.4. Molecular Diagnosis. No specific RT-PCR assays have been reported for equine encephalosis virus.

2.7. Vesicular Stomatitis Virus

2.7.1. Disease. Vesicular stomatitis virus (VSV) causes disease in cattle, horses, and pigs and is significant as it is clinically similar to foot and mouth disease (FMD). The initial incubation period is between two and eight days with a fever that often goes undetected. Early signs include drooling and frothing at the mouth. Blister-like lesions form in the mouth, on the dental pad, the tongue, the lips, the nostrils, the hooves, and the teats. Oral lesions can be sufficiently painful to cause the infected animal to refuse food and weight loss can occur. Infection is not fatal, although recovery from acute disease can take two weeks and ulceration can take months to heal.

2.7.2. Geographical Distribution. The virus is endemic in Central America and northern South America. Sporadic outbreaks occur in the USA and western regions of South America [85].

2.7.3. Vector. A range of haematophagus insects have been associated with transmission of VSV including Sand flies (Diptera: *Psychodidae*), black flies (Diptera: Simulidae), mosquitoes (Diptera: *Culicidae*), and *culicoides* midges (Diptera: *Ceratopogonidae*) [86]. Experimentally, only the sand fly (*Lutzomyia shannoni*) and the black fly (*Simulium vittatum*) have been shown to transmit the virus transovarially or to susceptible hosts. Serological evidence suggests that wild mammals can be infected with VSV but as yet there is no clear wildlife reservoir for the disease.

2.7.4. Molecular Diagnosis. Multiplex, real-time RT-PCRs have been devised to detect and differentiate different serotypes of VSV [86] and differentiate VSV from FMDV within the same assay [87].

3. Viruses Associated with Transmission by Mosquitoes

3.1. Eastern, Venezuelan, and Western Equine Encephalitis Virus

3.1.1. Disease. All three viruses cause disease in horses and humans [88]. This can range from asymptomatic infection

to acute, sometimes fatal, encephalitis. VEEV in particular has caused extensive epizootics in some regions of South America (Venezuala and Colombia).

3.1.2. Geographical Distribution. EEEV has been reported from the Eastern USA, Caribbean, South, and Central America. WEEV has been reported from North America and Cuba. VEEV is reported from many regions of South and Central America.

3.1.3. Vector

EEEV. The mosquito vector varies with climate and geography. In temperate zones, the ornithophagic *Culiseta melanura* is the main vector. In tropical regions, EEEV has been isolated from *Culex melanoconion*.

WEEV. In North America *Culex tarsalis* is considered the main vector of transmission between avian species. *Aedes* spp. have also been implicated in transmission to mammals. In South America *Aedes albifasciatus* has been reported as a vector.

VEEV. Culex melanoconion is associated with transmission of VEEV.

3.1.4. Molecular Diagnosis. TaqMan assays for North American EEEV and WEEV have been described [89]. Primer sets that detect a range of alphaviruses, including EEEV and VEEV have been described [90], however, this was linked to final detection using electrospray ionization mass spectrometry.

3.2. Japanese Encephalitis Virus

3.2.1. Disease. Japanese encephalitis virus (JEV) is asymptomatic in adult pigs but causes abortion, still-birth, and birth defects including central nervous system defects resulting in economic loss [91]. The virus also causes encephalitic disease in humans with over 50,000 cases reported annually [92]. Occasional cases in equines have been reported [93].

3.2.2. Geographical Distribution. JEV is found throughout Asia from Pakistan to Japan [94]. There is evidence that the virus is dispersing westwards through Asia.

3.2.3. Vector. The main vector of JEV is *Culex tritaeniorrhyncus*, which favours breeding in rice paddies. *C. gelidus* complex mosquitoes enable transmission to birds which assists in maintaining the virus in the environment [95].

3.2.4. Molecular Diagnosis. A real-time RT-PCR [96] that targets the 3 untranslated regions has been reported with detection to 112 $TCID_{50}$/mL. A multiplex real-time RT-PCR for detection of JEV, yellow fever virus, West Nile virus, dengue virus (serotypes 1–4), and St. Louis encephalitis virus has been reported [97] with a sensitivity of 2 PFU/mL. Real-time RT-LAMP [98, 99] assays have been developed for detection of JEV with detection levels as low as 0.1 PFU.

3.3. Rift Valley Fever Virus

3.3.1. Disease. Rift Valley fever virus (RVFV) affects ruminants with susceptibility influenced by age. Newborn animals are highly susceptible with adults showing less severe disease [100]. The incubation period ranges from one to three days followed by fever, recumbency, and haemorrhagic diarrhoea. Mortality can reach 70%. High rates of abortion are also associated with epidemics of Rift Valley haemorrhagic fever (RVHF), often described as abortion storms. The virus causes disease in humans ranging from uncomplicated influenza-like illness to haemorrhagic fever with liver damage and occasionally encephalitis.

3.3.2. Geographical Distribution. The virus is considered endemic throughout much of Africa, although clinical disease occurs infrequently. However, sudden outbreaks with high livestock mortality occur in many regions often following flooding. The disease was first reported in East Africa (Kenya and Tanzania) but is believed to have expanded its range north, west, and south, characterised by sudden epidemics [101]. There has been a well-documented outbreak of RVHF in the Arabian Peninsula associated with livestock movements from East Africa [102].

3.3.3. Vector. Aedes spp. are the principal virus vector for livestock and are believed to maintain the virus between epidemics although other species are capable of acting as bridge vectors enabling transmission during epidemics, such as *Culex* spp.

3.3.4. Molecular Diagnosis. Numerous real-time RT-PCR methods have been developed for rapid detection of RVFV [103–106]. The detection limit of these assays is typically between 10 and 100 genome copies. An alternative approach is the use of reverse transcription loop-mediated isothermal amplification (RT-LAMP) technique [107, 108]. The detection limits of RT-LAMP assays are comparable to real-time RT-PCR.

3.4. Wesselsbron Virus

3.4.1. Disease. Wesselsbron virus (WSLV) causes infection in sheep and goats and is associated with abortion and congenital abnormalities [109–111]. In adult animals, infection is usually subclinical, although in newborn animals clinical disease can result after a one to three day incubation period with fever and anorexia. Mortality can reach as high as 27%. Infection in humans has been reported following laboratory exposure and causes a mild influenza-like illness.

3.4.2. Geographical Distribution. Virus isolation and seroprevalence studies suggest that the virus is present across Africa [112, 113].

3.4.3. Vector. Aedes spp. are considered the main vector for Wesselsbron virus. In a recent study over 50 isolates of WSLV were isolated from *Aedes vexans* collected in Mauritania and Senegal [113].

3.4.4. Molecular Diagnosis. No specific RT-PCR tests have been reported, although the Wesselsbron virus genome has been published (NCBI Reference Sequence: NC_012735). Wesselsbron virus can be detected by RT-PCR using universal flavivirus primers and sequencing [114].

3.5. West Nile Virus

3.5.1. Disease. West Nile virus (WNV) causes encephalitic disease in horses, humans and some avian species [115]. In horses early disease consists of fever that is usually inapparent. Subsequent disease is neurological including ataxia, paresis and limb paralysis leading to recumbence. Muscle tremor and muscle rigidity may be observed. Mortality rates vary and may be particularly high in the USA reaching over 55%.

3.5.2. Geographical Distribution. Until recently, WNV was an Old World disease present throughout Africa and Asia with occasional incursions into Europe around the Mediterranean Basin [116]. However, in 1999, the virus emerged in the north east USA and spread throughout the Americas [117] and has remained endemic since that time [118].

3.5.3. Vector. The principal vector for WNV is the ornithophilic mosquito *Culex pipiens*. However, a wide range of mosquito species have been shown to support virus replication and transmit virus to mammalian species [119].

3.5.4. Molecular Diagnosis. Molecular detection of WNV has been reviewed recently by Shi and Kramer [120]. Such assays usually detect between 40 and 100 genome copies, although some suggest that sensitivity can be even lower [121]. Further developments have enabled multiplexing with other arboviral diseases [122, 123] and improved assay sensitivity [124] suggesting that the detection limit is 0.07 genome copies/mL. RT-LAMP assays have been developed for WNV [125] and used to detect the virus in mosquito samples [126]. The sensitivity limit of these assays has been reported to be approximately 0.1 PFU.

4. Viruses Associated with Transmission by Ticks

4.1. African Swine Fever Virus

4.1.1. Clinical Disease. African swine fever virus infects warthogs and bush pigs in Africa with no clinical disease, but domestic pigs can succumb to severe infection with an incubation period of five to fifteen days [127]. The disease can manifest in any one of four forms depending on the virulence of a strain. Highly virulent strains cause peracute and acute infections with clinical signs of high fever, anorexia, diarrhoea, recumbency, and general reddening of skin or discoloration on the ventral chest and abdomen, tips of the ears or tail, and on distal limbs. Death can occur within a day, sometimes before obvious clinical signs. The less virulent strains cause subacute and chronic infection. The subacute form manifests as a mild illness with an intermittent fever lasting approximately one month although pregnant animals might abort. In chronic cases, low fever, pneumonia and swelling of joints may occur. Some animals infected with low virulence strains can seroconvert without any clinical symptoms. Morbidity rates can reach close to 100% in herds that are naïve for ASFV whereas mortality rates vary, but can be as high as 100%, depending on virulence of the strain. Those animals that have survived acute or chronic disease can become persistently infected and act as carriers for the virus.

4.1.2. Geographical Distribution. African swine fever has been reported in Africa since the 1890's and is endemic in most parts of sub-Saharan Africa where transmission appears in three different forms; a sylvatic cycle, a domestic pig cycle and a pig-tick cycle [128, 129]. In 1958, ASFV emerged for the first time in Europe in Portugal before spreading to Spain in 1960, where it caused several outbreaks until the disease was finally eradicated [130]. In 1967, virus was detected in Italy and in 1978 ASF outbreaks occurred simultaneously in Malta and Sardinia. Whereas disease was eradicated from Malta and mainland Italy, ASF remains endemic in Sardinia where it is established in free-range pigs and wild boars. In 1998, Madagascar reported the first case of African swine fever [131], and from the year 2000 onwards virus has continued to spread into new territories including Georgia, Iran, and Mauritius [132–134]. The Georgian outbreak in 2007 demonstrated perfectly the emerging and transboundary characteristics of ASFV, as within a year virus spread from Georgia to several neighboring countries including Armenia, Azerbaijan, and the Russian Republic of Chechnya [132]. In the following year, virus spread to northwestern Iran where it caused an outbreak in wild boar [133]. Other short-lived, sporadic outbreaks have been reported from France, Belgium, Netherlands, Caribbean, and South America.

4.1.3. Vector and Transmission. The subclinical infection of ASFV in warthogs and bush pigs is maintained by soft ticks of the genus *Ornithodoros*. In Africa, the main vector is *O. moubata,* whereas in Southern Europe ASFV is transmitted by *O. erraticus* [135]. *O. moubata* is most abundant in eastern and southern Africa particularly in Cameroon, Central African Republic, and Sudan. The most prevalent viral hosts include warthogs, domestic pigs, and man. Although, ticks do play a part in virus transmission, the epidemiological role is thought to be low, especially in those areas where tick populations are small [134]. The more likely route of virus spread is through direct oronasal contact with an infected animal or indirectly via fomites and contaminated pig feed. The most recent outbreaks in previously ASFV-free areas could have been transmitted through feed products containing infected pig meat.

4.1.4. Molecular Detection. As there is no cure or vaccine for ASFV, slaughter is the only tool for control and eradication of this highly contagious virus in infected areas. Therefore, fast and reliable laboratory diagnosis is required to limit the socio-economic burden of outbreaks. Furthermore, highly specific, differential diagnosis of disease is necessary,

as clinical signs of African swine fever may resemble those of other infections, especially classical swine fever. The current OIE manual recommends Taqman real-time PCR such as the assay developed by King and coworkers [136]. As real-time PCRs are high throughput, sensitive, specific, and quick to run, recent development of these assays has focused on improvement of existing real-time assays by applying varying PCR chemistries. This has led to several modifications of real-time PCR including use of minor groove binding probes or molecular beacon assays [137, 138]. A recent method has been further developed with field diagnostics in mind. Two assays, a LAMP method and a linear-after-the-exponential PCR (LATE-PCR), have both been adapted for use in field conditions [139, 140]. These methods could considerably shorten the time between infection and diagnosis as there is no requirement for samples to be transported to the laboratory.

4.2. Crimean-Congo Haemorrhagic Fever Virus

4.2.1. Disease. Crimean-Congo haemorrhagic fever (CCHF) is one the most important and widespread diseases caused by tick-borne viruses. The causative agent, Crimean-Congo haemorrhagic fever virus does not cause disease in livestock, but vertebrates play a role in virus transmission as part of a tick-vertebrate-tick enzootic cycle [141]. Although there is no evidence of clinical disease in animals, contact with viremic animals, tick bite, or crushing ticks taken from infected animals can lead to human infection. In humans, CCHF virus causes a disease with four phases: incubation, prehaemorrhagic, haemorrhagic, and convalescence phases [141]. Following the short incubation period (3–7 days), sudden onset of fever, headaches, myalgia, and dizziness occur [142, 143]. A few days later, a rapidly developing haemorrhagic period occurs, with haematomas appearing on the skin and mucous membranes with mortality rates ranging from 3% to 30%. The surviving patients will go through the convalescence period lasting 9-10 days which can include variable symptoms such as tachycardia, temporary hair loss, poor vision, and loss of appetite. Infected humans can spread CCHF via close contacts resulting in community and nosocomial outbreaks [144–148]. Furthermore, individuals in certain occupations such as health carers, veterinarians, farmers, and abattoir workers are at increased risk of contracting Crimean-Congo haemorrhagic fever [141, 143, 149].

4.2.2. Geographical Distribution. The geographic distribution of CCHF virus is the widest amongst all tick-borne diseases. Currently CCHFV is endemic in Africa, Asia, Balkan countries, and Middle and Far East [142, 145, 150]. During the last ten years, CCHFV has been rapidly introduced into new, previously nonendemic areas; especially into eastern and southeastern Europe including Greece and Turkey [149, 151–153].

4.2.3. Vector. *Hyalomma* spp. are the vector and reservoir for CCHFV, particularly *Hyalomma marginatum*. The geographical distribution of these ticks closely match the distribution of CCHFV and covers southern Europe, southern Russia extending to southern Asia, and most of Africa [154]. The host range of these ticks varies from domestic animals (cattle, horse, sheep, and goats) for adults to small wild animals and birds for larvae and nymphs.

4.2.4. Molecular Detection. Since the mid 1990's several nested and real-time RT-PCR assays, which all target genomic S segment of the tripartite genome. These assays have been developed to achieve fast initial and differential laboratory diagnosis of CCHFV [104, 155–158]. Both the nested and real-time RT-PCR assays are comparable in terms of sensitivity, but the results can be achieved in two hours with one-step real-time RT-PCR, whereas it takes 4-5 hours to run nested RT-PCR without gel electrophoresis. Due to the high genetic variability of CCHFV isolates, the first real-time RT-PCR assay detecting CCHFV was based on a Sybr Green method that intercalates to any double stranded DNA and requires identification of the positive product using gel electrophoresis [104]. More specific assays can be developed by designing primers and probes directed at strains of interest, for example, those circulating in geographically defined areas. Strains from the Balkan region have been successfully identified by using a one-step real-time RT-PCR that is based on the fluorescence resonance energy transfer (FRET) probe technology employing the endonuclease ($5' \rightarrow 3'$) activity of *Taq* polymerase enzyme [158]. Furthermore, this assay can also be used for detection of CCHFV from ticks.

4.3. Nairobi Sheep Disease Virus

4.3.1. Disease. Nairobi sheep disease virus (NSDV) causes acute haemorrhagic gastroenteritis in sheep and goats. This is a severe and fatal disease with mortality rates reaching over 90 percent in non-immune animals. The short incubation period (2–4 days) is followed by high fever, diarrhoea, and collapse [159]. Watery to mucoid and bloody diarrhoea is seen 2–4 days after the onset of fever which itself can last up to 8 days. As the disease progresses nasal discharge and conjunctivitis are common features [160]. In pregnant animals, the infection can lead to abortion. In fatal cases, death often occurs in the early stages of a disease. A variant of NSDV, Ganjam virus has been isolated in India [161]. This virus is highly pathogenic in exotic and crossbred sheep and goats causing a disease with high fever, dullness, depression, and loss of appetite [162].

4.3.2. Geographical Distribution. Nairobi sheep disease has been reported since 1910 when it was first identified near Nairobi in Kenya [163]. NSDV is now mostly enzootic in Kenya with the majority of indigenous sheep and goats having protective antibodies, and outbreaks tend to occur when flocks from uninfected areas are brought into enzootic areas [164]. Further outbreaks have been reported from other countries in east and central Africa. Serosurveys suggest that the virus may be more widespread in countries of Southern Africa [165]. Ganjam virus, a variant of NSDV, circulates across southern India [166].

4.3.3. Vector. In Africa, the main vector for NSDV is the three-host Ixodid tick *Rhipicephalus appendiculatus*, which

is found between its northern limits of southern Sudan and Ethiopia through Eastern, central and Southern Africa. Livestock hosts for this tick include cattle, sheep, goats, and horses [164]. In wildlife, antelopes and buffalo are frequently infested. Ganjam virus has been isolated from *Haemaphysalis* spp. ticks, which have been collected from sheep and goats in India [161].

4.3.4. Molecular Detection. Primers for NSDV amplification have been reported [166, 167]. The complete genome sequences of the tripartite genome of NSDV have been published. No real-time RT-PCR has been reported.

4.4. Louping Ill Virus

4.4.1. Disease. The main forms of tick-borne encephalitis virus (TBEV) found in Europe and Asia do not cause clinical disease in livestock although human cases are on the increase in Europe [168]. However, a number of closely related variants of TBEV cause disease in livestock. These include louping ill virus (LIV), Spanish sheep encephalitis virus (SSEV), Greek goat encephalitis virus (GGEV), and Turkish tick-borne encephalitis virus (TTEV). Of these, LIV is the most studied virus as the cause of disease in sheep and red grouse. In red grouse, LIV causes rapid and fatal encephalitis whereas in sheep, disease is biphasic, especially in young nonimmune animals [169]. The initial clinical signs of louping ill include fever and weakness before animals develop neurological signs of disease. A range between 5 and 60% of infected animals develop clinical signs and death can occur between 24 and 48 hours following the development of neurological signs. During the neurological phase, the most damage is caused to the cerebellum. Clinically, the second phase is characterized by cerebral ataxia, hyperexcitability, and progressive paralysis [169]. Occasionally, natural infection of louping ill has also been reported from other species such as dogs, roe and red deer, and llamas [170–173]. The other variants are genetically distinct, but closely related to louping ill. They are much less studied, but appear to all cause disease that resembles louping ill [174].

4.4.2. Geographical Distribution. Tick-borne encephalitis virus is found in many areas throughout Europe and Asia. Transmission to humans is mainly through tick bites but can occasionally result from consumption of unpasteurized milk [175]. LIV is endemic within upland areas in the UK, particularly in Scotland, Wales, the northwest and southwest of England [169]. There have also been reports of LIV in Ireland and Norway [176]. Sheep infected with SSEV have been reported from the Basque region of Spain [174, 177]. The first isolation of GGEV was made in the village of Vergina in northern Greece [178]. Subsequent studies have suggested that GGEV circulates in the *I. ricinus* population in northern Greece, although at low density [179]. TTEV was first reported from northwestern Turkey (Anatolia) as encephalitis in sheep and was subsequent confirmed by nucleotide analysis to be related to tick-borne encephalitis virus [180–182].

4.4.3. Vector. Both TBEV and its variants are transmitted by the three-host hard tick *Ixodes ricinus* in Europe. These ticks are the most common species of ticks in northwest Europe and are also known as the common sheep tick, castor bean tick or wood tick. In addition to sheep and red grouse, the hosts for *I. ricinus* in the UK include mountain hares, red deer, roe deer and small mammals [183]. In the Far East of Europe and throughout Asia, TBEV is transmitted by *Ixodes persulcatus*.

4.4.4. Molecular Detection. The first nested one-step RT-PCR for louping ill detection was developed in mid-1990s. This assay, which targets the envelope (E) and membrane (M) genes of the virus genome, can be used for virus identification in ticks and other species [184]. As the E gene is the most conserved of the three LIV structural genes, a one-step Taqman RT-PCR assay has been developed with primers for this protein [185]. Brain and spinal cord samples were tested and detection limit of 1 PFU/mL was achieved, making this assay as sensitive as the traditional plaque assay. In recent years, real-time RT-PCR assay detecting eight different tick-borne flaviviruses (including louping ill) and West Nile virus has been developed in the UK [186]. The assay employs degenerate primers targeting nonstructural protein 5 and is based on incorporation of Sybr green. The assay is not as sensitive as species specific assays, but makes it an ideal choice when a single virus cannot be specified before testing [186]. A recently reported assay uses one-step real-time RT-PCR with pyrosequencing which enables the rapid differentiation between TBEV subtypes [187]. Although, this assay was developed to differentiate between the main TBE subtypes, it is also able to detect louping ill virus. Specific detection assays for SSEV, TTEV, and GGEV have not been reported.

5. Conclusions

The majority of the viruses reviewed have been thoroughly characterized and there are a wide range of molecular diagnostic tests available. These include bluetongue virus, AHSV, VSV, WNV, and ASFV. These tend to be those viruses that cause the greatest impact either to livestock health or as a consequence of spillover into the human population as a zoonotic agent. New serotypes and variants emerge and techniques may require modification or augmentation in response to genetic differences. Other viruses have molecular tests available, but these are not widely in use, either because many countries do not consider them necessary or do not have suitable containment facilities to handle the virus. This group includes EHFV, BEFV, Akabane virus, the equine encephalitides, JEV, RVFV, CCHFV, and variants of tick-borne encephalitis virus. Further effort is needed to establish molecular tests for these viruses more widely in preparation for potential outbreaks. Finally, there is a small group for which no molecular diagnostic tests have been reported. This includes equine encephalosis virus, Wesselsbron disease virus and Nairobi sheep disease virus. These viruses require some test development.

Test development and implementation could take two forms. The first option is to develop specific tests that detect

a single virus, often at high sensitivity. The alternative is the development of assays that detect a range of related pathogens in single step. This has been applied to groups including the flaviviruses, alphaviruses, and phleboviruses. The latter option has the benefit of screening for more than one pathogen. However, this approach is often less sensitive and may require further analysis to identify the exact virus involved.

Currently, rapid detection methods are dominated by nucleic acid amplification methods, particularly the polymerase chain reaction. By linking nucleic acid amplification to sequencing, it is possible to rapidly identify a large number of potential pathogenic viruses [114, 188]. This approach has been applied to detection of flaviviruses using universal primers ([186], and references therein). New technologies such as microarray [189] and next generation sequencing [190] are being applied to the investigation of infectious viruses. However, these technologies are expensive to establish and maintain for purely diagnostic or surveillance purposes. Future development is required in this area to make these technologies more accessible and affordable for use in the detection of arthropod viruses of livestock.

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Research Article

A Novel System for Rapid and Cost-Effective Production of Detection and Diagnostic Reagents of West Nile Virus in Plants

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The threat of West Nile virus (WNV) epidemics necessitates the development of a technology platform that can produce reagents to support detection and diagnosis rapidly and inexpensively. A plant expression system is attractive for protein production due to its low-cost and high-scalability nature and its ability to make appropriate posttranslational modifications. Here, we investigated the feasibility of using plants to produce two WNV detection and diagnostic reagents to address the current cost and scalability issues. We demonstrated that WNV DIII antigen and E16 monoclonal antibody are rapidly produced at high levels in two plant species and are easily purified. Furthermore, they are effective in identifying WNV and in detecting human IgM response to WNV infection. E16 mAb does not cross-react with other flaviviruses, therefore, is valuable for improving diagnostic accuracy. This study provides a proof of principle for using plants as a robust and economical system to produce diagnostic reagents for arboviruses.

1. Introduction

West Nile virus (WNV) is an arbovirus that belongs to the *Flavivirus* genus of the Flaviviridae family. It is a neurotropic, enveloped virus with a single-stranded, positive polarity, 11 kilobase RNA genome. The transmission cycles of WNV involve mosquitoes of the genus *Culex* and birds, while humans and other mammals are incidental hosts. Until 1999, WNV was found in the Eastern Hemisphere, with wide distribution in Africa, Asia, the Middle East, and Europe [1]. In 1999, WNV entered the Western Hemisphere through New York City. Since then, more than 30,000 humans with severe WNV infection in the continental United States have been diagnosed, with many more cases of infection remaining undiagnosed. Advanced age is one of the greatest risk factors for severe neurological disease, long-term morbidity, and death [2], although a genetic basis of susceptibility has also been recently identified [3].

Several methods have been developed for the detection of WNV and WNV infections. Specific work in mosquito vectors and infected avian or mammalian hosts has been successful and is protein or nucleic acid based [4–6]. Human WNV infection can be diagnosed by detecting host immune responses such as WNV-specific IgM or IgG production with enzyme-linked immunosorbent assays (ELISAs) or by directly identifying WNV from cell cultures of serum, cerebrospinal fluid, or tissues with WNV-specific monoclonal antibodies (mAbs) [4, 7, 8]. Reverse-transcriptase polymerase-chain-reaction- (RT-PCR-) based assays have also been developed to identify the presence of the RNA genome of WNV [9, 10]. Both protein and nucleic acid-based assays are being evaluated for identifying WNV or its genome in other mammals, mosquito pools, and avian specimens. However, due to the short viremic phase and low viral count of WNV in human blood and cerebrospinal fluid, protein-based assays such as ELISA have increasingly become the standard methods of detection and diagnosis [7]. Currently, WNV-specific mAbs, recombinant WNV antigens, and other protein-based detecting reagents are produced in mouse hybridoma, mammalian, insect, or bacterial cell cultures [11–13]. The high production costs and limited scalability associated with the bioreactor-based cell culture system may limit their application. The expanding epidemics of WNV demand the development of a technology platform that can rapidly produce reagents to accommodate the detection and diagnostic needs at a low cost.

Plants have been proposed as bioreactors for protein production because of their capacity to generate large volumes of proteins at low cost and their ability to make appropriate posttranslational modification of recombinant proteins [14, 15]. Traditionally, proteins are produced in transgenic plants that require an extensive time period to generate transgenic lines [16]. In contrast, transient expression of a target gene in plant material can produce the target protein within 1 to 2 weeks [17]. The recently developed transient expression systems based on plant viral vectors promote high-level accumulation of foreign protein due to their efficient replication, which results in high copy numbers of transgenes and their mRNA transcripts [18, 19]. An example of a viral-based vector uses tobacco mosaic virus (TMV) and potato virus X (PVX) genomes under the control of plant promoters (the MagnICON system) [20]. Another example is the geminivirus-based expression system: a DNA replicon system derived from the bean yellow dwarf virus (BeYDV) that allows rapid high-yield production of proteins in plants [21]. These plant-based transient expression systems provide the speed and flexibility of bacterial gene expression system coupled with the posttranslational protein modification capability and yield of mammalian cell cultures.

In this study, we demonstrate the feasibility of using plant transient expression systems to produce two groups of protein reagents that are required for the detection and diagnosis of WNV infection: a recombinant antigen derived from the domain III (DIII) of WNV envelope (E) protein and a mAb (E16) that specifically recognizes WNV DIII. Our plant transient expression systems permit high-level expression of WNV DIII antigen and E16 mAb in both *Nicotiana benthamiana* and lettuce plants. These detection and diagnostic reagents can be easily purified to greater than 95% purity. In addition, our results demonstrate their functional activity and utility in identifying WNV and in detecting human IgM response to WNV infections. Therefore, this study provides proof of principle for using plants as a robust, rapid, and flexible production system for protein reagents for the detection and diagnosis of WNV and can be broadly applied to other arboviruses.

2. Materials and Methods

2.1. Experimental Materials. The DIII protein of Dengue virus serotype 2 (DENV-2) was purchased from Genway Biotech (San Diego, Calif, USA). The *E. coli*-produced DIII protein of WNV was a gift from Dr. M. Diamond (Washington University School of Medicine, St. Louis, Mo, USA). The West Nile IgM capture ELISA kit was obtained from Diagnostic Automation Inc. (Calabasas, Calif, USA).

2.2. Construction of Plant Expression Vectors. The coding sequence of WNV E protein DIII 13.5 kDa ectodomain (amino acids 296–415 of WNV E protein of the New York 1999 strain, Genbank Accession number AF196835) was optimized *in silico* with *N. benthamiana*-optimized codons using an algorithm as described in Villalobos et al. [22]. An 18-bp sequence coding for a hexahistidine (His6) purification tag was added to the C-terminus of the DIII gene. Optimized DIII-His6 sequence was synthesized (DNA 2.0, Menlo Park, Calif, USA) and cloned into the 5′ modules of plant expression vector pICH11599 of the MagnICON system as described previously [20]. The geminiviral vector pBY-HL(hE16-no-KDEL).R for expressing E16, a mAb against WNV E protein [13, 23], was constructed by replacing the E16 heavy chain (HC)-KDEL sequence in vector pBY-HL(hE16).R [24] with the KDEL-less E16 HC sequence. The expression of the resulting pBY-HL(hE16-no-KDEL).R vector produces E16 mAb molecules in which the C-termini of the HC are not attached by the ER-retention signal KDEL.

2.3. Agroinfiltration of N. benthamiana and Lettuce. Plant expression vectors were individually transformed into *Agrobacterium tumefaciens* GV3101 by electroporation as previously described [25]. Wild-type *N. benthamiana* and lettuce (*L. sativa*) plants were grown in a greenhouse with 16/8 hr light/dark cycle at 25°C for 6 weeks. For DIII expression *N. benthamiana* leaves were co-agroinfiltrated with GV3101 strains containing the DIII 5′ module along with the TMV 3′ module, and an integrase construct, with a syringe as described previously [26]. For E16 expression in *N. benthamiana,* the syringe-agroinfiltration method was also used to deliver the geminiviral vector pBY-HL(hE16-no-KDEL).R into leaves. Lettuce heads were infiltrated with the GV3101 strain containing the geminiviral vector pBY-HL(hE16-no-KDEL).R for 2 × 5 min under vacuum (100 mbar) as described previously [27].

2.4. Extraction of Total Protein from Plant Leaves. Agroinfiltrated *N. benthamiana* or lettuce leaves were harvested 3–8 days following infiltration (dpi) for evaluating the temporal pattern of DIII and E16 mAb expression. For other protein analysis, plant leaves were harvested 4 dpi. Total leaf protein was extracted by homogenization in extraction buffer I (100 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1 mM phenylmethylsufonyl flouride) for DIII antigen or extraction buffer II (PBS, pH 7.4, 1 mM EDTA, 10 mg/mL sodium ascorbate, 10 μg/mL leupeptin, 0.3 mg/mL phenylmethylsufonyl flouride) for E16 mAb using a FastPrep machine (Qbiogene, Carlsbad, Calif, USA) following manufacturers instructions. Extraction buffers I and II were specifically developed based on the biochemical properties of DIII antigen and E16 mAb for the maximal extraction and stability of each target protein, while minimizing the coextraction of plant host proteins. The crude plant extract was clarified by centrifugation at 18,000 ×g for 30 min at 4°C. The concentration of leaf total soluble protein (TSP) was measured by using the Protein Bradford Assay (Hercules, Calif, USA).

2.5. SDS-PAGE and Western Blot Analysis. SDS-PAGE and western blotting were performed as described previously [28]. Protein samples were subjected to 12% or 4–20% gradient SDS-PAGE under reducing (5% v/v β-mercaptoethanol) or nonreducing conditions. Gels were then either stained with Coomassie blue or used to transfer proteins onto PVDF membranes. Horseradish-peroxidase- (HRP-) conjugated

antibodies against human-kappa light chain (LC) or gamma heavy chain (HC) (Southern Biotech, Birmingham, Ala, USA) were used for western blot analysis of E16 mAb. For DIII antigen, a rabbit anti-WNV DIII polyclonal antibody and an HRP-conjugated goat anti-rabbit IgG (Southern Biotech, Birmingham, Ala, USA) were used for the analysis.

2.6. Purification of DIII Antigen and E16 mAbs from Plant Tissue. *N. benthamiana* or lettuce leaves were harvested on 4 dpi and extracted with a blender in extraction buffer I (for DIII) or extraction buffer II (for E16), and the extract was clarified by filtering through Miracloth, followed by centrifugation at 17,700 ×g for 30 min at 4°C. For DIII purification, the pH of the clarified extract was adjusted to pH 5.0 and followed by centrifugation at 17,700 × g for 30 min at 4°C to precipitate the most abundant plant protein, the photosynthetic enzyme RuBisCo, and other host proteins. The supernatant was adjusted to pH 8.0 and respun to remove residue precipitates. The supernatant was then applied to a Ni-NTA His-Bind column and purified by metal chelation chromatography according to a protocol supplied by the manufacturer (Novagen, Madison, Wis, USA). The column eluate containing the purified DIII antigen was buffer-exchanged to PBS. For E16 purification, the clarified leaf extract was filtered through a 0.2-micron filter and loaded directly to a MAbSelect Protein A column (GE Healthcare, Piscataway, NJ, USA). The column was eluted with 50 mM sodium citrate, pH 2.5. Tris-base (1 M) was added immediately to the eluate to attain a final pH of 7.0. The purity of DIII or E16 was determined by quantitating Coomassie blue—stained protein bands on SDS-PAGE using a densitometer as described previously [26].

2.7. ELISA. E16 quantitative ELISA was designed to detect the assembled form of E16 mAb (with both LC and HC) as described previously [23]. Plates were coated with a goat anti-human-gamma HC antibody (1 : 2,000 dilution, Southern Biotech, Birmingham, Ala, USA). After incubation with plant protein extract, an HRP-conjugated anti-human-kappa LC antibody (1 : 10,000 dilution) was used as the detection antibody. A mammalian cell-culture-derived E16 was used as a reference standard [13]. The plates were developed with 3,3′,5,5′-tetramethylbenzidine (TMB) substrate (KPL Inc, Gaithersburg, Md, USA).

DIII expression analysis was performed by coating plates with a monoclonal mAb against WNV DIII (1 : 2,000 dilution) [13]. Clarified plant extract was then applied to the wells followed by detection with a rabbit anti-WNV DIII antibody (1 : 10,000 dilution) and an HRP-conjugated goat anti-rabbit IgG antibody (1 : 10,000 dilution).

Binding specificity of plant-derived E16 to DIII protein of various flaviviruses was performed using a previous published method [29]. An *E. coli*-derived WNV DIII [29] or DENV-2 DIII (100 uL, 2 ug/mL) was immobilized on microtiter plates. An HRP-conjugated anti-human-kappa LC antibody (1 : 10,000 dilution) was used as the detection antibody. A mammalian cell-culture-derived E16 and a generic human IgG (Southern Biotech, Birmingham, Ala, USA) were used as the positive and negative control, respectively.

The WNV IgM capture ELISA was performed according to the procedures provided by the manufacturer (Diagnostic Automation Inc. Calabasas, Calif, USA). Briefly, E16 mAbs derived from *N. benthamiana* and lettuce were conjugated to HRP with a commercial Lightning-Link HRP conjugation kit (Innova Biosciences, Cambridge, UK). Human WNV IgM positive control (PC) or negative control (NC) serum was incubated in microtiter wells coated with anti-human IgM antibodies. This was followed by incubation with a negative control antigen (NCA), a kit-supplied WNV recombinant antigen protein (WNRA), or plant-derived WNV DIII antigen (50 uL, 1 : 25 dilution). After washing, wells were incubated with either a kit-supplied WNRA-specific antibody (anti-WNRA) labeled with HRP or with the HRP-conjugated plant-derived E16 (50 uL, 1 : 100 dilution). High concentrations of plant-derived DIII and E16 mAb were used to saturate their respective target-binding sites, ensuring all WNV-specific IgM molecules captured on the plate are detected. After further washing, TMB substrate was used to develop the plate. The Immune Status Ratio (ISR) is calculated by dividing the average absorbance of the WNRA or plant-derived DIII antigen by the average absorbance of NCA (WNRA/NCA or Plant DIII/NCA). The quality control specifications for this ELISA are PC ISR > 5.66 and NC ISR < 4.47.

2.8. Flow Cytometry of Yeast Displaying WNV E Protein. Analysis of the detection of WNV E protein by plant-derived E16 was performed using a yeast strain that expresses WNV E protein on its surface. The staining of yeast cells and flow cytometry analysis were performed as described previously [13]. Briefly, yeast cells were grown to log phase, followed by an additional 24 h in tryptophan-free media containing 2% galactose to induce WNV E protein expression. The yeast cells were then incubated with E16 mAbs derived from *N. benthamiana* or lettuce. A generic human IgG was used as a negative control mAb. The yeast cells were stained with a goat anti-human secondary antibody conjugated to Alexa Fluor 488 (Invitrogen, Carlsbad, Calif, USA). Subsequently, the yeast cells were analyzed on a BD FACSCalibur flow cytometer (Franklin Lakes, NJ, USA).

3. Results

3.1. Expression of WNV DIII Recombinant Antigen with Transient Expression Vectors in N. benthamiana Plants. As an initial test of the feasibility of using plant to produce WNV diagnostic reagent, we examined the expression of DIII antigen in the plant host of *N. benthamiana*. This is a plant species that is related to the common tobacco plants and has been used extensively in producing recombinant proteins [30]. Western blot analysis showed that DIII antigen was detected in the plant sample infiltrated with DIII construct with the expected molecular weights of 13.5 kDa along with an *E. coli*-produced DIII positive control (Figure 1(a)). This band was not detected in negative control leaf samples that were infiltrated with infiltration buffer, confirming the specificity of the DIII band (Figure 1(a)). The slightly smaller molecular weight of plant-derived DIII reflects the fact that

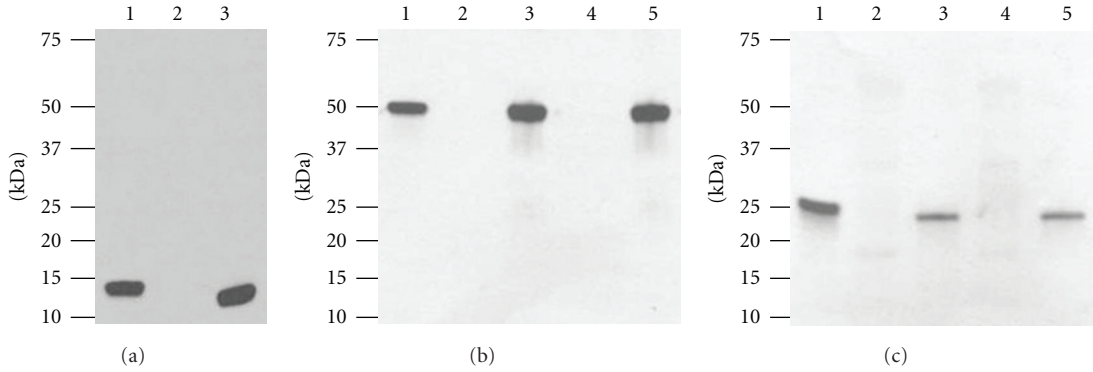

Figure 1: Western blot analysis of WNV DIII antigen and E16 mAb expression in *N. benthamiana* and lettuce. (a) WNV DIII. Leaf protein extracts were separated on a 4–20% SDS-PAGE gradient gel and transferred onto PVDF membranes. The membranes were probed with a rabbit anti-WNV DIII polyclonal antibody. Lane 1: *E. coli*-derived DIII standard; lane 2: protein extract from leaves infiltrated with buffer (negative control); lane 3: extract from DIII construct infiltrated *N. benthamiana* leaves. (b) and (c) Expression of E16. Wild-type laboratory-grown lettuce or *N. benthamiana* were infiltrated with dual-replicon geminiviral vector pBY-HL (hE16-no-KDEL). R and harvested on 4 dpi. Total leaf protein extracts were separated on 4–20% SDS-PAGE gradient gels under reducing conditions and transferred to PVDF membranes. The membranes were incubated with a goat anti-human-gamma chain antibody to detect HC (b) or a goat anti-human-kappa chain antibody to detect LC (c). Lane 1: human IgG reference standard; lane 2: extract from lettuce leaves infiltrated with buffer (lettuce negative control); lane 3: protein samples from lettuce infiltrated with geminiviral vector pBY-HL(hE16-no-KDEL). R; lane 4: extract from buffer-infiltrated *N. benthamiana* leaves (negative control); Lane 5: *N. benthamiana* leaf protein extract infiltrated with pBY-HL(hE16-no-KDEL). R vector.

it lacks the extra N-terminal peptide tags (His-Tag and T7-Tag) in the *E. coli*-derived DIII. Western blot confirmed the stability of plant-derived DIII as only the expected band was observed. The production of DIII was confirmed by a sandwich ELISA using two WNV specific antibodies. ELISA results also indicated that DIII reached a high expression level within 4 days after infiltration, with an average accumulation of 1% TSP (Figure 2), which corresponds to ~0.1 mg/g fresh leaf weight (FLW). This level is comparable to the high range of accumulation of other antigens in plants [25] and is the highest expression level of any flavivirus antigen ever reported in plants. This convincingly demonstrates that plants can rapidly express DIII antigen at high levels. Furthermore, in contrast to *E. coli*-produced DIII which requires a cumbersome solubilization and refolding process [11], plant-derived DIII is produced as a soluble protein and can be easily purified to greater than 95% purity (data not shown) by a metal chelation chromatography.

3.2. High-Level Expression and Assembly of a mAb against WNV in Wild-Type Plants. To further demonstrate the utility of plants as a platform for production of protein reagents, we examined the production of a mAb, a representative of another important group of relevant molecules used in the detection and diagnosis of WNV infection. To compare the expression of E16 mAb in two species of plant hosts (*N. benthamiana* and lettuce), we used the BeYDV-based geminiviral replicon vectors. In contrast to the MagnICON system, these vectors have been shown to direct transgenic protein expression in both tobacco and lettuce [24]. The Ag*robacterium* strain containing replicons of the LC- and HC-coding sequences of E16 were infiltrated into laboratory-grown lettuce and *N. benthamiana* leaves. Western blot analysis revealed that the LC and HC of E16 were expressed in both lettuce and *N. benthamiana* leaves with the expected molecular weights of 25 kDa and 50 kDa, respectively, (Figures 1(b) and 1(c)). The full tetrameric (2HC + 2LC) assembly of these mAbs was demonstrated by western blot (data not shown) and Coomassie blue staining analysis (Figure 3, Lanes 5–7) under nonreducing conditions. The assembly of pHu-E16 was corroborated by an ELISA that detects the assembled form of E16 (HC capture, LC probe, Figure 2). Since only the expected bands were observed on western blots, the results established that E16 is stable during biosynthesis in both plant hosts and proteolytic degradation of the LC or HC did not occur. Further ELISA analysis also showed that E16 was produced rapidly in both plant species and reached a high level of accumulation to 3.5% (in lettuce) or 5% TSP (in *N. benthamiana*) on 4 dpi (Figure 2). This expression level is comparable to the highest expression level of mAbs reported by geminiviral vectors in both lettuce and tobacco [24] and by MagnICON vectors in tobacco [26]. Overall, these results demonstrate that fully assembled E16 mAb can be produced rapidly at high levels in both lettuce and *N. benthamiana* plant hosts.

3.3. Purification of E16 mAbs from Plant Tissue. To examine whether plant-derived E16 mAbs can be recovered from plant tissue effectively, we extracted and purified the mAbs from infiltrated lettuce and *N. benthamiana* leaves using an affinity chromatography method based on Protein A resin. Coomassie blue staining analysis of SDS-PAGE showed that E16 mAbs produced in lettuce and *N. benthamiana* can be purified to >95% purity with intact HC and LC using this single-affinity chromatography step (Figure 3). A similar analysis under nonreducing conditions confirmed that mAbs

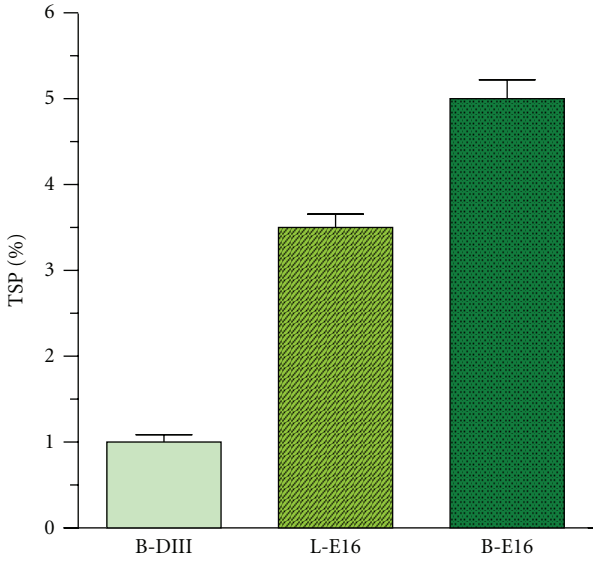

Figure 2: Accumulation of WNV DIII antigen and E16 mAbs in *N. benthamiana* and lettuce. Total proteins from plant leaves infiltrated with DIII or E16 expression vectors were extracted on 4 dpi and analyzed by ELISA with *E. coli*-produced WNV DIII or mammalian cell-produced E16 as the reference standard. For DIII ELISA, a monoclonal mAb against WNV DIII [13] was the capture antibody, and a rabbit anti-WNV DIII antibody was the detection antibody. For E16, goat anti-human gamma- and kappa-chain antibodies were used as capture and detection reagents, respectively, to detect the assembled form of E16 mAb. Mean ± SD of samples from three independent infiltrations are presented. B-DIII: WNV DIII produced in *N. benthamiana;* L-E16: lettuce-produced E16; B-E16: *N. benthamiana*-derived E16.

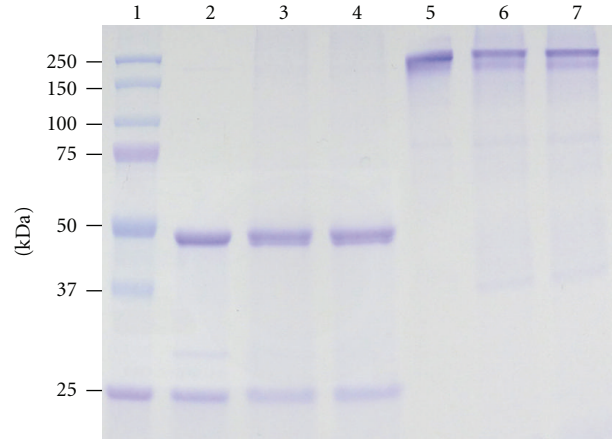

Figure 3: Purification of E16 mAbs produced in lettuce or *N. benthamiana*. Purified E16 mAbs were analyzed on a 12% SDS-PAGE gel using either reducing (Lanes 2–4) or nonreducing (Lanes 5–7) sample buffer. Lane 1: Molecular weight marker; lanes 2 and 5: mammalian cell-derived E16 as reference standard; lanes 3 and 6: E16 purified from lettuce leaves; lanes 4 and 7: purified E16 from *N. benthamiana.* One representative of several independent experiments is shown.

produced in both plant hosts remained fully assembled after purification (Figure 3 lanes 5–7). These results demonstrated that E16 mAb not only can be rapidly produced at high levels in two host plant species but also can be isolated and purified to high purity using a facile purification method.

3.4. Plant-Derived DIII WNV Antigen and mAbs Are Functional in Detection and Diagnosis of WNV Infection. Functional characterizations were performed to determine if DIII antigen and E16 mAbs produced in plants retained their biological activities and can be used in detection and diagnosis assays for WNV infection. As shown in Figures 1(a) and 2, plant-derived DIII antigen retains the ability to bind antibodies that specifically recognize DIII of WNV E protein. This binding specificity was confirmed by the lack of binding of plant-derived DIII to other non-DIII-specific mAbs (data not shown).

One of the issues facing the current immunobased WNV diagnosis is the cross-reactivity of antibodies against WNV with other flaviviruses. The E16 mAb produced from mammalian cells binds to a specific region of WNV DIII and does not cross-react with DIII of other flaviviruses [13]. To examine the binding specificity of plant-derived E16, various concentrations of this mAb were incubated with either DIII of WNV or DIII of DENV-2 that was immobilized on an ELISA plate. As shown in Figure 4, the binding to WNV DIII increased with the concentration of lettuce or *N. benthamiana*-derived E16 in the reaction in a similar manner as the mammalian cell-derived E16 positive control. In contrast, none of the E16s showed specific binding to DIII of DENV-2 (Figure 4). The negative control IgG (a generic human IgG) showed no specific binding to either WNV or DENV-2 DIII (Figure 4). These results indicate that the specific avidity for WNV DIII is retained by the plant-derived E16s. This high specificity makes it a valuable reagent in obtaining unambiguous diagnostic results for detecting WNV and WNV infection.

We first examined the application of plant-derived E16 mAbs in detecting the presence of WNV antigens by using a flow cytometry assay. In this assay, WNV E protein is displayed on the surface of yeast, and the ability of mAbs in detecting WMV E protein is measured by flow cytometry [13]. Our analysis showed that the percentage of yeast cells expressing WNV E protein and the mean fluorescence intensity of binding by lettuce or *N. benthamiana*-derived E16 were significantly higher compared to the negative control antibodies (Figure 5). This result indicates that plant-derived E16s are effective in specifically detecting WNV proteins. We further demonstrated the utility of plant-derived WNV DIII antigen and E16 mAb in a WNV IgM capture ELISA as another example of their potential application as diagnostic reagents. In the original commercial kit for diagnosis of WNV infection, controls and serum samples are incubated in microtiter wells which have been coated with anti-human IgM antibodies. The microtiter wells are then incubated with WNV recombinant antigen (WNRA) or a negative control antigen (NCA) and followed by incubation with WNRA-specific antibody labeled with HRP. The presence of IgMs against WNV is determined by whether the ratio of

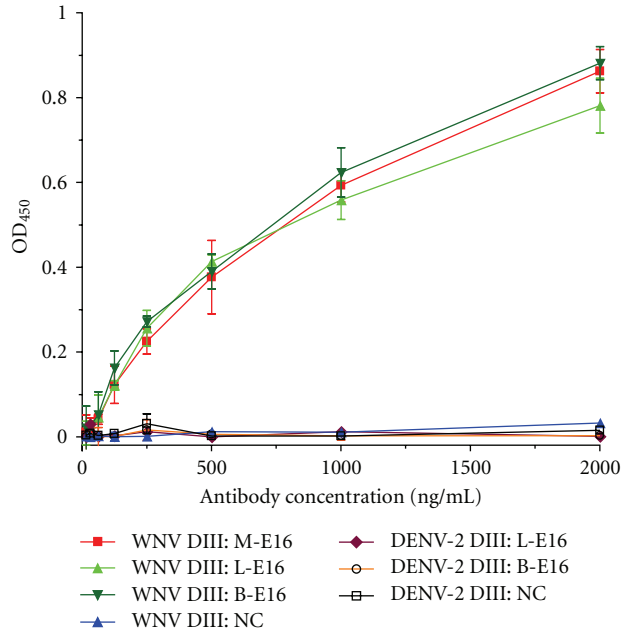

Figure 4: Highly specific binding of plant-derived E16 to WNV DIII antigen. WNV DIII or DENV-2 DIII antigen produced in *E. coli* was immobilized on an ELISA plate and incubated with increasing concentrations of lettuce or *N. benthamiana*-derived E16 mAb (L-E16 or B-E16), mammalian cell-derived E16 (M-E16, positive control), or a negative control generic human IgG (NC). A HRP-conjugated anti-human IgG was used to detect mAbs bound to DIII antigens. Mean ± SD of OD_{450} from three independent experiments are presented.

the absorbance of the WNRA and the NCA is above a preset threshold. In our modified ELISA, plant-derived E16 was conjugated to HRP first and then used to replace the WNRA-specific antibody in the original kit. Accordingly, WNRA was replaced by our plant-derived DIII antigen in the modified assay. Our results showed that the plant-derived DIII antigen and E16 mAb effectively differentiated the WNV human IgM-positive serum from the negative control serum in a similar manner as the reagents in the commercial kit (Figure 6). The ISRs of our modified assay for the negative control serum are 1.09 (lettuce-derived E16) and 1.24 (*N. benthamiana*-E16), which are similar to that of the original assay (1.61) and well within the required ISR range (<4.47). Similarly, ISR for the WNV IgM-positive serum is comparable between the modified and original assay (8.68 and 8.98 versus 13.48), which are also within the required specification range (>5.66). The positive IgM control serum showed a lower absorbance in the plant-derived DIII/E16 samples than that of WNRA/anti-WNRA (Figure 6). Since both WNRA and plant-produced DIII were added to the reaction in concentrations high enough to saturate all binding sites of WNV-specific IgMs that were captured on the plate by anti-IgM antibodies, it is unlikely that this is caused by the concentration difference of the two antigens. Instead, the potential difference in antigen-binding site and/or valency between the reference standard mAb and plant-derived E16 mAb is a more likely factor in causing the observed difference. It also remains possible that this result corroborates the previous observation that WMV DIII-specific IgM titers in the sera of infected mammals are generally lower than those of WNV E protein [29]. We are currently expressing a full-length WNV E protein in plants to address this issue. Nonetheless, this example clearly demonstrated the effectiveness of plant-derived antigen and mAb as reagents for detection and diagnosis of WNV infection and suggested their potential applications in other diagnostic assays.

4. Discussion

The rapidity of the spread of WNV and the potential threat to bird, mammal, and human populations demand the development of a technology platform that can rapidly produce a variety of protein reagents, flexibly and at low cost, to support the need of detection and diagnosis sufficient to address the potential public health crisis. Here, we investigated the feasibility of using plants to produce a WNV antigen and a mAb against WNV to address the cost and scalability issues associated with mouse hybridoma mammalian, insect, or bacterial cell cultures. Our results demonstrated that WNV DIII antigen and E16 mAb can be rapidly produced at high levels in two host plant species. Furthermore, these proteins can be easily isolated to high purity with simple extraction and purification methods and they retain the functionality and specificity of their original counterparts.

Plants are suitable for antigen and mAb production because they can produce large volumes of protein at low cost and be rapidly scaled up for commercial production without the high-capital investment associated with bioreactor facilities for mammalian, insect, or bacterial cell cultures [14, 15, 31, 32]. The WNV E glycoprotein is essential for membrane fusion and mediates binding to cellular receptors, and is a major target of host antibody responses [33]. DIII of WNV E protein contains the cellular receptor-binding motifs and the majority of the neutralizing epitopes that induce strong host antibody responses and/or protective immunity [13]. As a result, E protein and DIII have been extensively explored as WNV vaccine candidates and as detection and diagnostic reagents [7]. The WNV DIII protein has been produced in insect cell and bacterial cultures [11, 12]. These culture systems are difficult to scale up for large-scale protein production. In addition, production of recombinant DIII in bacterial cultures requires a cumbersome solubilization and refolding process due to the formation of inclusion bodies [11]. In contrast, DIII antigen was expressed rapidly in *N. benthamiana* plant as a soluble protein and accumulated to 1% of TSP or 0.1 mg/g FLW, the highest expression level of any flavivirus antigen ever reported in plants. In addition, plant-derived DIII can be easily purified to >95% purity without the needs of solubilization and refolding.

We previously investigated the possibility of producing E16 as a post-exposure therapeutic mAb in *N. benthamiana* with the TMV and PVX-based MagnICON system. In that investigation, the "KDEL" tetrapeptide ER-retention signal was added to the C-terminus of the HC [23], and/or E16 was expressed in "humanized" plant lines that can add

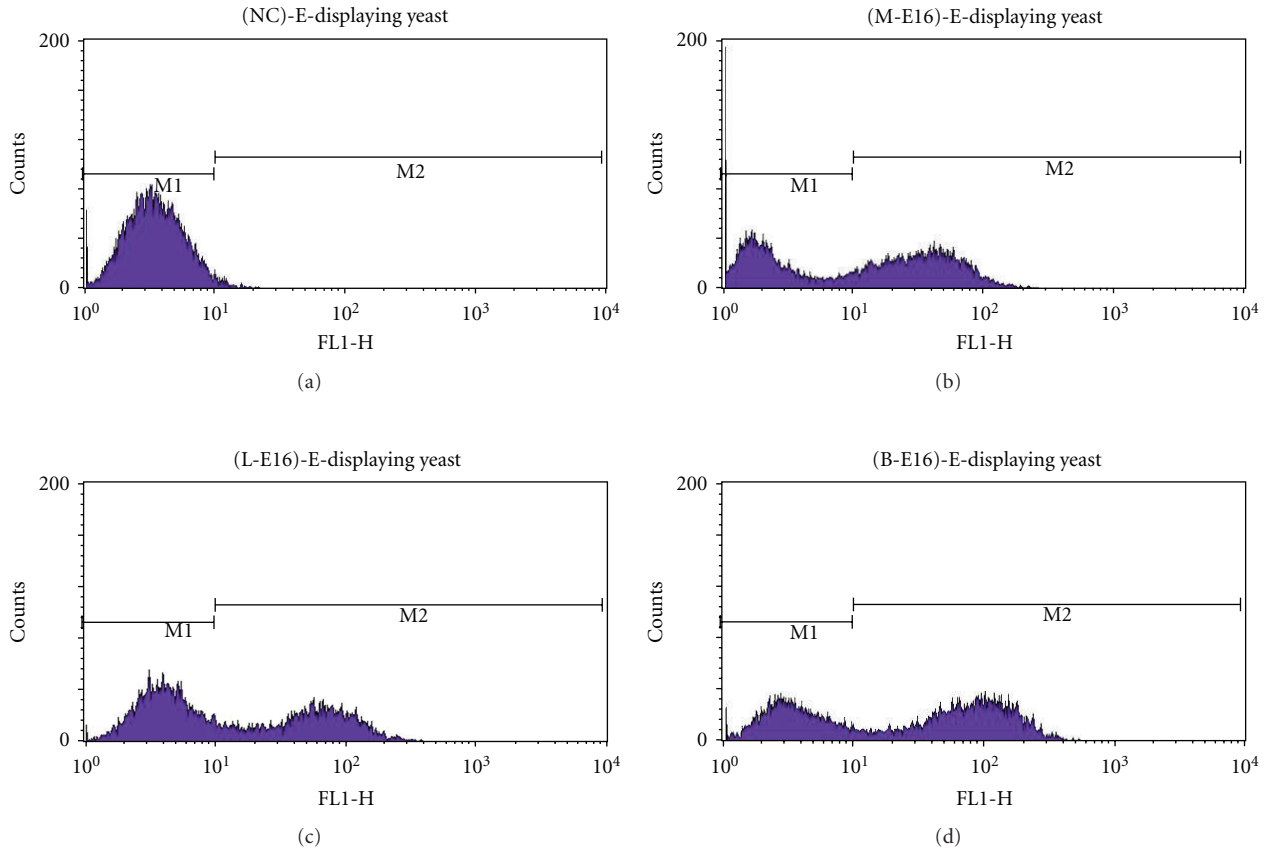

Figure 5: Identification of WNV E protein displayed on the cell surface of yeast by plant-derived E16 mAbs. Lettuce (L-E16) or *N. benthamiana*-produced E16 mAb (B-E16), mammalian cell-derived E16 (M-E16, positive control), or a generic human IgG (NC, negative control) was used to stain yeast cells displaying WNV E protein, which were then processed by flow cytometry. Representative data from three independent experiments are shown.

mammalian glycoforms to mAbs (Q. Chen, unpublished results). These extra procedures were performed to avoid the production of plant-specific glycans, which may trigger a potentially harmful immune response in human patients. Since diagnostic reagents are used *in vitro*, the concern for the potential adverse host immune response becomes irrelevant. Thus, in this study we examined the feasibility of using wild-type plants and HC without the extra-KDEL ER-retention peptide to produce E16. Furthermore, instead of using the MagnICON system of the previous study, we tested the ability of geminiviral replicon vectors for E16 production because it can direct recombinant protein expression in both tobacco and lettuce [24]. Our results showed that the KDEL-less E16 can be produced in wild-type plants with geminiviral vectors and accumulated to a level comparable to the highest expression level for mAbs in plants ever reported. The ease of using wild-type plants to produce E16 not only simplifies the experimental procedure, but also minimizes biosafety and regulatory concerns and costs associated with transgenic crops and genetically modified plants [15]. Furthermore, E16 mAbs produced from both wild-type *N. benthamiana* and lettuce can be easily purified to >95% purity and retain their highly-specific antigen binding avidity.

In addition to wild-type *N. benthamiana*, we also explored the possibility of using laboratory-grown lettuce to produce E16. Similar to *N. benthamiana*, lettuce is a robust-growing plant that produces large quantities of biomass rapidly. In contrast to tobacco, it produces negligible quantities of phenolics and alkaloids and, thus, can potentially simplify the protein purification process and reduce the overall cost of goods in commercial production. Our results demonstrated that lettuce-produced E16 mAb has equivalent structure and functionality as *N. benthamiana*-derived E16. Previously we showed that mAbs can be expressed in grocery store-bought lettuce [24]. This potentially would allow us to have access to unlimited quantities of inexpensive plant material for the large-scale commercial production of E16 as a detection and diagnostic reagent. Overall, the capability of performing posttranslational modifications by plant cells and the availability of the glycoengineered plant lines to produce recombinant proteins with mammalian glycoforms provide our plant system the advantage over bacterial cultures in producing mAbs and antigens that require posttranslational modification. Moreover, the rapid high-level production and assembly of E16 and a simple current good manufacture-practice- (cGMP-) compliant

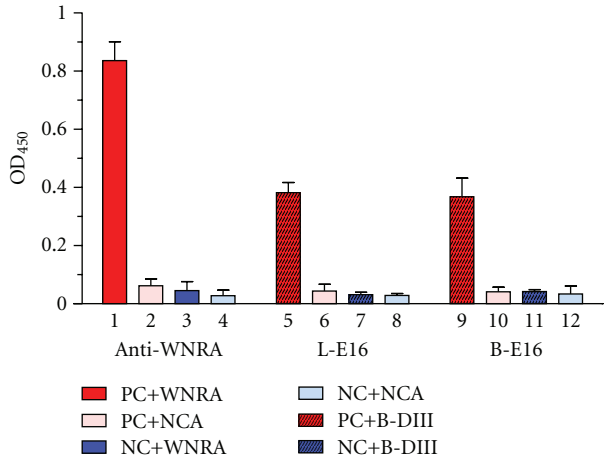

Figure 6: WNV IgM capture ELISA with plant-derived DIII antigen and E16 mAbs. Columns 1 and 2 (positive controls): WNV IgM-positive serum (PC) detected by kit-supplied positive control antigen WNRA (1) or negative control antigen NCA (2) with the kit-supplied WNRA-specific antibody (Anti-WNRA); Columns 3 and 4 (negative controls): WNV IgM negative serum (NC) detected by WNRA (3) or NCA (4) with Anti-WNRA; Columns 5 and 6: PC detected by *N. benthamiana*-derived DIII antigen (B-DIII) (5) or NCA (6) with lettuce-derived E16 (L-E16); Columns 7 and 8: NC detected by B-DIII (7) or NCA (8) with L-E16; Columns 9 and 10: PC detected by B-DIII (9) or NCA (10) with *N. benthamiana*-derived E16 (B-E16); Columns 11 and 12: NC detected by B-DIII (11) or NCA (12) with B-E16.

purification scheme—convincingly demonstrate the viability of this system for large-scale cost-effective production of mAbs compared to hybridoma and mammalian cell cultures.

We further demonstrated the utility of plant-derived antigen and mAbs in detection of WNV or host immune responses to WNV infection in a flow-cytometry-based and a WNV IgM capture ELISA assay. The results of these examples clearly indicated their value in the detection of WNV and diagnosis of WNV infection and suggest their potential application in other protein-based assays. One critical issue of immunobased WNV diagnosis is the cross-reactivity of antibodies among flaviviruses. The high specificity of plant-derived E16s may provide us a valuable tool in addressing such problems. For example, plant E16 can be used in a VecTest-like WNV antigen assay to rapidly detect WNV infection in wild bird and mosquito populations [34]. Since plant E16 does not recognize DIII protein from other flaviviruses, this high specificity will improve the accuracy of the current assay and reduce ambiguity. Sotelo and colleagues have recently developed a new epitope-blocking ELISA that has demonstrated utility in detecting WNV infection in a wide range of hosts, including humans, birds, and other mammals [35]. This ELISA utilizes a mouse hybridoma cell-derived neutralizing mAb that binds WNV E protein and requires very small volumes of sera, making it feasible to directly test small-size birds and mammals without harming their health. As a result, this new method promises to be helpful for both disease diagnosis and surveillance. Since plant-derived E16 is a neutralizing mAb that binds to WNV E protein, we speculate that its high specificity to WNV DIII will enhance the specificity of this assay. In addition, using plant-derived E16 for this assay will address the issue of production scalability of mouse hybridoma cells and greatly reduce the cost of mAb production.

The research of our laboratory and others has demonstrated the ability of plants in producing a variety of recombinant proteins at low cost [15, 31, 32, 36, 37]. This study extends the utility of plant expression system to the production of mAbs and antigens as detection and diagnostic reagents for arboviruses. One of the concerns of plant biotechnology has been whether the product yield can be high enough to allow large-scale production and advance the technology beyond the proof-of-principle stage. While this was a valid issue for traditional plant expression systems, the high-level product yield of new plant expression systems have suggested that such concern is no longer necessary. These new expression systems such as the MagnICON and geminiviral expression systems used in this study have allowed high and consistent level of target protein accumulation in plants sufficient for commercial-scale manufacturing [14, 15, 21]. For example, several of pharmaceutical proteins produced by these plant expression systems with similar product yield as WNV DIII and E16 mAb have not only been produced on a large scale under the United States Food and Drug Administration's (FDA) cGMP regulation but also have been tested in late-stage human clinical trials [14, 15, 38]. The high-level product yield of DIII antigen and E16 mAb and their simple cGMP-compliant purification schemes, demonstrate the viability of our plant production system for their large-scale cost-effective production and suggest a promising future beyond the proof-of-principle stage.

In addition to promoting high product yield, one of the other advantages of plant-transient expression systems is the rapid speed of target protein production [17]. With both MagnICON and geminiviral expression systems, our antigen and mAbs were produced within a week of vector infiltration. These rapid and high protein accumulation levels provide the flexibility and versatility of the plant production system that are superior over other production systems in accommodating the demands of new reagents by the fast-evolving diagnostic assays. For example, if a novel assay has been approved for viral detection and diagnosis, the plant-transient expression system can be rapidly adjusted to produce the new reagents within one to two months. This flexibility and versatility also give the plant production system an advantage in producing a virtually unlimited number of protein detection and diagnostic reagents for not only WNV but for many other arboviruses, such as dengue virus, Japanese encephalitis virus, St. Louis encephalitis virus, and yellow fever virus.

5. Conclusions

This research demonstrated the effective production of functional protein reagents in plants for the detection of WNV and diagnosis of WNV infection. The robustness, cost effectiveness, scalability, and flexibility of the plant

system will make it an attractive platform for the production of detection and diagnostic reagents for a broad range of arboviruses.

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Research Article

Plasma Cell Cerebrospinal Fluid Pleocytosis Does Not Predict West Nile Virus Infection

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Purpose. Diagnosis of WNV (WNV) relies upon serologic testing which may take several days after the onset of clinical symptoms to turn positive. Anecdotal reports suggest the presence of plasma cells or plasmacytoid lymphocytes in the cerebrospinal fluid (CSF) may be an early indicator of WNV infection. *Methods*. The CSFs of 89 patients (12 with WNV, 12 with other viral illness {OVI}, and 65 with nonviral illness{NVI}) were compared for the presence of either plasma cells or plasmacytoid lymphocytes. *Results*. Plasma cells were rarely seen in any of the patients. Plasmacytoid lymphocytes were more commonly seen in WNV (58%) and OVI (50%) than NVI (11%). The differences were significant for WNV versus NVI, but not WNV versus OVI ($P < 0.001$ and $P = 0.58$, resp.). *Conclusions*. A CSF pleocytosis with plasma cells or plasmacytoid lymphocytes was neither sensitive nor specific for the diagnosis of WNV infection.

1. Introduction

West Nile virus, a mosquito-borne flavivirus, first appeared in North America in the New York City metropolitan area in 1999. Since that time, the ArboNET surveillance system has documented the virus' dramatic spread across the United States with 30,662 cases of WNV disease reported from 47 states through 2010 [1, 2]. Over 11 000 cases were classified as West Nile neuroinvasive disease (WNND), and over 1000 of these cases died with their infection. WNV has now become the leading cause of arboviral encephalitis in the United States with likely ongoing seasonal epidemic transmission [2].

Symptoms of WNV infection are nonspecific, and definitive diagnosis rests on finding WNV RNA in CSF or blood, or more commonly, serologic evidence of recent infection as evidenced by the presence of IgM-specific antibodies to WNV antigens in CSF or blood. However, this test may not turn positive until 8–21 days after the first appearance of symptoms [3]. Finding earlier indicators for WNV would facilitate making a more rapid diagnosis. In the setting of meningitis or encephalitis, previous anecdotal reports have suggested the presence of plasma cells or plasmacytoid lymphocytes in CSF may be an early indicator of WNV infection [4–7]. Plasma cells originate in the bone marrow as B cells and after antigenic stimulation undergo differentiation in the lymph node from lymphocyte to plasmacytoid lymphocyte to the plasma cell capable of producing large amounts of antibodies. WNV is known to induce a brisk antibody response that is largely responsible for clearing of the virus [8, 9]. It is not clear that the cells previously observed in the aforementioned studies are truly plasma cells. In one study of transplant patients with WNV infection, five patients had cytology performed on the CSF that showed atypical lymphocytes and plasma cells. However, flow cytometry was performed on 3 of these patients which showed the cells were predominantly mature T cells with only negligible B cells present [7].

Table 1: CSF cell count findings in WNV-infected patients versus patients with other viral illness (OVI) or non-viral illness (NVI).

Variable	Display meaning	WNV infection	Other viral illness	Non-viral illness	*P* values for tests		
					Overall	WNV versus OVI	WNV versus NVI
Total	*N*	$n = 12$	$n = 12$	$n = 65$	—	—	—
Gender	M	50.0	50.0	50.8	0.98	—	—
Age	Mean (SD)	51.2 (17.5)	37.1 (16.1)	35.5 (27.1)	0.13	—	—
RBC	Mean (SD)	42.3 (53.3)	642.3 (2095.3)	28858.4 (179338.1)	0.74	0.99	0.55
WBC	Mean (SD)	236.7 (265.1)	325.0 (446.0)	174.9 (561.6)	0.64	0.68	0.71
% Lymphocytes	Mean (SD)	41.6 (27.1)	53.9 (28.9)	58.2 (30.7)	0.22	0.32	0.08
% Monocytes	Mean (SD)	12.0 (5.8)	25.8 (20.1)	18.8 (19.3)	0.19	0.07	0.24
% Neutrophils	Mean (SD)	44.4 (28.9)	18. 7(29.8)	23.1 (30.8)	0.07	0.04	0.03
% PC lymphocytes	Mean (SD)	1.8 (2.1)	1.1 (1.4)	0.1 (0.4)	<.0001	0.06	<.0001
% Plasma Cells	Mean (SD)	0.2 (0.6)	0.3 (0.7)	0.1 (0.4)	0.30	0.38	0.69
PC lymphocytes present	*N* (%)	7/12 (58.3)	6/12 (50.0)	7/65 (10.8)	<.0001	0.58	<.0001
Plasma Cells present	*N* (%)	1/12 (8.3)	3/12 (25.0)	5/65 (7.7)	0.18	0.17	0.94

We sought to determine the utility of the anecdotal finding of plasma cell CSF pleocytosis by calculating sensitivity and specificity among a larger group of patients undergoing lumbar puncture for a variety of indications.

2. Subjects and Methods

From June 2007 to August 2008, slides submitted to our laboratory for CSF cell counts were saved for future investigation. Patients were excluded if they were under the age of 1 month or had a known immunosuppressive condition. Eighty-nine slides had enough cells present to be able to do a 100-cell differential count. Subject's records were reviewed for the final diagnosis pertaining to the lumbar puncture, CSF white blood cell (WBC) count, red blood cell (RBC) count, WBC differential, and WNV test results (if done). Slides were then reviewed with a 100-cell differential count specifically looking for the presence of plasma cells or plasmacytoid lymphocytes based on standard morphologic criteria [10]. Only one person reviewed all the slides and was blinded to the patient's diagnosis.

Patients with WNV were compared for the presences of plasma cells or plasmacytoid lymphocytes in the CSF to patients with other viral illness and those without any presumed CNS infection. Differences between groups were tested using generalized linear methods (for binomial variables) or ANOVA methods (for continuous variables), with contrasts used to test differences between groups within the overall three-group difference. Differences were also examined using nonparametric methods, with equivalent results. Tests were performed using SAS V. 9.2 software.

3. Results

Twelve patients tested positive for WNV by IgM capture ELISA. Five of the WNV patients had meningoencephalitis, 7 had just meningitis. Twelve other patients were thought to have a viral illness other than WNV. Eleven of these patients had negative testing for the presence of WNV-specific IgM antibodies in serum, and 6 had specific other viral infections diagnosed (3 with enterovirus, 2 with herpes simplex type II, and one with Epstein-Barr virus). Details of the CSF findings for WNV patients versus patients with other viral illnesses versus patients with no presumed infection are summarized in Table 1. WNV patients were older and more likely to have neutrophils in their CSF. When compared to patients without presumed infection, they were more likely to have plasmacytoid lymphocytes in their CSF. However, the sensitivity of this finding was relatively low (58%), and the overall percentage of plasmacytoid lymphocytes was relatively low (mean of 1.8%). However, when patients with WNV were compared only to patients with other viral infection, finding plasmacytoid lymphocytes was not specific for WNV. Six out of twelve patients with other viral syndromes had plasmacytoid lymphocytes present, suggesting a specificity of only 50%. The presence of plasma cells was rare in WNV patients (only 1/12) and was actually more common in the other viral illnesses (3/12). Combining the presence of plasma cells and plasmacytoid lymphocytes did not enhance sensitivity or specificity. If we compared the WNV encephalitis patients with the WNV meningitis patients, plasmacytoid lymphocytes were seen rarely in the encephalitis cases (1/5) whereas they were frequently seen in the meningitis cases (6/7).

4. Discussion

Our study did not confirm earlier anecdotal findings that plasma cells or plasmacytoid lymphocytes may be an early marker for WNV infection. The overall sensitivity of this finding was only 58%. The sensitivity increased to 86% if one excluded the WNV encephalitis cases and only considered the WNV meningitis cases. However, this may be a spurious finding due to the low numbers of subjects in either group.

All of the encephalitis cases also had meningitis with nearly equivalent numbers of total WBCs in the CSF, so a distinction between these clinical entities which lie on a spectrum of illness would seem unlikely. Most importantly, this finding was seen in several of our patients with other forms of viral meningitis, thus suggesting a significant lack of specificity.

It is likely that the presence of plasma cells or plasmacytoid lymphocytes is simply a nonspecific marker of central nervous system infection. Of note is that, in our prior reported case series of 4 patients, all had underlying conditions causing variable degrees of immunosuppression: myelodysplasia, myeloproliferative disorder, Graves' disease, and renal/pancreas transplantation. This suggests that immunosuppression may have played a role in the development of the plasma cell pleocytosis.

It should be noted that plasma cells and plasmacytoid lymphocytes have been noted in the CSF of several other infections including HIV [11], tuberculous meningitis [12], neuroborreliosis [13], neurocysticercosis [14], secondary syphilis [15], herpes zoster meningoencephalitis [16], and African sleeping sickness [17]. Furthermore, the morphologic characterization of what constitutes a plasma cell or plasmacytoid lymphocyte is somewhat subjective and, therefore, technician dependent. One might expect further diminishing of specificity in real-life conditions where different laboratory technicians would be interpreting these cell differentials variably.

5. Conclusions

The finding of plasma cells or plasmacytoid lymphocytes in the CSF was neither sensitive nor specific for the diagnosis of West Nile virus infection. Other modalities or improvements in current laboratory testing will need to be sought if we hope to improve on the timeliness of diagnosing acute WNV infection.

Conflict of Interests

There is no conflict of interests to report among any of the authors, and all authors had full access to the data and participated in the preparation and review of the paper.

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