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Review

A review of successful flavivirus vaccines and the problems with those flaviviruses for which vaccines are not yet available☆

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ABSTRACT

Genus *flavivirus* comprises many important human pathogens causing public health problems worldwide. Some flavivirus infections are characterized by a relatively high mortality rate and/or high sequelae rate in survivors. Because most flavivirus life cycles are maintained between arthropod vectors and amplifying/reservoir hosts in the absence of humans, eradication of flaviviruses might be extremely difficult. Flavivirus vaccine development is considered a reasonable method to prevent flavivirus infections. Some vaccines have been successfully developed, but others have not, regardless of much effort. This review article describes currently available flavivirus vaccines against yellow fever, Japanese encephalitis, and tick-borne encephalitis. In addition, the current status of dengue and West Nile virus vaccine development is reviewed and problems regarding their development are discussed.

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1. Introduction

Genus *flavivirus* of the family *Flaviviridae* comprises over 70 different members including many important human pathogens causing public health problems worldwide [1]. Most flaviviruses are hematophagous arthropod-borne viruses. For example, yellow fever virus (YFV), Japanese encephalitis virus (JEV), West Nile virus (WNV), and four dengue viruses (DENVs: DENV-1 to DENV-4) are transmitted by mosquitoes, and members of tick-borne encephalitis viruses (TBEVs) are transmitted by ticks. The geographical distribution of each flavivirus is likely to be affected by their vector distributions. Therefore, climate change and development of transportation systems have allowed flaviviruses to invade new areas. Currently, many people are exposed to flaviviruses worldwide (Fig. 1). In addition, traveling through endemic countries increases the opportunity of exposure to flaviviruses. Because flaviviruses are maintained in nature between vectors and hosts without humans, eradication of flaviviruses is thought to be extremely difficult.

Flavivirus infection causes a variety of manifestations ranging from non-severe flu-like symptoms to severe/lethal symptoms (meningitis and encephalitis for JEV, TBEV, WNV infections, jaundice for YFV infection and hemorrhagic fever for DENV infection). The case fatality rate among symptomatic infections (~50%) and/or sequelae rate of survivors (~75%) are relatively high in some flavivirus diseases [2]. To date, no specific antivirals are available. Thus, vaccination is considered a reasonable method to prevent flavivirus infections.

Flavivirus genome, positive-strand RNA, encodes three structural proteins (capsid, precursor of membrane (prM), envelope (E)) and seven non-structural (NS) proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B, NS5). The major virion surface protein is E, which is involved in receptor binding and membrane fusion. Therefore, most neutralizing antibodies are induced against E protein [1], and all approved and most developing flavivirus vaccines contain E antigens. Effective flavivirus vaccines against YFV, JEV and TBEV infections have been developed, but WNV and DENV vaccines have not been licensed for human use. In addition, Wesselsbron virus and

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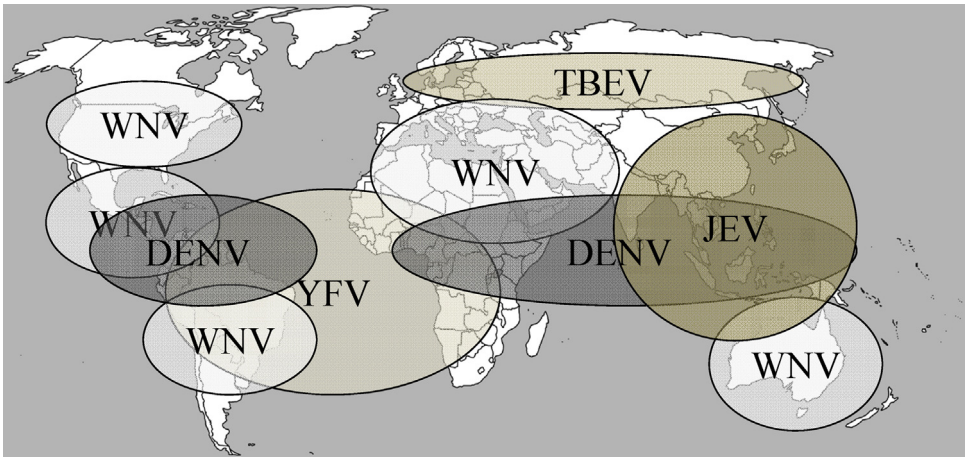

Fig. 1. Worldwide distribution of flaviviruses. Distributions of YFV, JEV, TBEV, DENV and WNV are indicated.

louping ill virus are important flavivirus pathogens, particularly in the veterinary field, where vaccines are also available. These viruses have been described previously [3,4].

In this article, the history and current status of successful YFV, JEV, and TBEV vaccines are reviewed. In addition, current progress/problems on the development of WNV and DENV vaccines are discussed.

2. Successful flavivirus vaccines

2.1. YFV vaccine

YFV circulates in urban and sylvatic cycles in Africa and South America [5] (Fig. 1). In the urban cycle, YFV is maintained between humans and *Aedes* spp. mosquitoes (mainly *Aedes aegypti*), and in the sylvatic cycle between non-human primates and mosquitoes (*Aedes* spp. in Africa and *Haemagogus* spp. in South America). In Africa, an intermediate cycle also exists, where non-human primates or humans are the vertebrate host and *Aedes simpsoni* is the main vector mosquito [6].

Human YFV infections exhibit three clinical stages [6]. Following a 3–6-day incubation period, illness begins with fever, headache, myalgia and viremia characterized as the "infection period", although the majority of cases are thought to result in asymptomatic infections. Following recovery from a 3–5-day "infection period", a "remission period" can occur in some patients with liver and renal failure. Finally, an "intoxication period" begins with hemorrhagic fever and multi-organ failure, where patients also develop jaundice and thrombocytopenia. Approximately 15% of cases develop moderate/severe manifestations. The World Health Organization (WHO) estimates 200,000 cases with 30,000 deaths occur annually, mostly in Africa [7].

Following the isolation of YFV in 1927 [8], two live-attenuated YFV vaccines were developed in the 1930s. French neurotropic vaccine derived from Dakar strain was used for human immunizations; however, neurological adverse events such as encephalitis were observed with unacceptably high rates, especially in children, and its production was discontinued in 1982 [9]. The 17D vaccine was developed in 1937 and obtained by 176 passages of Asibi strain in chicken embryo tissue to remove its neurotropic properties [10] (Fig. 2). The YFV-17D vaccine is the most successful live-attenuated human vaccine and has been used to develop other flavivirus vaccines by chimerization (see below).

Currently, substrains of 17D, 17DD and 17D-204 are used for vaccine manufacturing (Fig. 2). To date, four vaccine products were prequalified by the WHO, ensuring the United Nations and related agencies can provide these vaccines to developing countries [11]. The 17DD vaccine is manufactured in Brazil by Bio-Manguinhos and the 17D-204 vaccine in France (Stamaril®, Sanofi Pasteur), Senegal (The Institut Pasteur in Dakar), and Russia (Chumakov Institute of Poliomyelitis and Viral Encephalitides). Two additional 17D-204 vaccines, YF-VAX® (Sanofi Pasteur, USA) and Yellow Fever Vaccine Live (Beijing Tiantan Biological Products, China) are for local use. Comparative studies revealed that currently available vaccines possess equivalent immunogenicity [12,13]. A $\log_{10}$ neutralization index $\geq$0.7 is often used in clinical trials as a surrogate marker for protection [14]. Alternatively, a neutralization titer of 1:10 was evaluated as an equivalent titer to the $\log_{10}$ neutralization index 0.7, and thus was used as a surrogate marker for protection [15]. Generally, these vaccines are highly immunogenic, and thus a single immunization exhibits >98% seroconversion [12,13], and neutralizing antibodies persist for $\geq$30 years [16]. Although a repeat vaccination is recommended every 10 years for personnel at risk, in May 2013 the WHO decided to stop recommending the booster vaccination administered 10 years after the initial vaccination.

Most YFV endemic countries in Africa have faced obstacles for nationwide routine immunizations and only Gambia has a successful YFV vaccine immunization program. Following an initial mass vaccination campaign conducted in 1978/79 ($\geq$95% coverage for $\geq$6-month-old), $\geq$80% coverage has been maintained [17]. No confirmed cases have been reported since, although neighboring countries have experienced several outbreaks [17].

The incidence rates of general adverse events such as fever, rash, and injection-site pain were 38.3/100,000 doses [18]. Severe adverse events defined as hypersensitivity and YF vaccine-associated neurologic disease (YEL-AND) have been observed. YEL-AND is characterized as several clinical syndromes including meningoencephalitis, Guillain-Barré syndrome, and acute disseminated encephalomyelitis (ADEM). In addition, a novel severe adverse event, YF vaccine-associated viscerotropic disease (YEL-AVD), was first reported in 2001 [19–21]. YEL-AVD patients manifest jaundice, multi-organ failure, and shock, similar to manifestations observed in severe natural YFV infection. Several analyses revealed the incidence rates of overall severe adverse events (death, life-threatening illness, hospitalization, prolongation of an existing hospitalization, permanent disability including YEL-AND and YEL-AVD) observed in travelers were in the range of 1.1–4.7 per 100,000 doses [18,22], although these might have been calculated using overlapping databases. The incidence rates of YEL-AND and YEL-AVD were estimated at 0.4–0.8 and 0.3–0.4 per 100,000 doses, respectively [18,23]. In contrast, analyses in YFV endemic countries (Africa and Brazil) revealed the general

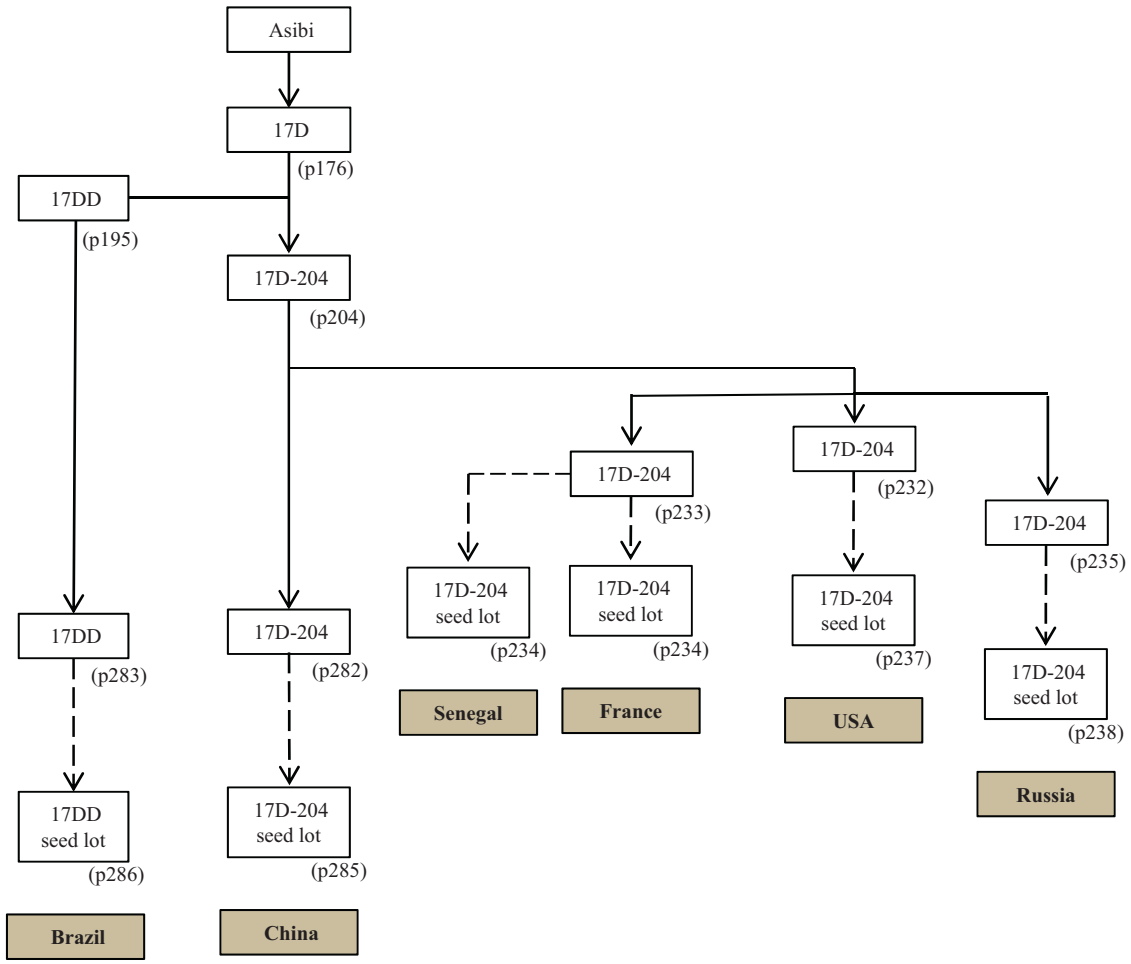

Fig. 2. History of YFV 17D and its related strains used for vaccines. Passage histories of YFV 17D, 17DD and 204 strains used in vaccine-manufacturing countries are shown. Arrows with a line indicate passages contaminated with avian leucosis viruses. Arrows with a broken line indicate passages without avian leucosis viruses.

adverse events rate was 8.2, and incidence rates of YEL-AND and YEL-AVD were 0.016–0.08 and 0.013–0.02 per 100,000 doses, respectively [22,24]. One possible explanation for lower incidence rates observed in YFV endemic countries is that pre-exposure to natural YFV in these regions could provide protection from adverse events [24].

In addition to the accumulation of the serious adverse event incidents, gelatin- and/or chicken egg-derived proteins are considered causative of adverse events. Therefore, as a potentially safer alternative, a Vero cell-derived inactivated vaccine was evaluated in a phase I clinical trial and 2-dose vaccination induced 100% seroconversion in 24 human subjects without severe adverse events [25]. Because a limited number of people were enrolled in this clinical study, the safety profile cannot be compared with live-attenuated vaccine.

2.2. JEV vaccine

In nature, JEV circulates between wild birds and vector mosquitoes. For human infections, swine act as an amplifier because they develop high viremia titers with long duration [26]; and *Culex tritaeniorhynchus* is considered a major vector mosquito [27]. JEV is distributed in East, Southeast and South Asia (Fig. 1), and was observed in Pakistan and Australia since 1983 and 1995, respectively [28]. Thus, the JEV distribution area has expanded westward and southward. Based on mosquito activities, two different seasonal prevalence patterns were identified. In the tropical region, human infections are observed throughout the year, although peak prevalence occurs after the start of the rainy season. In subtropical and temperate regions, JEV epidemics are observed from May/June to September/October because mosquito density increases during rice cultivation [29].

Most human JEV infections are asymptomatic. The symptomatic-to-asymptomatic infection rate is in the range of 1:25–1:1000 [29]. Following a 5–15-day incubation period, JEV symptoms include fever, headache, abdominal pain, and vomiting. In severe cases, neurological manifestations including meningitis, myelitis, and encephalitis develop after 2–4 days of febrile-like manifestations. Reported case fatality rates vary from 5 to 40%. Children and elderly populations are high-risk groups for severe cases. Psychoneurological sequelae are observed in approximately 20–50% of survivors [29]. According to the most recent estimation, approximately 67,900 cases occur annually, with up to 20,400 deaths [30].

JEV was isolated in 1935 and the first JEV vaccine, a mouse brain-derived inactivated vaccine using the Nakayama strain, was developed in Japan in 1954 (reviewed in [29]). Several purification processes have been modified to improve vaccine purity and/or immunogenicity, and the Beijing-1 strain that elicits immune responses against broader strains has been used in place of the Nakayama strain domestically since 1989. This technology was transferred to several countries and vaccines are now produced in South Korea, India, Taiwan, Thailand, and Vietnam (Table 1). This mouse brain-derived inactivated vaccine was the only internationally approved vaccine for a long time. A neutralizing antibody titer of 1:10 is the minimal recommended immunological correlate of

Table 1
List of currently licensed JEV vaccines.

Vaccine type	Manufacturer(s)	Strain	Status
Mouse brain-derived inactivated vaccine	Green Cross (South Korea), Central Research Institute (India), Adimmune corp (Taiwan), Government Pharmaceutical Organization (Thailand), Vabiotech (Vietnam)	Nakayama Beijing-1	Shifting toward cell culture-derived vaccines in many countries.
Vero cell-derived inactivated vaccine	Valneva SE (France), Biological E (India)	SA14-14-2	IXIARO® (Valneva) was approved in Europe, USA, Canada, Hong Kong, Singapore, and Israel, and JESPECT® in Australia and New Zealand for adult use. For pediatric use, IXIARO® was approved in USA, European Union, Norway, Liechtenstein, and Iceland. JEEV® (Biological E) was prequalified by the WHO for adult use in July 2013.
	Biken (Japan) Kaketsuken (Japan)	Beijing-1	JEBIKV® (Biken) and ENCEVAC® (Kaketsuken) were approved in Japan.
PHK cell-derived inactivated vaccine	Beijing, Shanghai, Wuhan and Changchun Institute of Biological Products (China)	Beijing-P3	Replaced by a live-attenuated vaccine.
Live-attenuated vaccine	Chengdu Institute of Biological Products (China)	SA14-14-2	CD. JEVAX™ was approved in China, Cambodia, North Korea, India, Laos, Myanmar, Nepal, South Korea, Sri Lanka and Thailand. CD. JEVAX™ was prequalified by the WHO for pediatric use in Oct 2013.
Live-attenuated chimeric vaccine	Sanofi Pasteur (France)	prM/E genes replaced YFV-17D genes.	IMOJEV® and THAIJEV® are approved in Australia and Thailand, respectively.

disease protection [31]. Case-control field trials revealed that the mouse brain-derived inactivated vaccine exhibited 81–95% efficacy [32,33]. Nationwide vaccine programs have been introduced in several Asian countries including Japan, South Korea, and Taiwan [34], and have helped to dramatically decrease the number of human cases since its introduction in the 1960s/70s [35–37]. However, Japanese manufacturers discontinued its production in 2005 following a decision by the Japanese government to suspend the strong recommendation of this vaccine for routine immunizations, because a 14-year-old girl developed severe ADEM (acute disseminated encephalomyelitis) following immunization and a next-generation JEV vaccine was expected to be approved soon.

Because of a potential problem in the use of mouse brains for a vaccine antigen source, efforts to develop cell culture-derived vaccines have been made. In 2009, two Vero cell-derived inactivated vaccines were licensed: IXIARO®, using the SA14-14-2 strain, formerly known as IC51, was launched by Intercell AG, Austria (now Valneva SE, France) and JEBIKV®, using the Beijing-1 strain, was launched by BIKEN, Japan (Table 1).

IXIARO® is composed of a formalin-inactivated Vero cell-grown SA14-14-2 strain, which has been used as a live attenuated vaccine as described below, with an aluminum hydroxide adjuvant. In clinical trials, a two-dose regimen of IXIARO® revealed a higher seroconversion rate (98%) than mouse brain-derived vaccine (95%) [38]. In addition, IXIARO® elicited higher geometric mean neutralizing antibody titers (GMT) at 1:244 than the mouse brain-derived vaccine (1:102). One year after initial immunization, 83% seroconversion rate was maintained [39]. Although a single-dose regimen is suitable for vaccination of travelers, the seroconversion rate induced by single immunization was only 65.8% and neutralizing antibody titers declined rapidly [40]. Thus, the 2-dose regimen of IXIARO® was licensed in 2009 as a traveler's vaccine and post-marketing analyses revealed an excellent safety profile [41]. Since the single-dose regimen did not show acceptable efficacy, a novel 2-dose regimen with an accelerated schedule that helps travelers complete the immunization schedule is currently under evaluation.

As of 2013, IXIARO® vaccine for adults (aged ≥18 years) was approved in Europe, USA, Canada, Hong Kong, Singapore, and Israel, and was approved under the JESPECT® trade-name in Australia and New Zealand. A clinical trial of children revealed ≥95% of seroconversion rates following a two-dose regimen [42]. Currently, IXIARO® is approved for pediatric use (≥2-month-old) in USA, European Union, Norway, Liechtenstein and Iceland. Furthermore, this technology was transferred to the Indian biopharmaceutical company, Biological E, as a traveler's vaccine and for routine vaccination in endemic countries. It is now available as JEEV® in India and was prequalified for adult use by the WHO in July 2013, as the first prequalified JEV vaccine worldwide.

In Japan, JEBIKV® was launched by BIKEN in 2009 [43]. A clinical trial of children (6–90-month-olds) exhibited 99.2% seroconversion rate (GMT of 1:263) and 100% seroconversion rate (GMT of 1:5834) following a second and third immunization, respectively [44]. In addition, another Vero cell-derived inactivated vaccine, ENCEVAC® produced by Kaketsuken, was approved in 2011 [45]. These two vaccines available in Japan contain the formalin inactivated Vero cell-grown Beijing-1 strain used in the mouse brain-derived vaccine, and do not contain an adjuvant unlike IXIARO®. Based on the safety profiles of these vaccines and their supply capacities, the Japanese government has restarted its strong recommendation for routine immunization since 2010. Although only a limited population (3-year-old infant) was recommended at that time, now all routine immunizations (3- and 9-year-olds) are recommended.

A live-attenuated vaccine using the SA14-14-2 strain has been available in China since 1988 [46] (Table 1). Since its approval, the live-attenuated vaccine has been extensively used, replacing the PHK cell-derived inactivated vaccine using the Beijing-3 strain approved since 1968. Several field evaluations demonstrated single immunization exhibited ≥95% efficacy [47]. Because it has a high efficacy combined with low cost compared with inactivated vaccines requiring multiple doses, this vaccine is now approved in Cambodia, North Korea, India, Laos, Myanmar, Nepal, South Korea, Sri Lanka, and Thailand under the CD.JEVAX™

trade-name (Chengdu Institute of Biological Products, China) [48]. Most recently, the CD.JEVAX™ was prequalified by the WHO for pediatric use in Oct 2013.

The YFV-17D vaccine was used to generate a chimeric live-attenuated JEV vaccine, ChimeriVAX™-JE or JE-CV (Sanofi Pasteur) containing the SA14-14-2 strain-derived prM/E genes in place of the corresponding YFV-17D genes [49]. Unlike the original YFV-17D vaccine, JE-CV was prepared in Vero cells. In clinical trials, ≥99% and 95% seroconversion rates were observed following single immunization in adults and children, respectively [49–52]. Since 2010, JE-CV has been approved in Australia and Thailand under the IMOJEV® and THAIJEV® trade-names, respectively (Table 1).

JEV comprises five distinct genotypes. Recently, genotype I has gradually become the dominant genotype in many countries, although genotype III was formerly dominant [53]. Because all current vaccine strains belong to genotype III, the change in genotype dominance questions whether currently available vaccines will be effective against other genotypes. Human sera vaccinated with genotype III antigens could neutralize all four genotypes (genotype V was not tested), indicating that the current vaccine is effective in areas where any JEV genotype is dominant [54].

2.3. TBEV vaccine

TBEV is divided into three distinct subtypes. The European subtype (TBEV-Eu) is distributed in Western, Central, Northern, and Eastern Europe. The Far Eastern subtype (TBEV-Fe) circulates in China, Japan, and eastern Russia. The Siberian subtype (TBEV-Si) is present in Siberia and some parts of Russia (Fig. 1). In the Baltic region and Finland, multiple subtypes circulate concurrently [55]. TBEV is maintained in nature between mammals, particularly rodents, and vector ticks [56]. Rodents act as maintenance, amplifying and reservoir hosts. For human infections, the viruses are transmitted by tick bites (adult and nymph) or intake of non-pasteurized dairy products. *Ixodes ricinus* is the main vector for TBEV-Eu and *Ixodes persulcatus* is the main vector for TBEV-Fe and TBEV-Si, although TBEV can be transmitted by 11 tick species. Once infected, the tick is infective for its lifespan. The peak prevalence of human infections is observed between May and June, the height of tick activity [55]. Another peak (between September and October) is only observed for *Ixodes ricinus*.

About 10–30% of human infections are symptomatic with an incubation period of 2–28 days (generally 7–14 days). Although infection with TBEV-Fe tends to exhibit a monophasic clinical course, typical TBEV infection is biphasic, particularly in severe cases [56]. In typical cases, disease begins with febrile-like symptoms including fever, vomiting, fatigue, and headache for 1–8 days, followed by an asymptomatic period in the range of 1–20 days. If the infection involves neurological organs, the second phase includes myelitis, meningitis, and encephalitis. For encephalitis, up to 40% of cases result in neurological sequelae. Fatality rates are reported to be ≥20%, 6–8%, and 1–2% in TBEV-Fe, TBEV-Si, and TBEV-Eu infections, respectively [57]. Approximately 6000–12,000 cases have been reported annually worldwide, which is probably underestimated [58,59]. Because of climate change, increased outdoor activities and improved surveillance systems, numbers of reported cases have increased in recent decades.

TBEV vaccines have been developed since TBEV isolation in 1937. The first TBEV vaccine, FSME-IMMUN® (Immuno AG, now Baxter AG, Austria), is a primary chicken embryo cell (PCEC)-derived inactivated vaccine using the Austrian Neudörfl strain of the TBEV-Eu serotype, and was approved in Austria in 1976 [55]. The mouse brain-derived virus was used as a seed virus for inoculation onto PCECs. To improve the vaccine, the seed virus was changed to the PCEC-derived form, so antigenic contents were limited to a narrow range. In addition, thiomersal and human serum albumin (HSA), a preservative and stabilizer, respectively, were removed from the vaccine formulation. This vaccine was re-licensed under the TicoVac® trade-name in 2000. However, adverse events such as fever were unexpectedly increased, particularly in infants and young children [60]. TicoVac® induced high levels of TNF-α and low levels of IL-1β, which were likely causally related to the high fever, but the addition of HSA to the vaccine formulation restored the cytokine levels to normal. Therefore, HSA was re-incorporated into the vaccine formulation and re-licensed under the FSME-IMMUN® (new) trade-name in 2001 [61] (Table 2). For pediatric use, FSME-IMMUN® (Junior) was launched in 2003. Although the surrogate maker for seroprotection has not been defined for TBEV infection, over 1:10 of neutralizing antibody titers is generally used [57]. In clinical trials, the new formulated vaccines exhibited ≥95% and 100% seroconversion rates against TBEV-Eu in all age groups following second and third immunizations, respectively, and seropositivity persisted for at least 5 years [62].

Encepur® (Chiron Behring, now Novartis, Switzerland), a PCEC-derived inactivated vaccine using the German K23 strain of TBEV-Eu subtype, was licensed in 1991 [55]. For pediatric use, Encepur® K was approved in 1994. Although these vaccines initially contained polygeline (gelatin) as a stabilizer, new formulations without polygeline, Encepur adults® and Encepur children®, have been available since 2001 [55] (Table 2). Like FSME-IMMUN®, Encepur exhibited high immunogenicity against TBEV-Eu. Seroconversion rates of 100% were observed in all age groups after third immunization [63,64] and long-term persistence of neutralizing antibodies was confirmed [65,66].

Two additional vaccines are only available in Russia and some neighboring post-Soviet Republics [55] (Table 2). TBE-Moscow® produced by the Chumakov Institute of Poliomyelitis and Viral Encephalitides, a PCEC-derived inactivated vaccine using the Sofjin

Table 2
List of currently licensed TBEV vaccines.

Vaccine	FSME-IMMUN® (new)	Encepur®	TBE-Moscow®	EnceVir®
Strain	Neudörfl	K23	Sofjin	205
Subtype	European	European	Far Eastern	Far Eastern
Licensed	2001	2001	1982	2001
Production	PCEC	PCEC	PCEC	PCEC
Adjuvant	$Al(OH)_3$	$Al(OH)_3$	$Al(OH)_3$	$Al(OH)_3$
Preservative	None	None	None	None
Stabilizer	HSA	Sucrose	HSA Sucrose BSA	HSA Sucrose
Availability	26 European counties and Canada	18 European countries	Russia, Ukraine, Belarus, and Kazakhstan	Russia, Ukraine, Belarus, and Kazakhstan

PCEC: primary chicken embryonic cell; HSA: human serum albumin; BSA: bovine serum albumin.

strain of TBEV-Fe subtype, was approved in 1982. EnceVir®, licensed in 2001, is also a PCEC-derived inactivated vaccine using the 205 strain of TBEV-Fe subtype, and is produced by Microgen. These vaccines contain HSA as a stabilizer. Both TBE-Moscow® and EnceVir® exhibited 100% and 88% seroconversion rates following a third immunization, respectively [67]. Two years after immunization, approximately 94% and 84% of participants immunized with TBE-Moscow® and EnceVir® remained seropositive, respectively [67].

In several European countries, nationwide or partial vaccination programs have been conducted. Among these, Austria has maintained high vaccination coverage (~85%) [68]. Because of the successful introduction of TBEV vaccine into the routine program, the incidence rate of TBEV infection in Austria has declined from 5.7/100,000 population (average from 1972–1981) to 0.9/100,000 (average of 2002–2011).

As described above, there are three distinct subtypes concurrently circulating in the Baltic region. Therefore, it is important to determine whether one subtype-based vaccine can induce protective immunity against other subtypes. The TBEV-Eu-based vaccine elicited protective immunity against TBEV-Fe and TBEV-Si, and the TBEV-Fe-based vaccine elicited protective immunity against TBEV-Eu and TBEV-Si in mice [69]. In addition, cross-neutralizing antibodies against TBEV-Fe and TBEV-Si were detected in humans immunized with FSME-IMMUN® containing TBEV-Eu antigens [70]. Thus, it is assumed cross-protective immunity against all three serotypes can be induced by any vaccine.

3. Flavivirus vaccines not yet available

3.1. DENV vaccine

The four serotypes of DENVs (DENV-1 to DENV-4) are taxonomically distinct species distributed throughout tropical and subtropical locations worldwide, where approximately 2.5 billion people are at risk of infection [71] (Fig. 1). The taxonomic rank of DENVs species is termed “serotype”. DENV is maintained in both sylvatic and urban cycles. In the urban environment, DENV circulates between humans and *Aedes* spp. mosquitoes (mainly *Aedes aegypti*), whereas DENV circulates between non-human primates and mosquitoes (mainly *Aedes luteocephalus*, *Aedes furcifer*, *Aedes niveus* spp.) in the sylvatic cycle. DENV in the sylvatic cycle can cross into humans, primarily by *Aedes furcifer* (in Africa) and *Aedes albopictus* (in Southeast Asia) [72].

Dengue fever (DF) and its severe form, dengue hemorrhagic fever (DHF)/dengue shock syndrome (DSS), can be caused by infection with any DENV serotype. Following a 2–7-day incubation period, DF patients develop self-limited febrile illness accompanied by headache and muscle and joint pain, and cases are normally not fatal. In contrast, following DF-like manifestations, DHF patients develop plasma leakage and hemorrhagic manifestations, which may lead to fatal shock [73]. An estimated 50–100 million infections including 250,000–500,000 DHF cases with more than 20,000 deaths occur annually [71]. These include autochthonous cases in endemic regions and imported cases of travelers in Europe and the USA. Thus, extremely large numbers of traveling people are at risk in addition to people living in DENV circulating regions. Among severe/fatal DHF cases, children are particularly affected with a mortality rate of ~2.5%. The most recent estimation using cartographic methods calculated 390 million DENV infections per year, three times higher than previously thought [74]. Thus, DENV infection is the most important arthropod-borne viral disease worldwide.

Detailed pathogenic mechanisms of DHF development are not fully understood. Both viral and host factors are thought to be involved. Viral factors, such as virulence of each DENV strain are considered responsible for DHF development and genetic background of infected individuals is considered a host factor. Several single-nucleotide polymorphisms have been implicated as determinants for DHF development. Furthermore, immunological responses are recognized as one of the major host factors. Contributions of cellular immunity to protection and/or DHF development have been well studied [75]. However, humoral immune responses have also been extensively characterized. Despite several proposed mechanisms (factors), it is generally accepted that DHF often results from high levels of viremia [76]. One of the most important hypothetical mechanisms to increase viremia levels is antibody-dependent enhancement of infection (ADE), which causes a significant increase in infected monocyte/macrophage numbers (DENV target cells in humans) in the presence of cross-reactive, sub- or non-neutralizing antibodies (enhancing antibodies) by an Fcγ-receptor-mediated mechanism [77].

Following infection with one serotype of DENV, individuals are protected from subsequent homotypic infections [78]. Cross-protection against heterotypic infections only occurs over a short period. Epidemiological studies indicate that primary infection with one DENV serotype usually causes DF, and the risk for deteriorating to DHF caused by a secondary heterotypic infection is 15–80 times higher than that of primary infection [79].

Thus, effective dengue vaccines should be tetravalent vaccines, composed of four monovalent vaccines corresponding to each DENV serotype. While effective and safe vaccines for monotypic flaviviruses such as YFV, JEV, and TBEV have been successfully developed (see above), a requirement of tetravalent formulation for DENV has made vaccine development complicated [80]. Regardless of many efforts over 70 years, an approved vaccine is not available. To date, various types of vaccines have been developed, and some are currently under preclinical or clinical evaluation.

The first dengue tetravalent vaccine evaluated in clinical trials was a classical live-attenuated form developed in Thailand (Mahidol University, licensed to Aventis Pasteur, currently Sanofi Pasteur) (Table 3). This vaccine was composed of strains obtained by sequential passages through primary dog kidney (PDK) cells or primary green monkey kidney cells [81]. Another classical live-attenuated vaccine was developed in the USA (Walter Reed Army Institute of Research: WRAIR) and licensed to GlaxoSmithKline (GSK) in the United Kingdom (Table 3). The GSK vaccine was composed of strains obtained by sequential passages through PDK cells and fetal rhesus lung (FRhL) cells [82]. When these vaccines were examined as monovalent vaccines, individual attenuated vaccines induced high seroconversion rates. However, when these vaccines were examined by tetravalent formulations, interference between four individual attenuated viruses was a big obstacle for their development. Although seroconversions against all four serotypes following the first dose were as low as 30% [81,82], additional doses improved them to 60–70% [81,83]. Imbalanced antibody induction resulting from viral interference is a problem because of vaccine effectiveness but also the potential risk for increased disease severity [84]. It is difficult to assess accurately the interference/interplay properties of individual vaccines and to find an appropriate formulation that may vary between individuals [85]. In addition to viral interference, dengue-like syndrome was observed in a large number of the participants [81,85]. Therefore, evaluation of the Sanofi vaccine was discontinued in a phase II clinical trial. Most recently, development and evaluation of the live-attenuated vaccine was resumed by Mahidol University collaborating independently with Chemo-Sero Therapeutic Research Institute (Kaketsuken, Japan) and Serum Institute of India (SII, India). Pre-clinical evaluations are planned to start in 2014. GSK vaccine strains were subjected to three additional passages on FRhL cells to improve purity, and the

Table 3
Tetravalent dengue vaccines in clinical trials.

Type	Developer	Strategy	Trial place	Clinical trial	Status
Classical attenuated vaccine	MU Sanofi Pasteur	Attenuated in cultured cells	Thailand	Phase II	On hold
	WRAIR GSK	Attenuated in cultured cells	USA	Phase II	On hold
Attenuated chimeric vaccine	Sanofi Pasteur	prM/E genes from each DENV replaced YFV-17D genes	Colombia, Mexico, Honduras, Puerto Rico, Brazil, Thailand, Indonesia, Malaysia, Viet Nam, Philippines	Phase III	On going
	CDC Inviragen Takeda	prM/E genes from each DENV replaced DENV2 genes	Puerto Rico, Colombia, Singapore, Thailand	Phase II	On going
	NIAID NIH	Attenuated by 3′-UTR deletion	Brazil, Thailand	Phase II	On going
Protein vaccine	Merck	Subunit vaccine including 80% of E proteins	Australia	Phase I	On going
	WRAIR GSK	Purified inactivated vaccine	USA, Puerto Rico	Phase I	On going
DNA vaccine	NMRC Vical	A naked DNA plasmid encoding the prM/E genes from each DENV	USA	Phase I	On going

MU: Mahidol University, Thailand; WRAIR: Walter Reed Army Institute of Research, USA; GSK: GlaxoSmithKline Biologicals; prM: premembrane; E: envelope; YFV-17D: Yellow Fever 17D vaccine strain; CDC: Centers for Disease Control and Prevention, USA; NIAID: National Institute of Allergy and Infectious Diseases, USA; NIH: National Institute of Health, USA; UTR: untranslated region; NMRC: Naval Medical Research Center, USA.

new version was evaluated in a phase II clinical trial [86]. Approximately 40% of naïve volunteers were seroconverted against all four serotypes following a first immunization and the seroconversion rates reached approximately 70% with additional doses. However, the GSK vaccine is also on hold, and has been the subject of development of a purified inactivated vaccine (see below).

Gene engineering technology has been incorporated to generate chimeric vaccines. The most advanced dengue vaccine being developed is Sanofi Pasteur's ChimeriVAX™ dengue tetravalent vaccine (CYD-TDV), which contains prM/E genes derived from each serotype of DENV in place of corresponding genes from the YFV-17D backbone [49] (Table 3). Preclinical, phase I and phase II trials demonstrated that CYD-TDV induced high levels of seroconversion in the range of 77–100% and robust neutralizing antibodies against all four serotypes following a third immunization in flavivirus naïve volunteers [49,87]. Although promising, a recently published field efficacy test from a phase IIb clinical trial conducted in Thailand exhibited an unexpectedly low efficacy to DENV-2 infection [88] (see below for details).

Another chimeric tetravalent vaccine, DENVax initially developed by CDC (In Viragen, USA and Takeda, Japan), was constructed using an attenuated DENV-2 strain (16681-PDK53) as the backbone [89] (Table 3). The prM/E genes of other DENV serotypes replace the corresponding DENV-2 parental attenuated-strain genes. Preclinical evaluations revealed this tetravalent vaccine is highly safe and immunogenic [89]. Recently, phase I clinical trials were completed and a phase II clinical trial is underway in Puerto Rico, Colombia, Singapore, and Thailand.

Another attenuated vaccine produced by nucleotide deletion at the 3′-untranslated region (UTR) was developed by the National Institute of Allergy and Infectious Disease (USA) and the National Institutes of Health Laboratory of Infectious Diseases (USA) (Table 3). DENV-1 and DENV-4 containing a 30-nucleotide deletion in the 3′-UTR (rDEN1Δ30 and rDEN4Δ30, respectively) were attenuated but retained immunogenicity in rhesus monkeys and humans [90]. The Δ30-deletion caused a partial attenuation of DENV-2 and almost no attenuation of DENV-3 when tested in rhesus monkeys; thus, a chimerization strategy was used to create new candidates. The prM/E genes from DENV-2 and DENV-3 replaced corresponding genes of the rDEN4Δ30 backbone (rDEN2/4Δ30 and rDEN3/4Δ30, respectively) [90]. Although the rDEN2/4Δ30 vaccine was well attenuated and exhibited safe and immunogenic properties in humans, over-attenuation was observed in rDEN3/4Δ30 [90]. To improve the DENV-3 vaccine candidate, an additional 31-nucleotide deletion was performed in rDEN3Δ30 (rDEN3Δ30/31), and exhibited tolerable attenuation, safety, and immunogenic profiles in rhesus monkeys [90]. These individual candidates were proven to elicit seroconversion without serious adverse events in humans [90,91]. To evaluate a tetravalent vaccine (Tetra-VAX-DV), various tetravalent formulations were examined in a phase I clinical trial. A formulation containing each of 3 $\log_{10}$ plaque-forming units (pfu) of rDEN1Δ30, rDEN2/4Δ30, rDEN3Δ30/31, and rDEN4Δ30 induced the most balanced antibody responses. Seroconversion rates of DENV-1, DENV-2, DENV-3, and DENV-4 were 100%, 50%, 85%, and 100%, respectively [92]. Based on this initial clinical trial, a further clinical evaluation using the same formulation is underway in Brazil and Thailand.

As a non-replicable protein vaccine, recombinant DEN-80Es (Hawaii Biotech, now part of Merck & Co, USA) containing full-length prM and 80% of E proteins derived from each of four serotypes of DENV is produced in Drosophila S2 cells [93] (Table 3). The advantage of the subunit protein vaccine as a multivalent vaccine is the minimization of interference among monovalent vaccines, which is often observed in live-attenuated vaccines. A tetravalent formulation examined in mice and monkeys revealed DEN-80Es elicited seroconversion against all four serotypes [93]. Thus, a phase I clinical trial using a monovalent formulation was recently completed [94].

In addition, a tetravalent purified inactivated vaccine (TDEN-PIV, WRAIR and GSK) has been developed [94] (Table 3). The individual viruses were prepared in Vero cells. TDEN-PIV, combined with an adjuvant system, was evaluated in a phase I clinical trial in the USA and Puerto Rico.

A naked DNA-based tetravalent vaccine comprising plasmids encoding prM/E genes of four DENV serotypes was developed by the US Naval Medical Research Center and Vical Inc, USA (Table 3). In the phase I clinical trial evaluating a monovalent

DENV-1 DNA vaccine, approximately 42% of volunteers receiving the higher dose elicited detectable but low titers of neutralizing antibodies [95]. Recently, a new tetravalent formulation with the lipid-based adjuvant Vaxfectin® increased the immunogenicity in non-human primates [96]. Thus, Vaxfectin®-adjuvanted tetravalent DNA vaccine has been further evaluated in a phase I clinical trial.

Unlike other flaviviruses, appropriate animal models that reproduce disease manifestations in humans have not been established. In addition, immunological correlates such as neutralization antibody titers of 1:10 or greater that are often used as surrogate markers in other flavivirus vaccine evaluations have not been defined for DENV infection. Therefore, human field trials are required to evaluate vaccine efficacy. The first efficacy evaluation was performed using CYD-TDV in a phase IIb clinical trial in Thailand [88]. Unfortunately, the overall protective efficacy (30.2%) was not statistically significant and individual protective efficacies were 55.6% to DENV-1, 9.2% to DENV-2, 75.3% to DENV-3, and 100% to DENV-4 following a third immunization. Despite the low efficacy against DENV-2, neutralizing antibodies to DENV-2 were detectable in most volunteers and excellent immunogenicity was shown by the CYD-TDV against all four serotypes [88,97]. These results suggest a neutralizing antibody is not always a surrogate marker for evaluating protection in humans.

Although there are several possible explanations for the low efficacy against DENV-2 observed in the CYD-TDV clinical trial [88,98–100], we provide a summary of the conclusion from the WHO advisory group of experts [101]. Essentially, this phase IIb clinical trial was not performed to evaluate serotype-specific vaccine efficacy, since the number of subjects enrolled for this study was too small. The advisory group thought that the clinical trial exhibited satisfactory safety profiles and the issue of vaccine efficacy was inconclusive. The follow-up studies should be informative regarding the evaluation of long-term safety and immunogenicity. In addition, multicenter phase III trials enrolling much larger number of participants are ongoing in several Asian and Latin American countries. Indeed, an initial report from the phase III trials revealed excellent safety and immunogenicity profiles as observed in the phase IIb trial in Thailand [102]. Further results obtained from these trials will provide some insights to help understand the relationship between immune responses elicited by the vaccine and protective efficacy.

In addition to the ongoing clinical trials, basic research is critical for developing safe and effective DENV vaccines. First, appropriate evaluation systems are required. Although neutralizing antibody is used to evaluate vaccines, Vero cells and LLC-MK2 cells commonly used for neutralization tests are not Fcγ-receptor-bearing cells. Use of Fcγ-receptor-bearing monocytes or monocyte-derived cell lines, such as U937, THP-1, or K562, or Fcγ-receptor-expressing CV-1 and BHK cells [103,104] may reflect *in vivo* states more properly. Second, epitope analyses will be required. Indeed, DNA vaccine candidates in which cross-reactive immunodominant epitopes relevant to the induction of enhancing antibodies were manipulated, successfully reduced ADE activities in vaccinated mice [105,106]. Third, establishment of animal models for evaluating protective capacity of vaccines could be helpful. Genetically modified and immunocompromised mouse models have been developed to reproduce human clinical symptoms including viremia, fever, and hemorrhage, although they still have limitations [107]. Non-human primates have also been used, but viremia levels were much lower than those observed in humans. Recently, common marmosets demonstrated high-level viremia comparable to that in humans, suggesting this model is promising [108]. In addition, a human challenge model was recently reported [109]. Despite arguments on ethical and safety matters, information obtained from this model is useful to reduce the size of phase III trials [110].

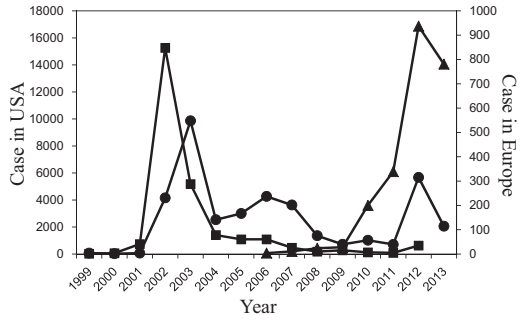

Fig. 3. Yearly changes of WNV disease cases in the USA and Europe. Human cases in the USA (●), equine cases in the USA (■) and human cases in Europe including neighboring countries (▲) are shown. Data of human cases in the USA were obtained from CDC (as of 29th October 2013). Data of equine cases in the USA were obtained from USDA animal health monitoring and surveillance. Data of human cases in Europe were obtained from ECDC (as of 24th October 2013). These data contained confirmed and probable cases.

3.2. WNV vaccine

Since the isolation of WNV in 1937 in Uganda, its distribution has expanded [111]. In 1999, WNV invaded the North American continent and spread rapidly. Today, WNV is widely distributed through Africa, Europe, Asia, Australia, North America, Central America, and South America [111] (Fig. 1). WNV is circulated/maintained by an enzootic cycle between wild birds and vector mosquitoes. Humans and horses are dead-end hosts. For human infections, many mosquito species, mainly *Culex* mosquitoes, can transmit WNV [112]. *Culex pipiens*, *Culex univittatus*, and *Culex antennatus* are considered important vectors in Europe and Africa. *Culex tritaeniorhynchus*, *Culex vishnui*, and *Culex pseudovishnui* are major vectors in Asia. In Australia, where WNV exists as Kunjin virus, *Culex annulirostris* is a major vector. *Culex pipiens*, *Culex restuans*, *Culex tarsalis*, and *Culex quinquefasciatus* are the principal vectors in the USA. Most recent phylogenetic analyses proposed that WNV is classified into seven distinct genetic lineages [113].

Approximately 80% of human WNV infections are asymptomatic. Following 2–14 days of incubation, most symptomatic cases exhibit mild febrile illness characterized as West Nile fever [114]. In mild non-neuroinvasive infections, the case fatality rate is estimated as 0.5% [115]. Only 1% of symptomatic infections cause neuroinvasive diseases including meningitis, encephalitis, and acute flaccid paralysis [114]. Based on surveillance in the USA since 1999, case fatality rates of <40-year-old, 40–59-year-old, and ≥60-year-old among neurological diseases are 0.8%, 3%, and 17%, respectively [115]. Although the overall case fatality rate is 4%, the actual case fatality rate is considered lower than 4% because of many unreported cases [115].

Until the mid-1990s, human WNV cases were sporadic with mild manifestations. Since the mid-1990s, epidemiological trends have changed; incidents with severe neurological outcomes accompanied by large human outbreaks have increased. Finally, WNV emerged in the USA in 1999 and rapidly spread across the Western Hemisphere [111]. Drastic increases of human cases have been observed in Europe and neighboring countries since 2010 and the USA experienced large human and horse outbreaks in 2012 (Fig. 3). Regardless of the many efforts to develop WNV vaccines, no approved vaccine for human use is available. To date, several veterinary vaccines have been licensed.

The first veterinary WNV vaccine, WEST NILE-INNOVATOR®, an inactivated vaccine developed by Fort Dodge Animal Health (now distributed by Zoetis, USA), was approved in 2001 [116]. In 2004, Recombitek® Equine WNV, a chimeric live canarypox virus expressing prM/E proteins of WNV, was launched by Merial

(now part of Sanofi) [116]. In 2005, the WEST NILE-INNOVATOR DNA®, a naked plasmid DNA encoding prM/E genes of WNV, was approved. However, this product is no longer commercially available, since production of this vaccine was recently discontinued [117]. The chimeric live attenuated vaccine PreveNile™ (Intervet), encoding WNV prM/E genes in place of the corresponding YFV-17D genes, has been used since 2006. Because of increased numbers of unexpected adverse events such as acute anaphylaxis, colics and respiratory distress, PreveNile™ was recalled in 2010 [117]. The following year, an inactivated chimeric YFV-17D vaccine encoding WNV prM/E genes, Equi-Nile™, was launched by Merck Animal Health (USA). Another inactivated vaccine, Vetera™ WNV vaccine (Boehringer Ingelheim Vetmedica, USA), containing the E159™ strain isolated from a horse in 2005, has been available since 2009.

Although there is currently no licensed vaccine for human use, several clinical trials have been conducted. ChimeriVAX-WN02 (Sanofi Pasteur), a chimeric YFV-17D vaccine encoding WNV prM/E genes in place of the corresponding 17D genes, is a promising candidate [49]. In a phase II clinical trial, ≥96% seroconversion rates were observed in all age groups examined [118]. Although an immunological correlate for seroprotection has not been established for WNV infection, 1:10 of neutralizing antibody titers was used as a surrogate marker. Another phase II clinical trial enrolling only ≥50-year-old observed ≥92% seroconversion rates in all tested doses [119]. As for other vaccine candidates, a phase I clinical trial of WNV-DENV chimeric vaccine (WN/DEN4Delta30) was completed. A single dose as low as 10^3 pfu of WN/DEN4Delta30 elicited 74% seroconversion [120]. A naked DNA vaccine encoding WNV prM/E genes was evaluated in a phase I clinical trial [121]. A three-dose-regimen using a needle-free injector demonstrated 96.5% seroconversion. As a subunit vaccine, WN-80E containing full-length prM and 80% E proteins (Hawaii Biotech, now part of Merck & Co) elicited protective immunity in monkeys [122]. A phase I clinical trial of WN-80E has been completed.

As described above, several vaccine candidates have exhibited good immunogenic properties in humans. One of the major reasons for the lack of available vaccines is cost effectiveness. An evaluation of cost effectiveness conducted in 2006 concluded that introduction of WNV vaccine into the routine immunization program would not be beneficial because of relatively low incidence rates [123] and that WNV activity is not consistent. In addition, the evaluation of vaccine efficacy by field trial is considered difficult because of the low incident rates in humans and a lack of defined immunological correlates for protection. In the absence of available vaccines, the number of human cases has unexpectedly increased and decreased, probably owing to local climate and mosquito activity. Therefore, it is difficult for pharmaceutical companies to determine the importance of human vaccines. However, the distribution of WNV is expanding, resulting in increased populations at risk. In addition, human cases have increased in recent years both in Europe and USA (Fig. 3). Therefore, the importance and/or requirement of developing a WNV vaccine has increased. Thus, re-evaluation of its cost effectiveness should be considered.

4. Conclusion

This article provided an overview of currently available flavivirus vaccines against YFV, JEV, and TBEV infections. Importantly, all these vaccines are neutralizing antibody-inducible vaccines. For these infections, a neutralizing antibody titer of 1:10 is considered an immunological correlate for protection. In addition, neutralizing antibody is considered important for protection from WNV infection, although an immunological correlate has not been defined. Thus, neutralizing antibody titers are used as a surrogate marker in vaccine evaluation.

Immunological correlates have not been defined also for DENV infections. Because the viremia level correlates with disease severity, neutralizing antibodies that reduce viremia can contribute to prevention from disease and/or death. In addition, neutralizing antibody-inducible vaccines have other benefits. All flaviviruses described here are hematophagous arthropod (mosquito and tick)-borne viruses. Thus, lower viremia titers because of neutralization by vaccine-induced antibodies can reduce viral transmission to arthropod vectors, indicating neutralizing antibody-inducible flavivirus vaccines can provide social protection. This is an important benefit, particularly for YFV and DENV, because the human-mosquito-human transmission cycle maintains the viruses.

The epidemiological facts described above clearly indicate that flaviviral diseases are vaccine-preventable diseases and can be controlled by successful nationwide vaccine programs. Thus, it is worth developing new DENV and WNV vaccines. Regarding DENV vaccine development, concerns for vaccine-related DHF and a lack of appropriate animal models are major obstacles. In recent decades, much evidence relating to DENV pathogenicity and mechanisms of DHF development has accumulated, and this evidence should be incorporated into vaccine development. Similarly, several animal models have been proposed. Although no animal models currently reproduce human manifestations completely, each animal model is useful in some aspects. By using a suitable model or some in combination, more appropriate vaccine evaluations can be conducted. Alternatively, a human challenge model to evaluate DENV vaccines is being considered. Regarding WNV vaccine development, cost effectiveness, which is considered a major obstacle, is expected to be higher than estimated because of the recent increase in human cases. In addition, difficulty in conducting field case-control studies is another obstacle to human vaccine development. Indeed, many candidate vaccines have been under evaluation in clinical trials. Because several veterinary WNV vaccines have been available for decades, it is expected that safe and effective human vaccines will be available once the need for a human WNV vaccine is recognized.

Even though several flavivirus vaccines are available, the coverage rates are insufficient in many countries at risk. Safer, more effective and inexpensive vaccines are required for these countries to improve their coverage. Therefore, new technologies including vaccine types, adjuvants, and immunization devices need to be incorporated into the development of next generation flavivirus vaccines.

Conflict of interest

The authors have no conflict of interest with this paper.

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