EXPERT REVIEWS

Current trends in West Nile virus vaccine development

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West Nile virus (WNV) is a mosquito-borne flavivirus that has become endemic in the United States. From 1999–2012, there have been 37088 reported cases of WNV and 1549 deaths, resulting in a 4.2% case-fatality rate. Despite development of effective WNV vaccines for horses, there is no vaccine to prevent human WNV infection. Several vaccines have been tested in preclinical studies and to date there have been eight clinical trials, with promising results in terms of safety and induction of antiviral immunity. Although mass vaccination is unlikely to be cost effective, implementation of a targeted vaccine program may be feasible if a safe and effective vaccine can be brought to market. Further evaluation of new and advanced vaccine candidates is strongly encouraged.

KEYWORDS: antibody • epidemiology • flaviviruses • immunity • immunocompromised • neurotropic • vaccination • West Nile virus

West Nile virus (WNV) is a member of the genus *Flavivirus*, which includes arthropod-borne viruses belonging to the *Flaviviridae* family. Besides WNV, there are several clinically significant human pathogens within this group, including St Louis encephalitis virus, dengue virus (DENV), tick-borne encephalitis virus (TBEV), Japanese encephalitis virus (JEV), Murray Valley encephalitis virus and yellow fever virus (YFV) [1,2]. These single-stranded positive-sense RNA viruses have a relatively small genome of approximately 11 kb and form enveloped mature infectious particles that are approximately 50 nm in diameter. The genomic RNA of flaviviruses contains a single open reading frame, which is translated into a large polyprotein that is processed by both cellular and viral proteases into three structural proteins (C [capsid], prM [premembrane] and Env [envelope]) and seven non-structural proteins (NS1, NS2a, NS2b, NS3, NS4a, NS4b and NS5).

Following its initial discovery in Uganda in 1937, WNV was generally considered a minor public health threat, though sporadic outbreaks were occasionally noted [3]. During the 1990s, more severe outbreaks with increased neuroinvasive disease were seen in North African and Southern European countries [3]. Neuroinvasive disease has also been the hallmark of WNV in the Western hemisphere, starting with a cluster of viral encephalitis cases in New York City in 1999 [2,4]. Since that time, the number of WNV cases has grown rapidly throughout the USA (FIGURE 1). Although there was hope that WNV would eventually decrease in incidence, epidemiological data suggest that WNV has become endemic throughout the continental USA, with periodic peaks and lulls in disease incidence. For example, after dropping to a postendemic low of 720 reported WNV cases and 32 deaths in 2009, WNV jumped to 5674 cases and a record 286 deaths in 2012.

While a large number of flaviviruses represent important human pathogens, they are also distinguished by a number of successful vaccines to control disease. Licensed human vaccines for flaviviruses include formaldehyde-inactivated vaccines against TBEV and JEV as well as a live, attenuated vaccine against YFV. Although cellular immunity plays an important role in clearing primary WNV infection [5–9], memory $CD8^+$ T cells are dispensable if high levels of antiviral antibody are present [7] and vaccine-induced memory T cells may not play a substantial role in controlling flavivirus infection in humans [10,11]. Moreover, a number of studies using passive immunization have shown that transfer of neutralizing antibodies to naïve animals is sufficient for protection against lethal WNV infection [12–16]. Accordingly, neutralizing antibody titers are generally correlated with protection against disease for licensed flavivirus

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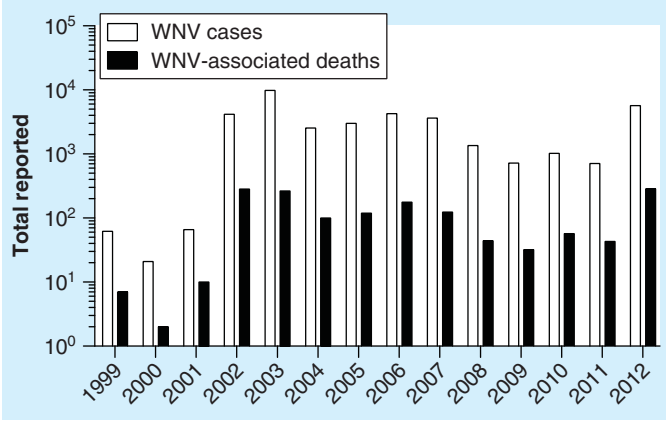

Figure 1. Sustained prevalence of West Nile virus in the USA. The annual number of reported WNV cases and associated deaths from 1999 to 2012 are shown. The highest number of deaths was reported in 2012, with 286 fatalities.
WNV: West Nile virus.
Data taken from [34].

vaccines. For example, YFV vaccine recipients with a serum antibody log neutralizing index ≥0.7 are considered protected against clinical disease [17]. This was based on vaccination studies performed in rhesus macaques [18] and is an efficacy benchmark that has continued to be used in YFV clinical trials [19,20]. Similarly, the protective threshold for the JEV vaccine is correlated to neutralizing antibody titers, with a serum plaque reduction neutralization ($PRNT_{50}$) ≥1:10 considered protective by vaccine manufacturers [21] as well as by a WHO recommendation panel [22]. While the TBEV vaccine does not have a specific, established level for achieving protective immunity, neutralizing antibody titers are still considered the key to vaccine efficacy [23]. Based on the track record with related flaviviruses, such as JEV, TBEV and YFV, a WNV vaccine should be feasible, and a large number of WNV vaccines are in various stages of development. Although several veterinary vaccines against WNV have been licensed for use in horses, a human vaccine is still not available. In this review, we will discuss the range of WNV vaccines that have been developed and tested at both the preclinical and clinical level. We will also review current challenges to vaccine licensure, including technical limitations in late-stage efficacy trials and concerns regarding cost–effectiveness, and propose alternate approaches toward development of a safe and effective WNV vaccine.

WNV epidemiology & surveillance

WNV is considered the most geographically widespread arbovirus in the world, reaching into every continent except Antarctica [3]. The virus was first identified in Africa in 1937 from a patient experiencing fever, which later resolved without incident [24]. In the decades following this initial identification, sporadic rural outbreaks linked to WNV were recorded throughout the world, though reports of severe neurological disease were limited [25]. Starting in the 1990s, more frequent and severe outbreaks were seen in countries bordering the Mediterranean Sea, with further movement of the virus north and west into countries such as Romania, Russia and Israel [3]. During this same time frame, WNV reached the shores of North America, starting with a cluster of viral encephalitis cases in Queens, New York in 1999 [2,4,26]. Sequencing of WNV RNA indicated close homology with an Israeli isolate of WNV [4], and the current theory holds that WNV may have been introduced from Israel, with subsequent transmission to mosquito populations and spread into the local ecosystem [27]. Following this initial introduction, WNV spread across the continent, reaching the West Coast of the USA by 2003. The rapid spread of WNV in North America has been linked to the flyways of migratory fowl, with the virus able to take advantage of yearly migration routes for rapid transit and spread into local bird populations [26]. Other aspects attributed to the spread of WNV include a wide range of vertebrate hosts and mosquito species that can carry the virus [26], though the transmission cycle between *Culex* spp. of mosquitoes and various bird species is considered the primary enzootic maintenance pattern. Perhaps the most important aspect underlying increased virulence and spread are the genetic differences between strains of WNV. Presently, WNV is categorized into as many as seven lineages depending on the classification scheme, though most isolates fall into lineage 1 or lineage 2 categories [28]. Lineage 1 strains (such as WNV-NY99) are considered emerging diseases, generally associated with increased virulence in humans [28]. By comparison, lineage 2 strains of WNV (including the founding Uganda 1937 strain) are usually less severe, though recent outbreaks in Europe with pathogenic lineage 2 strains may modify this position [29]. This type of divergence within lineages is not unprecedented with even the lineage 1 strain of WNV split into multiple clades (1a–1c), representing a broad spectrum of pathogenicity [30]. For example, while WNV–Kunjin (an Australian clade 1b) shows approximately 98% amino acid identity to WNV-NY99 (clade 1a) [5], WNV–Kunjin is highly attenuated in humans, typically resulting in either mild or clinically asymptomatic infections [31]. Importantly, vaccination with several licensed veterinary vaccines based on lineage 1 strains (WNV-NY99) has shown good cross-protection against pathogenic lineage 2 strains in both mouse [32] and horse [33] challenge models. This indicates that human vaccine candidates based on lineage 1 strains may likewise protect against a broad range of circulating WNV lineages, though this will be an important ongoing consideration during further clinical development.

Since its introduction in North America in 1999, surveillance shows that WNV has now become endemic throughout the continent. In the USA, 37,088 cases have been reported from 1999 to 2012 (Figure 1) with 16,196 classified as neuroinvasive disease [34]. During this time frame, there were 1549 deaths associated with WNV, yielding a case fatality rate of 4.2%. The impact of WNV has also extended north into Canada, with a total of 5094 cases and 71 deaths (equaling an estimated 1.4% case fatality rate) reported from 2002 to 2012 [35]. After a peak in reported cases and deaths in the USA

from 2002 to 2003, WNV activity generally waned from 2006 to 2011, reaching a low of 720 cases and 32 deaths in 2009 (Figure 1). However, 2012 saw an explosion in WNV incidence, with 5674 reported cases and 286 deaths, the most WNV-associated fatalities on record in the USA (Figure 1). A similar spike in reported cases was also seen in Canada [35], suggesting that North America will continue to have WNV outbreaks into the foreseeable future. A rise in WNV incidence has also been observed recently in Southern Europe, with Greece experiencing a significant outbreak in 2010 [29]. Following this outbreak, the EU along with other groups, has begun efforts to improve surveillance across affected European countries and neighboring regions [36]. Surveillance from 2010 to 2012 demonstrated a total of 2414 WNV cases with 127 associated deaths (Table 1) for a case fatality rate of 5.3%, similar to the rate observed in the USA. While Figure 1 shows the number of reported cases and deaths in the USA, the overall disease burden is likely much higher. For instance, North Dakota reported approximately 1300 cases of WNV from 1999 to 2008 [37], but seroprevalence studies indicated that more than 40,000 were infected in the same time frame [38], suggesting that at least 30 undiagnosed cases of WNV occur for every case reported. This is not unique to North Dakota; recent evidence suggests that across the USA, there have been nearly 3 million WNV infections resulting in an estimated 780,000 illnesses [39]. Based on these estimates, WNV outbreaks and disease incidence are far greater than previously realized, and this could have profound consequences on the economic impact of WNV disease and cost–effectiveness calculations for a WNV vaccine [40], which were made prior to publication of this study in 2013 [39].

WNV disease

Most cases of WNV infection are clinically inapparent but approximately 25% will present as West Nile fever [41], and 1 in 150 to 1 in 250 will develop more severe West Nile neurotropic disease (WNND) [39,42]. West Nile fever symptoms include fever of >38°C, general fatigue, headache, muscle pain, malaise, and in some cases, gastrointestinal symptoms and rash [43,44]. In some patients, symptoms may last more than a month after disease onset [44,45]. WNND manifests as encephalitis, meningitis or flaccid poliomyelitis-like paralysis that may result in respiratory failure [46–52]. Although the overall case fatality rate is 4.2%, there is a 9.6% case fatality rate among patients with WNND [53]. Unfortunately, neuroinvasive disease is likely to be under-reported since only 40% of meningitis or encephalitis patients are tested for WNV, even during well-publicized WNV outbreaks [54]. WNND is not only accompanied by a high rate of acute mortality [55] but survivors also often experience long-term neurological dysfunction [51] with many requiring assistance with daily activities after hospital discharge [55,56]. Following recovery from WNV encephalitis, up to 77% of patients continue to have neurological complications including impaired gait, muscle weakness, hearing loss and tremors lasting ≥3 years after infection [57]. Moreover, WNV survivors demonstrated a 2.5- to threefold higher age- and sex-adjusted mortality rate within the first 2 years following hospitalization compared with controls [58].

Although the main focus of WNV pathogenesis has been neurological complications, recent evidence suggests that chronic kidney disease (CKD) may be a previously underappreciated complication of WNV infection [59]. Persistent WNV infection has been described in several animal models [59], and WNV may be shed in the urine of infected Golden hamsters for up to 8 months [60,61]. A strain of WNV isolated from hamster urine at 274 days postinfection was found to have lost neurovirulence but induced persistent renal infection in mice [62]. In humans, WNV RNA has been detected in urine during the acute stages of infection [45,59,63], and one study found WNV RNA in urine from 25% (5/25) of patients between 1.6 to 6.7 years after infection [64]. However, another report was unable to identify WNV RNA among a cohort of 40 patients examined at >6 years postinfection [65]. Acute renal failure has been reported in WNV patients suffering from encephalitis [66,67] but CKD may be more common than previously thought; a recent study found that 40% of WNV patients had evidence of CKD within 4–9 years after infection, and the presence of detectable WNV RNA in the urine was associated with more severe renal disease [68]. Although not confirmatory, this is consistent with a study that found 21% of deceased WNV patients had documented renal failure listed as a cause or underlying condition at the time of death [69]. While more studies are needed, if CKD proves to be an important clinical outcome of human WNV infection, then this could greatly alter the economic impact of WNV infection and further emphasize the need for development of a safe and effective vaccine.

Vaccines in preclinical development

DNA-vectored vaccines

Following the initial outbreak of WNV in the USA, a large number of vaccine candidates have been developed (Table 2). These approaches can be divided into several broad categories including DNA-based vaccines, live chimeric/recombinant vaccine constructs, live attenuated virus and inactivated or subunit vaccines. DNA-vectored vaccines have offered the promise of rapid vaccine development through the power of modern genetic tools [70]. However, to date, no DNA vaccines have been licensed for use in humans. DNA vaccines expressing the prM and Env proteins from WNV [71–73] or the domain III (DIII) region of the Env protein [74] have been developed and tested in both mice and horses. Other approaches to DNA vaccines have included constructs encoding for single-round infectious particles [75,76] or a full-length cDNA copy of the attenuated Kunjin strain of WNV [77]. Candidates expressing the prM and Env proteins elicited PRNT titers against WNV-NY99 in both mice (range: 1:320–1:640) and horses (range: 1:40–1:320) after a single dose [71], and a vaccine based on this technology was developed into a licensed veterinary vaccine (though later discontinued by Pfizer) and eventually pursued in human clinical trials.

Table 1. Surveillance of West Nile virus in the Southern Europe and neighboring countries from 2010 to 2012.

	Country	National surveillance system	Reported cases	Deaths
Southern Europe	Greece	Permanent	523	62
	Romania	Seasonal	83	7
	Italy	Seasonal	45	8
	Bulgaria	Permanent (starting 2011)	2	0
	Spain	Permanent (specific areas)	2	0
	France	Permanent + seasonally enhanced	0	0
	Malta	Permanent (starting 2012)	0	0
The Balkans	Serbia	Seasonal (starting 2011)	71	9
	Albania	Permanent	50	0
	Republic of Macedonia	Permanent	10	0
	Croatia	Permanent	6	0
	Kosovo	Permanent	6	0
	Bosnia–Herzegovina	None	1	0
	Montenegro	Permanent (starting 2012)	1	0
	Cyprus	Permanent	0	0
	Slovenia	Permanent	0	0
North Africa and the Middle East	Israel	Permanent	255	10
	Tunisia	Permanent + seasonally enhanced	92	12
	Turkey	Permanent	65	13
	Palestine	Permanent + seasonally enhanced	3	0
	Algeria	None	1	0
	Egypt	Unavailable	0	0
	Morocco	Permanent	0	0
	Jordan	Permanent	0	0
	Lebanon	None	0	0
	Libya	Permanent	0	0
	Syria	None	0	0
Neighboring regions	Russia[†]	Unavailable	1152	6
	Hungary	Unavailable	26	0
	Ukraine	Unavailable	20	0
Total			2414	127

[†]Of cases reported in Russia, 40–74% occurred in the southwestern Volgograd oblast.
Data taken from source: EpiSouth cofunded by the European Union DG SANCO/EAHC and DEVCO/EuropeAid [36].

Live chimeric/recombinant vaccines

Chimeric/recombinant vaccines have been another active field for the development of WNV vaccines (Table 2). One well-studied approach uses the YFV-17D vector backbone expressing WNV prM and Env (ChimeriVax-WN, Sanofi), with testing performed across several animal species including mice, hamsters, horses and nonhuman primates (NHPs) [78–80]. The construct is based on the genetic backbone of the vaccine strain of YFV (YFV-17D) in which the YFV prM and Env genes have been replaced by the WNV-NY99 prM and Env proteins, with several point mutations engineered into the Env to reduce potential neurovirulence [78]. This vaccine platform (ChimeriVax™) has been the basis for a number of related flavivirus vaccine candidates including JEV and all four serotypes

Table 2. Preclinical approaches to West Nile vaccine development.

Vaccine approach	Animal models	Ref.
DNA-vectored vaccines		
DNA plasmid expressing WNV prM and Env	Mice, Horses	[71–73]
DNA plasmid expressing WNV EDIII	Mice	[74]
Pseudoinfectious DNA vector (C deletion mutants)	Mice, Horses	[75,76]
DNA plasmid expressing the attenuated Kunjin strain of WNV	Mice	[77]
Live chimeric/recombinant vaccines		
YFV-17D backbone expressing WNV prM/Env	Mice, Hamsters, Horses, NHP	[78–80,82]
DV4 backbone expressing WNV prM/Env	Mice, Geese, NHP	[84,85]
Canarypox vector expressing WNV prM/Env	Cats, Dogs, Horses	[86,87]
Adenovirus vector expressing WNV C, prM, Env and NS1 proteins	Mice	[93]
WNV Env expressed by multiple vesicular stomatitis virus vectors	Mice	[92]
HIV-based vectors expressing WNV Env protein	Mice	[88,89]
Measles vector expressing WNV Env protein	Mice, NHP	[90,91]
Live attenuated or pseudoinfectious vaccines		
Neutralizing mAb escape variant following serial passage in cell culture	Mice, Geese	[94]
Molecularly cloned lineage 2 WNV strain	Mice	[95]
Attenuating mutations in the glycosylation sites of WNV Env and NS1	Mice	[96]
Attenuating point mutations in the WNV Env and 3'-untranslated region	Mice	[97]
Attenuating point mutations in the WNV NS2A or NS4B proteins	Mice	[98,99]
Pseudoinfectious WNV achieved through C protein deletion mutations	Mice, Hamsters, NHP	[100,101]
Recombinant subunit vaccines		
WNV virus-like particle	Mice	[103]
Recombinant WNV Env protein	Mice, Horses, Birds, NHP	[108,113,149,170–173]
WNV EDIII constructs with different conjugate and adjuvant approaches	Mice	[105,108,111,112,174]
Env peptide vaccine derived from the EDIII domain	Mice	[175]
Recombinant NS1 protein	Hamsters	[115]
Inactivated whole virus vaccines		
Formalin-inactivated WNV	Mice, Geese, Hamsters, Horses	[80,102,122–124]
Hydrogen peroxide-inactivated WNV	Mice	[5,130]

Env: Envelope; NHP: Non-human primates; prM: Premembrane.

of DENV [81]. In preclinical studies of the ChimeriVax-WN candidate, vaccinated rhesus macaques reached average $PRNT_{50}$ titers against homologous vaccine virus of 1:381 by 30 days postvaccination, declining to 1:193 by day 63 [79]. Vaccinated mice demonstrated a low level of immunity with neutralizing titers in the range of 1:20–1:37 at 4 weeks following vaccination. This vaccine has since moved into several clinical trials and was licensed as a veterinary horse vaccine under the trade name, PreveNile® [82]. However, the horse vaccine was later recalled in 2010 after reports of increased adverse events in horses following vaccination [83]. An alternate chimeric flavivirus platform, using an attenuated DENV serotype 4 (DENV4) backbone expressing WNV prM and Env, has also been developed [84,85]. In NHP, peak $PRNT_{60}$ titers against WNV-NY99 of 1:324 were observed at day 28 postvaccination, dropping to 1:170 by day 42 [85]. A number of other live, attenuated recombinant virus vector vaccines have also been developed. A veterinary vaccine using a canarypox-based vector-expressing WNV prM and Env has demonstrated efficacy in preventing viremia across several animal species including horses, cats and

dogs [86,87]. An HIV-based lentiviral vector expressing the WNV Env protein was shown to induce a protective immune response in mice within 1 week of a single immunization [88]. A nonintegrative version of this same vector system was later developed in an attempt to reduce safety concerns and was also shown to induce protective immune responses in mice [89]. A WNV candidate based on an attenuated strain of the measles virus (Schwarz strain) expressing WNV Env was tested in both mice [90] and squirrel monkeys [91], with protection demonstrated against death and viremia, respectively. Similarly, a vesicular stomatitis virus vaccine vector has also been used to express the WNV Env protein, with 90% protection achieved against lethal WNV challenge in mice following a two-dose, intranasal vaccination schedule [92]. A multiantigen adenovirus-vectored vaccine expressing the WNV C, prM, Env and NS1 proteins induced robust $PRNT_{50}$ serum titers against WNV-NY99 (average = 1:2816) following a two-dose vaccination schedule in mice [93]. While several of these approaches showed promising results with regard to immunogenicity and protective efficacy in animal models, some practical constraints may limit their clinical utility. For vaccine approaches using lentiviral vectors [88,89] or vesicular stomatitis virus [92], can safety and biocontainment issues be adequately addressed? For candidates using adenovirus [93] and measles virus vectors [90,91], will pre-existing immunity in humans limit their use? These questions may need to be addressed if these vaccine platforms are to move forward into clinical development.

Live attenuated or pseudoinfectious vaccines

A number of preclinical studies have been reported using attenuated strains of WNV [94–99], created either through classic cell culture methods or targeted genetic mutations. For example, through a targeted deletion in the WNV C protein, one group has developed a WNV vaccine candidate that is limited to a single round of infection (RepliVAX WN, [100,101]). Using this approach, investigators were able to induce neutralizing antibodies and protective immune responses in mice, hamsters [100] and NHP [101]. Another group demonstrated that concurrent mutations in the Env and NS1 proteins were able to dramatically reduce the virulence of replication-competent WNV-NY99, increasing the 50% lethal dose from 5 PFU with wild-type virus to >1,000,000 PFU in the attenuated vaccine candidate [96]. Despite these mutations, the attenuated virus could elicit high $PRNT_{50}$ neutralizing responses against WNV-NY99 in mice (range: 1:320–1:2560) with as little as a 10^3 PFU dose. Results such as these hold promise as an additional avenue for development of a WNV vaccine, but live viral vaccines have been somewhat unpredictable in the past and may pose potential regulatory concerns for the elderly and immunocompromised, representing two vulnerable populations with the greatest need for a safe and effective WNV vaccine.

Recombinant subunit vaccines

In terms of protein vaccines, three main types have been developed including chemically inactivated whole virus, virus-like particle (VLP) and recombinant WNV Env subunit formulations (Table 2). The first successful veterinary vaccine, licensed in 2003, was a formaldehyde-inactivated preparation of WNV-NY99, shown to protect against WNV challenge [102]. Though this vaccine was not developed for human use, it has provided an important proof of principle for this class of nonreplicating protein vaccines against WNV disease. Another early WNV vaccine candidate within this general class of vaccines was a VLP expressing the prM and Env proteins [103]. Vaccination elicited neutralizing antibody titers in mice and although responses were relatively low (average: 1:37), they could be boosted with a monophosphoryl lipid A, (a detoxified form of lipopolysaccharide) and saponin-based liposomal adjuvant (average: 1:75) [103]. Several groups have developed vaccine candidates based on the DIII of the WNV Env protein, since this region of the Env has been shown to harbor potent neutralizing antibody epitopes in mice [104]. Using a 13 kDa DIII-recombinant protein for vaccination, mice mounted significant $PRNT_{50}$ serum titers (1:1000) against the lineage 2 WNV Sarafend strain following three immunizations with 100 μg of antigen per dose [105]. One caveat is that recent studies indicate that DII is the immunodominant domain in humans following WNV infection [106,107]. In particular, a study of convalescent sera collected from 30 human subjects at 4–7 months postinfection demonstrated that only 7.3% (range: 0.6–50.5%) of the total WNV-specific antibody response was directed toward DIII [108]. However, this immunodominance pattern was observed in the context of live viral infection, and it is unclear if humans will be able to respond effectively to DIII-based recombinant WNV vaccines. While beyond the scope of this review, the reader is directed to a number of excellent reviews discussing WNV structural immunology and the nature of protective epitopes [104,109,110]. Other groups have pursued vaccines based on VLP platforms that incorporate fusion proteins engineered to display the WNV Env DIII on their surface, but results have varied [111,112]. In one study using an HIV-based VLP, only one out of five mice seroconverted ($PRNT_{50}$ ≥1:10) against the lineage 1 WNV–Kunjin strain following vaccination [112]. Using a bacteriophage expression system, another DIII-based VLP vaccine was shown to induce neutralizing responses against WNV-NY99 in mice following a single immunization and partial protection against lethal WNV challenge ($PRNT_{100}$ ~1:20–1:30, 60% survival), but higher neutralizing titers and full protective immunity ($PRNT_{100}$ ~1:1000, 100% survival) were achieved after three doses [111]. A recent report exploring the subunit WNV Env vaccine approach has provided further insight into the role of different adjuvants in this vaccine model system [113]. In this study, mice were immunized on days 0 and 28 with 10 μg of WNV Env protein alone or adjuvanted with Matrix-M™, a saponin-based adjuvant. At 21 days after vaccination, $PRNT_{90}$ titers (against the lineage 1 WNV Eg101 strain) in the Env group averaged approximately 1:250. By comparison, the Matrix-M-formulated vaccine induced neutralizing antibody titers of approximately 1:8000, a 30-fold increase in immunogenicity. Additional

studies demonstrated that Matrix-M was also approximately fourfold more immunogenic than an aluminum hydroxide-based adjuvant. It is possible that subunit WNV vaccines will require the use of advanced adjuvants in humans to elicit high immunogenicity and although these approaches may bring additional regulatory complexity, the Matrix-M adjuvant looks very promising since it has been used in >10 million horses vaccinated with Equilis®Prequenza and has been shown to be safe and effective in a human influenza vaccine Phase I clinical trial [114].

In addition to Env-based recombinant vaccines, candidates using nonstructural proteins have also been explored. In one study, purified WNV NS1 was used to immunize hamsters followed by lethal challenge [115]. While not as effective as a recombinant Env comparator, the vaccine was able to reduce viremia and modestly increase survival (87% survival) compared with unvaccinated control animals (47% survival). In humans, T cell and antibody responses to nonstructural proteins do not appear to provide protection against flavivirus reinfection [11]. For instance, although YFV-17D-immune subjects are fully protected against viremia following homologous YFV-17D challenge, there was no observable protection against infection by recombinant strains of YFV-17D expressing the structural proteins (Env and PrM) from JEV [116] or DENV2 [117], despite previous studies demonstrating robust T cell responses [118–121] and presumably antiviral antibodies to the YFV-17D nonstructural proteins present in the recombinant YFV-17D virus vector. This indicates that despite having pre-existing antiviral immunity to up to eight YFV-17D nonstructural proteins, recombinant YFV-17D virus strains expressing JEV or DENV surface antigens replicated as efficiently in the YFV-17D-immune subjects as they did in YFV-17D-naïve subjects [116,117].

Inactivated whole-virus vaccines

Inactivated whole-virus vaccines represent another important class of vaccine candidates (Table 2). The first licensed veterinary vaccine was based on this approach, using a formalin-inactivated crude viral harvest of WNV-NY99, formulated with a squalene-based adjuvant, to induce protective immunity in horses [102] as well as other animal models [80]. A formalin-inactivated vaccine based on a pathogenic lineage 1 strain of WNV (ISR98) has also been described and was shown to be protective in a goose challenge model [122]. Two other groups have developed formalin-inactivated vaccines both based on the virulent WNV-NY99 strain of virus [123,124]. In one study, mice immunized with a two-dose schedule (1 μg per dose, alum adjuvanted) achieved virus-neutralizing titers of approximately 1:250 against WNV-NY99 at 2 weeks after final vaccination and were protected from intranasal challenge with virulent WNV [124]. In a second study, mice were also given a two-dose schedule of experimental, nonadjuvanted vaccine provided by the Research Foundation for Microbial Diseases of Osaka University (BIKEN, Osaka, Japan) [123]. At 4 weeks postboost, vaccinated mice demonstrated a neutralizing titer of 1:70 (WNV-NY99) and were protected against lethal WNV challenge. From a clinical perspective, one concern for these formalin-inactivated WNV vaccine candidates is that they are based on pathogenic strains of WNV. The use of highly pathogenic strains of virus for inactivated vaccines creates logistical issues associated with the handling of BSL3 pathogens during large-scale manufacturing, in addition to safety concerns if complete inactivation is not achieved. For instance, one of the worst vaccine-related tragedies in the USA came from the improper inactivation of virulent poliovirus during vaccine manufacturing in 1955 (i.e., 'The Cutter Incident') [125]. This resulted in 120,000 doses of vaccine that contained live poliovirus, leading to the inadvertent infection of 40,000 children, with 56 developing paralytic poliomyelitis and five who died [126–128]. While modern manufacturing practices ensure the safety of the inactivated polio vaccine, there has still been a push to further increase the safety margin of inactivated polio vaccine by switching from current virulent poliovirus strains to attenuated virus vaccine strains [129]. As an alternative to traditional formaldehyde-based vaccines, a novel hydrogen peroxide (H_2O_2) inactivation approach has been developed to produce a first-generation whole-virus vaccine against WNV [5,130]. Mice immunized with two 10 μg doses of H_2O_2-inactivated WNV formulated with aluminum hydroxide plus monophosphoryl lipid. A demonstrated high serum-neutralizing titers, with $PRNT_{50}$ values reaching 1:14,400 against WNV-NY99, and vaccinated mice showed complete protection against lethal WNV challenge [130]. Using this same H_2O_2 inactivation platform, a single aluminum hydroxide adjuvanted 10 μg dose of WNV vaccine (using H_2O_2-inactivated WNV–Kunjin) induced $PRNT_{50}$ titers against WNV–Kunjin of 1:6958 at 90 days postimmunization [5]. Following two immunizations, the H_2O_2 WNV vaccine demonstrated 90% protection in a robust intracranial challenge model involving 1,000,000-times the 90% lethal dose for WNV-NY99 [5]. In light of these promising preclinical results, Najít Technologies, Inc. has recently produced a clinical lot of H_2O_2-inactivated WNV vaccine and plans to initiate a Phase I clinical trial in 2014.

Vaccines in clinical development

DNA-vectored vaccines

Since the introduction of WNV into the USA in 1999, significant research efforts have been expended to create a viable vaccine for disease prevention in humans. To date, there have been eight published clinical trials assessing safety and immunogenicity across multiple vaccine platforms (Table 3). One of the first candidates to reach the clinic was a single-plasmid, recombinant DNA vaccine, VRC-WNVDNA017–00-VP, encoding the WNV prM and Env [131]. The vaccine consisted of a closed, circular plasmid DNA vector incorporating a CMV promoter, with the WNV-NY99 prM and Env coding sequences expressed downstream from a modified JEV signal sequence. Vaccine material was provided by Vical (San Diego, CA, USA), with the National Institute of Allergy and Infectious Diseases sponsoring the clinical trial. The trial was performed as an open-label Phase I study in

Table 3. West Nile virus vaccine clinical trials.

Sponsor and phase	Vaccine approach	Seroconversion[†] (%)	Neutralizing titer (Range)[‡]	Ref.
DNA-vectored vaccines				
Vical/NIAID Phase I	DNA plasmid-expressing WNV prM/Env	96.6–100	50 (16–128)[§]	[131,133]
Live chimeric/recombinant vaccines				
Sanofi Phase I	YFV-17D backbone expressing WNV prM/Env	100	11,392[¶]	[78]
Sanofi Phase II	YFV-17D backbone expressing WNV prM/Env	95.4–97.3	3309 (1727–6342)[¶]	[139,140]
NIAID Phase I	DV4 backbone expressing WNV prM/Env	75–89	161 (8–1530)[§]	[138]
Recombinant, subunit vaccines				
Hawaii Biotech Phase I	Recombinant WNV Env protein	100	~10–100[§,#]	[150]

[†]Seroconversion definitions may vary between trials. From each clinical trial, the group with the highest reported response is highlighted in the table.
[‡]Peak geometric mean titers with range are given for neutralizing antibody responses as determined by PRNT, when available.
[§]PRNT titers were assessed against the wild-type WNV-NY99 strain.
[¶]PRNT titers were performed against the homologous vaccine virus. For chimeric flavivirus vaccines, PRNT titers are higher when using homologous vaccine virus in the assay, in comparison to wild-type virus targets [138,141,142].
[#]Summary data on human results can be found in US patent application No. US20120141520 A1, Figure 3 [150].
Env: Envelope; NIAID: National institute of allergy and infectious diseases; prM: Premembrane; PRNT: Plaque reduction neutralization; WNV: West Nile virus; YFV: Yellow fever virus.

healthy subjects aged 18–50 years old. The vaccine was administered at 4 mg per dose intramuscularly using a needle-free injection system on days 0, 28 and 56. Fifteen vaccinees were enrolled with a total of 12 vaccinees completing the entire three-dose regimen. The most common side effects were limited to local injection site reactions, with no reports of serious adverse events. All vaccinees that completed the full three-dose regimen demonstrated seroconversion by serum ELISA titers but $PRNT_{50}$ titers were variable and generally low. At week 12 (~1 month following the final immunization), $PRNT_{50}$ titers ranged from 1:16 to1:128, with a group geometric mean of 1:50. By comparison, subjects infected with live WNV demonstrated a $PRNT_{50}$ of about 1:1400 at 1 year following infection [130]. An alternative WNV reporter virus particle (RVP) neutralization assay was also utilized to assess immunogenicity, with RVP neutralization titers ranging from 1:100 to 1:1000. The reason for the differences between the two assays is uncertain, though studies using WNV-specific monoclonal antibodies (mAbs) indicate that the maturation state of the virus used in the RVP assay may play a role [132].

In an effort to improve immunogenicity, a modified DNA plasmid construct incorporating an additional regulatory element from the human T cell leukemia virus type 1, in conjunction with the previously used CMV promoter, was tested in a Phase I clinical trial [133]. The clinical protocol closely matched the previous study [131] in terms of vaccination dose and booster regimen, but included both a young (ages: 18–50) and older (ages: 51–65) cohort, with 15 subjects enrolled per group. As with the prior DNA construct, side effects were mild and generally limited to the site of injection. Neutralizing antibody titers against RVP indicated a trend toward higher antibody responses with the modified vector although this was not statistically significant. At 12 weeks (~1 month following the final dose), RVP-based seroconversion was demonstrated in 28/29 (96.6%) subjects. However, $PRNT_{50}$ serum titers against WNV were not directly assessed in this second clinical trial, limiting the ability to make comparisons to other clinical studies. Both DNA vaccine candidates are similar to a veterinary horse vaccine formerly produced by Wyeth's Ft Dodge Animal Health division (West Nile-Innovator DNA) that was licensed by the United States Department of Agriculture in 2005. However, this veterinary vaccine has since been discontinued following Pfizer's acquisition of Wyeth in 2009 [134]. Further development of the WNV DNA vaccine platform is unclear, with the most recent human clinical trial completed in 2007 and the results published in 2011 [133]. In general, DNA vaccines have suffered from concerns over immunogenicity and potential safety issues such as DNA integration into the genome [135]. However, in this instance, the WNV DNA vaccine was able to induce a measurable WNV-specific immune response and was well tolerated without any serious adverse events [136]. Improvements in DNA delivery technologies are continuing to occur [137] and these innovations, combined with this promising vaccine candidate, may offer a path toward further development of a successful WNV-specific DNA vaccine.

Live, attenuated chimeric/recombinant vaccines

To date, two live, attenuated chimeric flavivirus vaccine candidates have been tested in humans [78,138–140]. The first WNV chimeric vaccine to enter clinical trials, ChimeriVax-WN02, was originally developed by Acambis, a company later acquired

by Sanofi Pasteur. In an initial Phase I study, subjects were immunized with either 10^3 (n = 15) or 10^5 (n = 30) PFU of ChimeriVax-WN02, as well as five control subjects who received the standard YFV-17D vaccine [78]. Viremia, as measured by the area under the curve (AUC), was significantly higher with the lower dose (312 vs 173 PFU/ml/day), a trend that has since been observed in other chimeric flavivirus vaccines [138]. Peak antibody titers for both the 10^3 and 10^5 PFU dose groups were recorded at 21 days postvaccination, reaching 1:11,392 and 1:6241 respectively, with 100% seroconversion in both groups. These titers fell to 1:1218 and 1:1280 by day 28. At 12 months, 97% (35/36) of tested subjects remained seropositive, with antibody titers of approximately 1:600 in both dose groups. One caveat is that the experimental approach for determining $PRNT_{50}$ titers was based on neutralizing homologous vaccine virus (ChimeriVax-WN) rather than a wild-type or reference strain of WNV. Using a similar vaccine approach, others have indicated that differences in the target virus used in the neutralization assays may have a substantial impact on the outcome of PRNT titers when assessing clinical samples [138]. In addition, prior studies in NHP vaccinated with chimeric DENV or JEV constructs demonstrated that $PRNT_{50}$ titers ranged from about 2- to 64-fold lower when using wild-type strains of target virus compared with the homologous chimeric vaccine construct [141,142]. It is unclear if the WNV-specific neutralizing titers described in the ChimeriVax-WN Phase I trial would likewise be reduced if a strain of WNV was used instead of ChimeriVax-WN vaccine virus in the PRNT assay, but this highlights the need for consensus in the WNV vaccine field in terms of how immunogenicity and neutralizing assays should be measured in clinical studies.

Following the Phase I results, two Phase II studies were performed using a plaque-purified derivative of ChimeriVax-WN02, which had been developed in an effort to increase attenuation and reduce viremia in vaccinated subjects [139,140]. The first of these trials was divided into two parts, assessing safety and immunogenicity between adult (part 1, ages, 18–40) and older patient populations (part 2, ages, 41–64 or ≥65). In part 1, a total of 112 subjects were enrolled, receiving 3.7×10^3, 3.7×10^4 or 3.7×10^5 PFU of ChimeriVax-WN02 or placebo. In part 2, 96 subjects were enrolled and received only the 3.7×10^5 PFU dose or placebo. As with the Phase I trial, the vaccine was generally well tolerated across dosages and age groups. Peak viremia was reduced in comparison to the Phase I study, though lower doses of virus still resulted in higher levels of viremia. In part 2 of the study, increased viremia was shown to be associated with advanced age. AUC measurements demonstrated a value of 181 PFU/ml/day in those ≥65 years of age compared with 115 PFU/ml/day in those aged 41–64 years [143]. Although there were no severe adverse events observed in this small study, this result indicates that close monitoring of viremia should be continued in the future since the elderly represent the primary target population for a successful WNV vaccine. Seroconversion rates in both parts 1 and 2 reached >95% by day 28, regardless of dose or age group. Immunogenicity (as judged by neutralization against the homologous vaccine virus) increased with virus dose in part 1, and group $PRNT_{50}$ titers reached 1:3309 by day 28 after vaccination with the highest dose of virus (3.7×10^5 PFU). In part 2 (3.7×10^5 dose only, subjects aged ≥41 years), $PRNT_{50}$ titers peaked at day 28, but only ranged between 1:883 and 1:965. It is unclear why there was a difference (~3.5-fold) in peak serum antibody titers between Parts 1 and 2 of this clinical study, though part 2 was limited to older subjects. Subjects in part 2 were followed for up to 1 year, at which point neutralizing titers had declined to an average of 1:116. A second Phase II study focused on subjects ≥50 years of age and was performed to collect additional safety and immunogenicity data in this older demographic [140]. In this study, a total of 479 subjects were enrolled and received vaccine at 4×10^3, 4×10^4 or 4×10^5 PFU/dose or placebo. At 28 days postvaccination, seroconversion rates ranged from 92 to 95%, increasing slightly with each dose level. $PRNT_{50}$ titers at day 28 were not statistically different between vaccine groups and averaged between 1:600 and 1:688, somewhat lower than that observed in elderly subjects in the prior trial [139]. Additional immunogenicity time points were not assessed. While peak viremia remained lower compared with the Phase I study (prior to further cell culture attenuation of the chimeric vaccine virus), AUC measurements were similar ranging from 234 to 309 PFU/ml per day across all dose groups [143]. Why viremia levels increased in this clinical trial compared with the prior Phase II study is unclear, though the demographics of the study population were more narrowly focused on older individuals. Further development of this WNV vaccine is uncertain since this program was suspended by Sanofi Pasteur after acquisition of Acambis in 2008 [144].

Another chimeric flavivirus vaccine based on a DENV4 backbone vector expressing the WNV prM and Env proteins has also completed early-phase clinical testing (Table 3) [138]. The vaccine, WN/DEN4Δ30, originated from the NIH Laboratory of Infectious Diseases and had previously been tested for safety and efficacy in preclinical animal studies [84,85]. The backbone was developed as a DENV4 vaccine candidate (rDEN4Δ30) attenuated through a 30-nt deletion in the 3′-untranslated region of the viral genome [145], which was then further engineered to express the WNV-NY99 prM and Env. Two current good manufacturing practices lots were produced, with the second lot modified to contain additional noncoding point mutations in an effort to increase virus production from cell culture, though the amino acid sequence of the polyprotein did not differ between lots [146]. A total of 82 subjects, aged 18–50 years, were enrolled into the study, which was divided into three subcutaneous dose levels (10^3, 10^4 and 10^5 PFU/dose) with 20 vaccine recipients per dose and a total of 22 placebo controls. For the low-dose groups (10^3 and 10^4 PFU/dose), only a single dose was administered, whereas the 10^5 high-dose group received a booster immunization at 6 months. The vaccine was found to be safe and well tolerated, with no statistically significant differences in injection site or systemic adverse events between vaccine and placebo groups. rWN/DEN4Δ30 viremia

was detected in eight subjects, generally starting within 1–2 weeks postvaccination, and lasting for 1–3 days. Interestingly, the lower doses of virus led to a higher percentage of viremic subjects (16–20%), while the 10^5 dose resulted in detectable viremia in only 5% of the subjects. This continued the trend of an inverse relationship between virus dose and subsequent viremia levels previously established with the YFV-17D-based ChimeriVax platform [147]. Levels of viremia appeared to mirror seroconversion rates, with a range of 74–75% seroconversion in the two low-dose groups (≥fourfold rise in serum $PRNT_{60}$ at day 28 or 42) compared with only 55% seroconversion in the 10^5 high-dose group by 42 days after vaccination (note: two more subjects also seroconverted by study day 180). Group geometric mean $PRNT_{60}$ titers also varied with dose. At the 10^3 dose level, $PRNT_{60}$ titers peaked at day 42 with an average of 1:161 (range: 1:8–1:1530), while the 10^4 dose demonstrated a peak of 117 (range: 1:5–1:3218) on day 28. By day 180, the 10^3 and 10^4 group titers had dropped to 1:76 (range: <1:5–1:290) and 1:35 (range: <1:5–1:232), respectively. The 10^5 high-dose group peaked at day 28 with a group average $PRNT_{60}$ of only 44 (range: 1:18–1:183), which dropped to 15 (range: <1:5–1:120) by day 180. Boosting of the 10^5 group on day 180 increased the rate of seroconversion to 89%, with a concomitant increase in average neutralizing antibody titers to 1:57 (range: 1:17–1:134). The design of clinical studies enrolling older subjects (>50 years of age) is underway [138], indicating that this vaccine will continue to be evaluated as a novel approach to WNV vaccination.

Recombinant, subunit vaccines

The only WNV subunit vaccine candidate that has been tested in clinical trials is WNV-80E, a recombinant form of the WNV-NY99 Env protein produced in *Drosophila* S2 cells. This vaccine was developed by Hawaii Biotech and is based on a truncated WNV Env protein formulated with aluminum hydroxide. Preclinical studies in multiple species, including mice, birds and NHP, demonstrated the induction of WNV-specific neutralizing responses after vaccination [148,149]. In a Phase I clinical trial, a total of 24 subjects were enrolled to assess immunogenicity and safety [150]. Subjects were divided into four groups, receiving 5, 15 or 50 μg of WNV-80E adjuvanted with aluminum hydroxide or 50 μg of vaccine without adjuvant. Immunizations were performed intramuscularly at weeks 0, 4 and 8 for a total of three inoculations per subject. The vaccine was well tolerated with most side effects limited to injection site reactions. At 4 weeks following primary immunization, $PRNT_{50}$ titers against WNV were negative (<1:10) in all groups. Following the second dose of vaccine, the aluminum hydroxide adjuvanted 15 μg and 50 μg doses elicited average $PRNT_{50}$ titers ranging between 1:10 and 1:100. After the third immunization, mean titers in these two groups increased but appeared to remain within the range of 1:10–1:100. The low-dose group (5 μg) reached seropositive status ($PRNT_{50}$ ≥1:10), but only after receiving a third dose of vaccine. These relatively low neutralizing antibody responses may be due to lack of appropriate tertiary or quaternary structure. For example, following natural infection with DENV, human serum samples were collected and tested for their ability to bind intact virions or the ectodomain of purified DENV Env protein, similar in composition to the WNV-80E candidate [151]. These studies demonstrated that the majority of DENV-specific neutralizing mAbs recognized and bound the complex quaternary structure of the virion, but not the recombinant Env protein. The Phase I clinical trial of this WNV vaccine ended in 2009 and further clinical development appears to have stalled. While WNV-specific immunogenicity seems to be generally low, the use of advanced adjuvant systems (such as saponin derivatives [113]) may improve vaccine potency and are worth exploring in future studies.

In the clinical WNV field, interlaboratory variations in serological techniques make it difficult to directly compare neutralizing antibody results. In other vaccine models, international serum standards have been useful for bridging the results obtained from different research groups [152,153] and it may be worthwhile to consider standardizing WNV-neutralizing assays with a defined target virus (e.g., WNV-NY99), a highly characterized reference serum standard, and perhaps a standard approach to presenting the neutralizing titers (e.g., $PRNT_{50}$, $PRNT_{60}$ or $PRNT_{90}$) to further aid in comparisons of immunogenicity within the field. Since WNV-NY99 represents a BSL3 infectious agent and it is not always feasible for organizations to accommodate this level of biosafety, another option might be to use WNV–Kunjin as a reference standard since it is a closely related Lineage 1 strain of WNV but can be handled under BSL2 containment. However, if WNV–Kunjin is to be used for these purposes, then a bridging study comparing neutralizing antibody responses of WNV–Kunjin to a virulent strain of WNV, such as WNV-NY99, may also be needed.

Challenges for future clinical development & licensure

Before a vaccine can be licensed for commercial use, it must be shown to be both safe and effective at reducing the disease that it is designed to prevent. One of the biggest hurdles in reaching licensure for a human WNV vaccine is the limited feasibility to perform field efficacy trials. Although WNV is endemic throughout the continental USA, the relatively low incidence and sporadic nature of WNV outbreaks pose challenges for study design and implementation. However, with over a decade of detailed epidemiology available from active WNV surveillance programs, it may be possible to identify long-term trends in WNV outbreaks and look for 'hot spots' of more sustained or predictable WNV activity. While some periodicity of WNV incidence has been found in states such as California and Texas, the overall incidence tends to be low (<1 case per 100,000). In contrast, Midwestern states have relatively high incidence of WNV disease (Figure 2A) and an older population at risk for WNV infection (Figure 2B). For example, South Dakota has recorded an average incidence of approximately 20 cases of WNV per 100,000 since 2002, resulting in one of the highest rates in the nation [37]. When focusing locally at the county

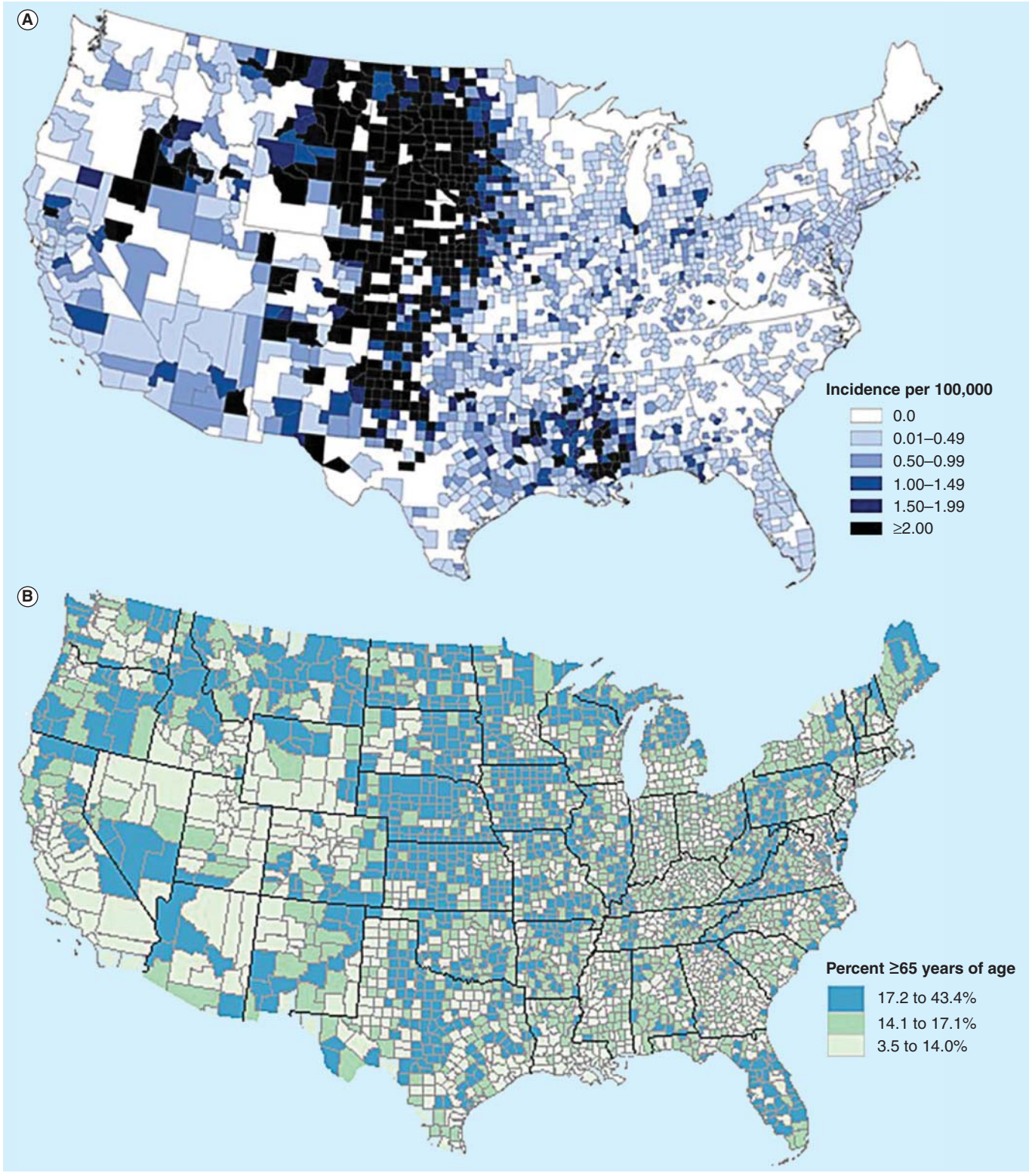

Figure 2. Maps of West Nile virus neuroinvasive disease incidence and age distribution in the USA. (A) The average annual incidence of human West Nile virus neuroinvasive disease in the USA, 1999–2012, reproduced with permission from the national Arbo-NET surveillance system conducted by the Centers for Disease Control and Prevention [34]. **(B)** Distribution of Americans aged 65 years and older based on the 2010 Census.

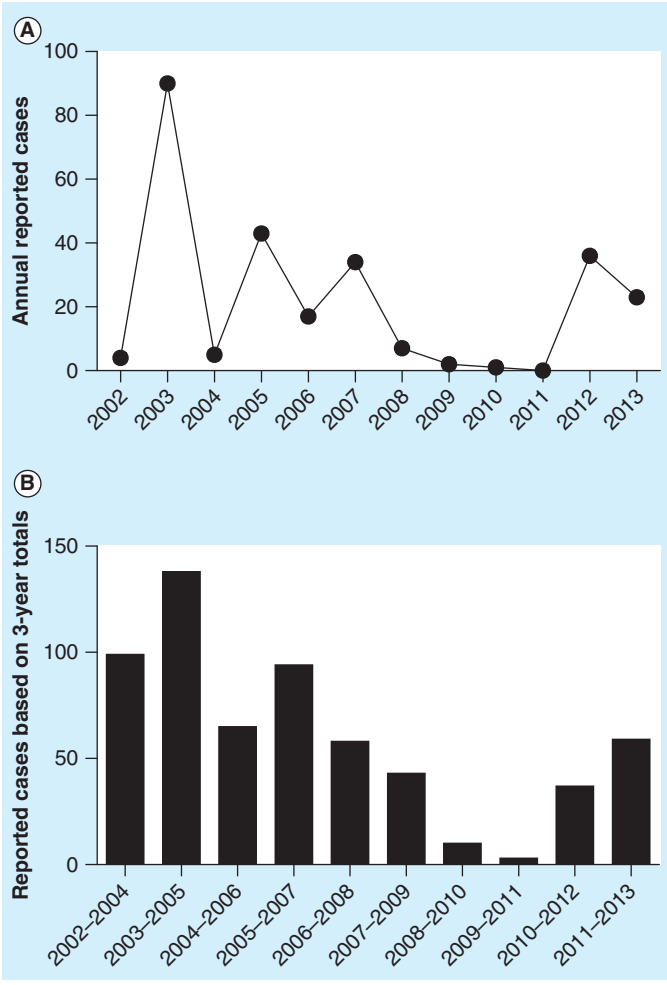

Figure 3. High WNV disease burden in localized regions of the USA. The annual number of reported cases for WNV **(A)** or the total number of cases for each overlapping 3-year period **(B)** are shown for Brown County, South Dakota from 2002 to 2013 (population size ~37,000). Data taken from [37]. Reported WNV cases declined during 2008–2011, but a substantial increase was observed in 2012 and 2013, indicating that relatively high incident, recurrent WNV may be likely to continue in certain at-risk geographical areas.
WNV: West Nile virus.

level, northeastern Brown County, SD (Figure 3A) demonstrated an annual incidence of approximately 60 cases per 100,000 during that same time frame, and a total number of 262 reported WNV cases from 2002 to 2013 despite a small population size of approximately 37,000. One complicating factor is that even in these endemic locations, WNV disease activity varies greatly from year to year. However, if a systematic effort is put forth to test the efficacy of an advanced WNV vaccine, then it may be possible to vaccinate an at-risk population and monitor WNV disease activity over the course of 2–3 seasons or until enough WNV cases have accumulated to provide statistical significance between vaccine and placebo groups. Using Brown County, SD as an example, if the cumulative number of reported WNV cases is calculated for each overlapping 3-year period from 2002 to 2013, then for 8 of the 10 (80%) of these blocks of time, there were between 37 and 138 reported cases of WNV (Figure 3B). By vaccinating the subpopulation at highest exposure risk and by performing an efficacy trial at more than one location with historically high WNV incidence, the risk of failing to identify a statistically significant decrease in disease incidence may be further mitigated. Albeit logistically challenging, this long-term approach to disease monitoring during a WNV Phase III field trial may provide the feasibility necessary to determine vaccine efficacy and move an effective vaccine closer to licensure.

As an alternative to field efficacy trials, some have suggested that the US FDA Animal Rule may provide a route for licensure [143]. The Animal Rule was put forward to allow FDA evaluation of drug effectiveness based on the evidence from appropriate animal studies in instances where human efficacy studies are considered unethical or unfeasible (21 Code of Federal Regulations 601 Subpart H, 21 Code of Federal Regulations 314 Subpart I for New Drugs). However, the Animal Rule appears to have been primarily implemented in the context of bioterrorism threats wherein no other practical alternative exists [154]. Since initial publication of the Animal Rule in 2002, two drug products for the treatment of nerve gas poisoning have been approved following this mechanism, but no vaccines have advanced to licensure [154]. As noted by the FDA, the Animal Rule does not provide a shortcut to licensure and may actually take much longer than standard clinical testing [155]. As another method to supporting innovations in medicine, the FDA has recently developed its Advancing Regulatory Science initiative, which has been suggested as an avenue for WNV vaccine development [143], though how this would work in practice remains to be seen.

Vaccine-induced antibody-dependent enhancement (ADE) of infection is a potential concern for some flaviviruses such as DENV [156–160], but enhanced risk of disease following secondary infection with WNV has not been described [104]. While it is clear that WNV-specific ADE can be demonstrated *in vitro*, it is less obvious if these results translate to a clinical concern under *in vivo* conditions. For instance, in one study examining a poorly neutralizing WNV-specific mAb, the authors were able to demonstrate enhancement of infection *in vitro* [161]. However, when tested prophylactically *in vivo*, the mAb was protective in mice and resulted in decreased viremia and significantly increased survival following lethal WNV challenge. A more recent clinical study involving a suboptimal human DENV vaccine candidate has also provided new insight into the general debate of flavivirus vaccine-associated ADE [162]. In this Phase IIb clinical trial, a tetravalent DENV vaccine was used to immunize over 2600 children who were monitored for 2 years after vaccination (i.e., >5200 person-years). Although the vaccine was shown to be immunogenic, the overall efficacy of the vaccine was low (30.2%). However, there were no signs of increased rate or severity of DENV disease in the vaccinated children compared with placebo controls. These results indicate that in a high-risk setting (e.g., low neutralizing antibody titers

coupled with high-level endemic exposure), ADE may not represent as significant of a concern for flavivirus vaccine development as previously thought, though postmarketing evaluation of any vaccine will still be needed to further support this conclusion.

Will a vaccine against WNV be cost-effective?

Outbreaks of WNV cause considerable morbidity, mortality and disease-associated economic loss. Although the economic impact of a successful vaccination program should not be the sole determinant involved in making public health decisions, it is nevertheless an important parameter in determining overall feasibility. If the financial cost to a society is reduced through decreased disease burden, there will be more support for implementing a particular vaccine policy rather than when there is little or no cost advantage. In 2002, there were 4156 reported cases of WNV in the USA (Figure 1), with 329 cases identified in Louisiana [163]. The estimated cost associated with these 329 cases was US$20.1M (i.e., US$61,094/case) [163] and if applied to all 4156 cases, this would suggest a cost of US $254M in 2002 dollars. Albeit optimistic, if we assume that the earliest date that a vaccine could be commercially available is in 5 years (e.g., 2019), then a similarly sized outbreak in 2019 would have an estimated economic impact of US$356M after adjusting 2% per year to 2019 dollars. Even though the outbreaks in 2006 and 2012 were larger than that encountered in 2002 (Figure 1), over the past 10 years (2003–2012), the average annual number of reported cases of WNV in the USA is 3278. Based on this average number of annual WNV cases, the total financial impact of WNV in 2019 would be estimated at US$281M.

In contrast to these rough estimates of cost, a formal analysis of the cost–effectiveness of WNV vaccination was performed in 2006 [40], and this study is frequently used as the basis for estimating the costs associated with more recent WNV outbreaks [164,165]. Using simulations and sensitivity analysis, the healthcare costs per case of WNV were estimated at US $36,000 (range: US$20,000–US$59,000/case) in 2004 dollars [40]. If adjusted to 2019 dollars (US$48,451/case) and an estimated incidence of WNV at 3278 cases/year, then the direct societal cost of WNV outbreaks would be approximately US$159M/year. The Zohrabian *et al.* study [40] concluded that universal vaccination against WNV would be unlikely to result in societal monetary savings and this has led many to believe that the development of a WNV vaccine will not be feasible. However, this model was based on several assumptions including the implementation of mass vaccination of 100 million people with a vaccine regimen costing US$100/person. Results from their sensitivity analysis indicated that the probability that a WNV vaccine would provide societal cost savings changed from 0 to 76% as the cost of vaccination decreased from US $150 to US$10. Since it may be challenging for a new vaccine to have an appreciable profit margin when initially launched at a price of US$10/vaccinee, commercial enthusiasm for development of a safe and effective WNV vaccine has subsequently waned.

One key parameter for determining economic cost is loss of productivity due to death or short-term and long-term disability [40]. However, since WNV disease disproportionally afflicts the aged population, there is consequently a lower base productivity profile and decreased lifespan potential. Together, this indicates that diseases that target aged and/or low-income populations will simply not have the same financial impact that is associated with diseases of younger people or individuals from high-income communities based on mathematical modeling estimates alone. The lack of a more humanitarian component to cost–effectiveness projections is a challenging issue, especially in cases in which cost–effectiveness is borderline or not cost-effective, despite providing a mechanism to reduce clinical disease burden [166]. Another key point with the Zohrabian study [40] is that it is based only on direct WNV-related health care costs and does not take into account the costs of WNV surveillance, vector control and outbreak prevention and response costs, which can be large [163,165]. Others have also argued that cost–benefit analyses should include the more 'intangible' value of a successful WNV vaccine program by taking into account the broader economic costs associated with the impact of WNV outbreaks on travel, tourism and local economic growth [167]. Further studies that incorporate these factors will be important for gauging the full economic impact of WNV outbreaks in the USA and abroad.

Instead of implementing mass vaccination, a more feasible and cost-effective approach to preventing WNV outbreaks in the USA might be to perform targeted vaccination of the populations at greatest risk for WNV disease (Figure 2). For instance, although it may be unlikely for a vaccine manufacturer to develop a new WNV vaccine for 100 million people at an initial price of US$10/each [40], there is higher potential for commercialization of a vaccine developed to provide targeted immunization to 10 million people at a price of US$50–100/each and this would still provide a favorable cost–benefit ratio. A targeted vaccine campaign could be designed based on vaccinating specific regions with the highest WNV disease incidence or the highest total number of reported WNV cases. Alternatively, the targeted vaccine campaign could be based on age, with the elderly representing the most at-risk population. WNV activity has been reported in all 48 contiguous states but the risk of contracting WNND varies substantially both between states and even within each state when monitored at the county level (Figure 2A). Interestingly, the Midwestern states and individual counties with the highest incidence of WNND are also enriched for an older population of people aged ≥65 years (Figure 2B), representing the group that bears the most severe short-term and long-term WNV disease manifestations and WNV-associated mortality. The low population density in the regions most greatly impacted by WNV provides further support for the approach of a targeted regional vaccine program instead of implementing mass vaccination of the nation at large, which will invariably include large populations at low historical risk for WNV disease. There are eight states (CO, LA, MI, MT, NE, ND, SD and WY) with an incidence of

WNND of >1/100,000 (annual incidence from 1999 to 2012; Figure 2A). Based on 2012 Census estimates (Source [168]), there are approximately 24.6 million people living in these states, with a subpopulation of 2.3 million people over the age of 65 years who could be the focus of targeted vaccination. Alternatively, instead of basing a targeted vaccine campaign only on states or counties with the highest disease incidence, one could focus vaccine programs in states/counties with the highest total number of reported WNV cases. In this scenario, there are eight states (AZ, CA, CO, IL, LA, MI, OH and TX) which have had between 604 and 2357 reported cases of WNND from 1999 to 2012 and account for more than half of all reported WNV neurotropic disease in the USA [34]. The total population of these states is 115 million, making mass vaccination a potentially challenging prospect from a cost–effectiveness perspective. On the other hand, if focused only on the aged population, then a nationwide vaccine plan could be implemented. In 2012, approximately 40.5 million people in the USA were ≥65 years of age, and with a targeted vaccine developed for 10 million aged individuals, it would be possible to vaccinate one-fourth of the people in this age group who are at the highest risk for exposure and complications from WNV infection. Alternatively, in 2012, there were approximately 102 million people at ≥50 years of age and another approach would be to vaccinate 10% of this age group who are at greatest risk for WNV infection. By using a targeted vaccine program aimed at the most at-risk populations across the nation at either the state or county level, a safe and effective WNV vaccine could sharply reduce disease burden and mortality while still providing substantial societal cost savings.

Expert commentary

WNV is an emerging/re-emerging pathogen that has become endemic in the continental USA and appears to be on the rise in Southern Europe and neighboring regions. While several promising WNV vaccines have been evaluated in clinical trials over the last decade, a licensed human vaccine remains elusive. One concern for future vaccine development is the feasibility of performing Phase III efficacy trials for a zoonotic disease like WNV that is known for sporadic outbreaks that are often difficult to predict. However, with detailed WNV epidemiologic data from across the USA, it may be possible to identify locations for potential vaccine trials, especially in Midwestern regions with relatively low population density but high WNV disease incidence. Although universal WNV vaccination is unlikely to be cost-effective, further studies are needed to determine if targeted vaccine campaigns focused on at-risk age groups or geographical regions will provide a favorable cost: benefit ratio, especially in light of recent evidence indicating that almost 3 million Americans have likely been infected with WNV and most cases continue to go unreported. The recognition of much larger WNV incidence, coupled with increasing evidence for long-term disability and decreased quality of life among WNV survivors, further indicates a compelling need for a safe and effective WNV vaccine.

Five-year view

The next 5 years may be pivotal in terms of the continued successful development of a safe and effective vaccine against WNV. Several vaccine products are in various stages of clinical development, and new vaccine technologies are still entering the pipeline. A targeted vaccine program has the potential to be cost-effective, and there is renewed interest in WNV vaccine technology, especially since the outbreak of 2012 represented not only the largest outbreak since 2003, but also resulted in the most WNV-associated deaths on record. Despite the continued dedication to WNV-related research spanning the last 15 years, several key challenges remain. For instance, a WNV vaccine will likely represent a 'niche market' compared with other blockbuster vaccine products and steps may be needed to incentivize industry leaders to move forward with further clinical development. Another important challenge to these efforts will be to identify a feasible path forward for conducting Phase III efficacy trials or implementing the FDAs 'Animal Rule' to demonstrate efficacy when human efficacy studies are not ethical or feasible [155]. Due to the sporadic nature of WNV outbreaks and many mitigating factors (e.g., bird density/diversity, urban/agricultural landscape, temperature, rainfall, human population density/socioeconomics) [39,169], further advances in WNV epidemiology and outbreak prediction may be necessary in order to quickly implement local vaccine trials in areas either undergoing an early-stage WNV outbreak or those with a high likelihood of an upcoming WNV outbreak. Through the combined and collaborative efforts of WNV epidemiologists, virologists, vaccine manufacturers, state and county health officials and regulatory agencies, it is possible that a safe and effective vaccine against WNV can become a reality and provide protection to the vulnerable populations within our communities that need it most.

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Key issues

- Based on more than a decade of surveillance in the USA, it is expected that WNV will continue to threaten vulnerable populations for the foreseeable future.
- The severity of WNV disease is associated with advanced age and often results in long-term health issues including potentially severe neurological sequelae and a higher mortality rate after recovery from acute infection. More studies are needed to determine if WNV infection is linked to chronic kidney disease.
- Several early-stage vaccine clinical trials have been completed, but none has advanced to licensure.
- Reference standards for performing WNV-specific neutralization assays, including highly characterized serum standards and reference strains of WNV, should be considered.
- Due to the sporadic nature of WNV outbreaks, concerns remain regarding the feasibility of Phase III vaccine field efficacy trials. However, this may be mitigated, at least in part, by maintaining intense surveillance efforts, performing trials in locations of high/continued WNV incidence and monitoring for vaccine efficacy over a prolonged period of time (possibly 1–3 years).
- A formal cost–benefit analysis of targeted WNV vaccination should be performed, preferably including not only direct healthcare costs but also costs associated with WNV surveillance, prevention and outbreak response.

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