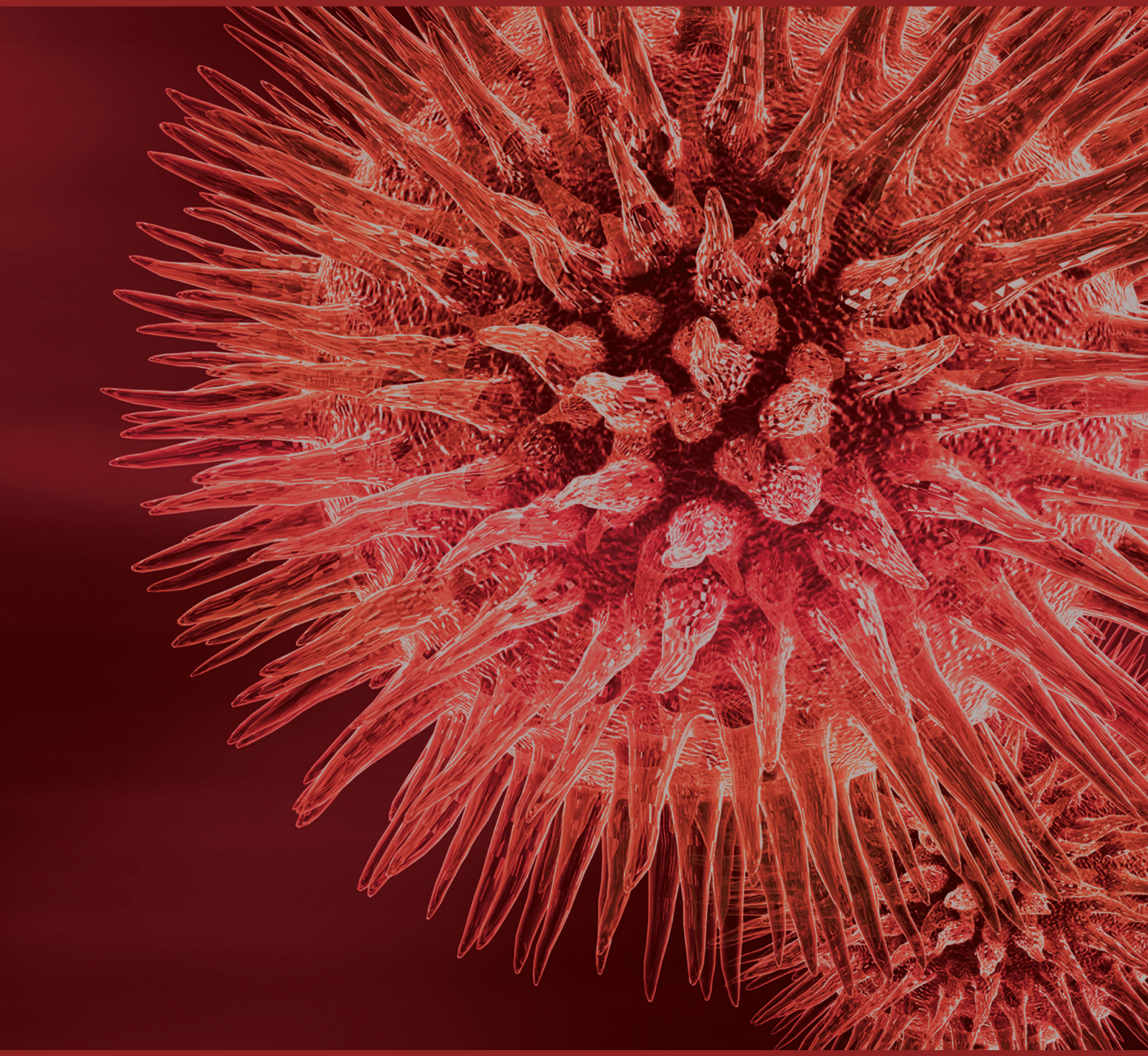
BioMed Research International

Vector-Borne Viral Diseases

Guest Editors: Penghua Wang, Fengwei Bai, Gong Cheng, Jianfeng Dai, and Michael J. Conway



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Vector-Borne Viral Diseases

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In this special issue, we have solicited review articles on West Nile virus epidemiology and research articles on dengue diseases.

N. J. Samanta et al. report the occurrence of West Nile virus antibodies in wild birds, horses, and humans serum samples collected between 2010 and 2014 from different areas of Poland. By using multiple ELISA screening, they found West Nile virus positive serum in wild birds, patients, and horses. They suggest that West Nile virus is already present in their climate zone and in their ecosystem.

By using a meta-analysis, H. Zhang et al. study the association between 11 clinical symptoms and the outcome of dengue diseases. They found five symptoms demonstrating an increased risk for severe dengue diseases (SDD), including bleeding, vomiting/nausea, abdominal pain, skin rashes, and hepatomegaly. They concluded that bleeding (hematemesis/melena), vomiting/nausea, abdominal pain, skin rashes, and hepatomegaly may predict the development of SDD in patients with DF.

J. Cime-Castillo et al. report the role of mosquito sialic acid in the life cycle of dengue virus. They identified a putative enzyme involved in sialic acid synthesis and evaluated its function in vitro. Sialic acid interactions were found to be important for dengue virus binding and perhaps transmission.

C. Napoli et al. describe the West Nile virus surveillance system of Italy, which integrates data from human and animal infections.

C. Chancey et al. review the global epidemiology of West Nile virus.

D. Di Sabatino et al. review the main epidemiological findings on WNV occurrence in Europe and the Mediterranean Basin from 2009 to 2013 and discuss potential future spread patterns.

Penghua Wang
Fengwei Bai
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Jianfeng Dai
Michael J. Conway

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Research Article

Vector Borne Infections in Italy: Results of the Integrated Surveillance System for West Nile Disease in 2013

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The epidemiology of West Nile disease (WND) is influenced by multiple ecological factors and, therefore, integrated surveillance systems are needed for early detecting the infection and activating consequent control actions. As different animal species have different importance in the maintenance and in the spread of the infection, a multispecies surveillance approach is required. An integrated and comprehensive surveillance system is in place in Italy aiming at early detecting the virus introduction, monitoring the possible infection spread, and implementing preventive measures for human health. This paper describes the integrated surveillance system for WND in Italy, which incorporates data from veterinary and human side in order to evaluate the burden of infection in animals and humans and provide the public health authorities at regional and national levels with the information needed for a fine tune response.

1. Introduction

The epidemiology of arboviral zoonoses is influenced by multiple ecological factors and, therefore, integrated and comprehensive surveillance systems are needed for early detecting the infection and activating consequent control actions. West Nile virus (WNV) is a *Flavivirus* belonging to the Japanese encephalitis antigenic complex of the family Flaviviridae. The genus *Flavivirus* also includes other arboviruses, such as St Louis encephalitis virus, Japanese encephalitis virus, Murray Valley virus, Usutu virus, and Kunjin virus. WNV is maintained in nature by birds and is transmitted primarily by the bite of infected mosquitoes acquiring the virus by feeding on infected birds [1]. Mosquitoes can also acquire the virus by transovarial transmission or by mating [2]. Migratory birds are strongly suspected to play a significant role in the introduction of WNV from endemic areas into new regions [3]. Humans, horses and other mammals are considered incidental dead-end hosts [1].

West Nile disease (WND) is a zoonosis. The infection in humans mainly occurs asymptomatically or with mild febrile illness [4]. Less than 1% of patients show severe neurological symptoms classifiable in three main syndromes: meningitis, encephalitis, and poliomyelitis (acute flaccid paralysis) [5]. In horses the disease is usually subclinical, although sometimes they may show neurological symptoms [1].

In the Western Hemisphere WNV was first detected in New York City in 1999 [6]. After that episode, the virus spread dramatically westward across the United States of America, southward into Central America and the Caribbean, and

northward into Canada, resulting in the largest human epidemic of a neuroinvasive disease ever reported [7].

The first large human outbreak of WND in Europe was recorded in 1996 in Romania with 393 confirmed cases [8]. After that episode, the number of WND reported cases in horses and humans increased significantly. This apparent rise of reported cases is partly due to the improvement of both surveillance systems and diagnostic methods [3, 9, 10], and also to the introduction and rapid spread of WNV lineage 2 in Europe [11, 12]. In the last recent years, virus circulation was observed in different Mediterranean countries with an increasing number of human cases in the Eastern Europe [13]. Some countries (i.e., Greece, Spain, Russia, Israel, Hungary, and Romania) were affected by the virus circulation for several consecutive years, supporting the hypothesis of a possible local endemisation of the infection.

As various animal host species take part, with different epidemiological roles, to the transmission of WNV, a multispecies surveillance approach is required. Some European countries focused their surveillance program only on human population (i.e., Albania, Kosovo, Montenegro) [14], whereas others integrated these activities with general and/or targeted surveillance in equines (such as Croatia, Spain, Greece, Portugal, France, Romania, Cyprus, and Morocco) [14–16]. Moreover, in some European nations (i.e., in Italy, Greece, United Kingdom, Spain, Germany, Hungary, and Serbia) the surveillance on mosquito populations is added to that addressing humans and equines, with the aim of detecting the WNV early during the season [11, 12, 17]. In this regard, the highest percentage of mosquito pools tested positive for WNV was reached in Serbia in 2013 (5.5%) [12].

In Italy, the first outbreak of WND was identified in horses in Tuscany region during the late summer of 1998. Fourteen animals showed neurological disorders [18], but no cases of human encephalitis were reported [9]. Following this epidemic, a national veterinary surveillance plan was put in place in 2001 to identify the geographical areas at risk for reintroduction of the WNV infection. This surveillance plan was coordinated by the National Reference Centre for the study of Exotic Animal Diseases (CESME) and carried out in 15 Italian wetlands. It was based on entomological monitoring and periodical serological testing of sentinel chickens and equines [19, 20]. The surveillance system did not detect any relevant circulation of WNV in animals until 2008, when the virus was detected in mosquitoes, birds, equines, and humans in the area surrounding the Po river delta, involving eight provinces in three northern regions: Emilia Romagna, Veneto, and Lombardy [20–22].

Based on what happened during 2008, the national WNV veterinary surveillance system was revised and new activities were added, aiming at identifying as early as possible the virus circulation all over the country and implementing measures for the prevention and control of human infections [20].

In light of the increasing number of confirmed cases in animals and the circulation of the virus in a wider geographical area, a human surveillance plan for the West Nile neuroinvasive disease (WNND) was put in place in 2008 in Emilia Romagna and Veneto regions, which allowed to detect the first eight indigenous cases of WNND in humans [9].

In 2009, the results of the veterinary surveillance system showed the resurgence of infection mostly in the same geographical areas of previous year, but new foci were reported in central Italy, in Tuscany and Latium, rather far from the areas infected in 2008 [22]. Cases of human WNND increased to 18 in 2009 (nine cases in Emilia Romagna, seven in Veneto, and two in Lombardy regions), occurring in the same geographical areas where WNV circulation was detected in mosquitoes and animals (chickens and equine) [9].

In August 2010, new foci of infection were observed in Sicily and Molise regions, respectively, in southern and central Italy. These outbreaks confirmed the WNV ability of spreading to new areas, affecting new host populations [20, 23]. In 2011, WND outbreaks were confirmed in six regions: Sardinia, Sicily, Friuli Venetia Giulia, Veneto, Basilicata, and Calabria, where clinical cases in horses and neurological signs in birds were observed.

Between 2010 and 2011 seventeen new human cases of WNND were reported from three regions (Veneto, Friuli Venetia Giulia, and Sardinia) with a 23.5% case-fatality rate [9].

Following the geographical spread of WNV westward, the Directorate General for Prevention of the Italian Ministry of Health (MoH) issued on the spring of 2010, a national plan for WNND human surveillance that integrated human and veterinary surveillance [24]. Since then, the national surveillance plan on imported and autochthonous human vector-borne disease (chikungunya, dengue and West Nile disease) was issued and revised annually [25–27].

In 2012 WNV continued to circulate in Italy causing infection in humans, horses, birds, and vectors in Sardinia, Friuli Venetia Giulia, Veneto, and Latium regions [10, 28, 29].

The main objective of this paper is to describe the components of the integrated surveillance system for WNV established in Italy with the aim of evaluating the burden of the disease in animals and humans and providing the Public Health local and national authorities with the needed information to fine tune response. To this aim, the existing data exchange flows between veterinary and human systems and the results of 2013 surveillance are presented.

2. Materials and Methods

2.1. WNV Circulation Surveillance Plan in Animals. The Italian national veterinary authority annually revises the WNV veterinary surveillance plan in line with the observed epidemiological changes. The surveillance plan for 2013 delimits three different geographical areas (Figure 1):

(i) with virus circulation (AVC): the geographic areas affected by the circulation of WNV in the previous years (since 2008),

(ii) surveillance zones (SZ): the territories surrounding the AVC with an extension of 20 km,

(iii) at risk (AR): wetlands characterized by the presence of a significant number of migratory water fowls.

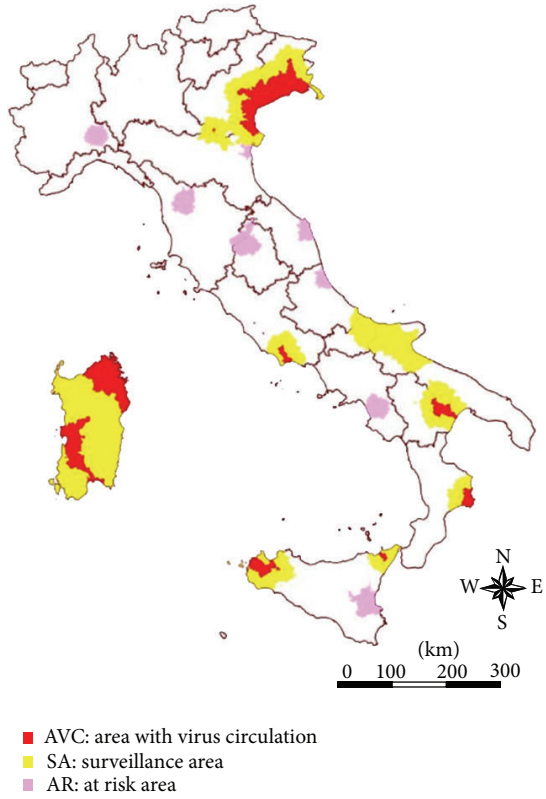

Figure 1: WND surveillance areas in 2013.

Target species of the veterinary surveillance activities include migratory and resident birds, horses, and poultry. The entomological surveillance is based on a certain number of mosquito collection sites placed in the three above mentioned areas for identifying possible WNV vector species and determining their abundance and spatiotemporal distributions [30].

Active bird surveillance is focussed on the following species: Magpie (*Pica pica*), Hooded Crow (*Corvus corone cornix*), and Jay (*Garrulus glandarius*), which are sampled and virologically tested in AVC. Serological testing of sentinel chickens and backyard poultry is foreseen as a possible alternative in case the planned activities on resident birds could be not carried out. Passive surveillance on birds mortality is carried out throughout the country, and target species include Blackbird (*Turdus merula*), Starling (*Sturnus vulgaris*), Jackdaw (*Corvus monedula*), Magpie (*Pica pica*), Jay (*Garrulus glandarius*), Hooded Crow (*Corvus corone cornix*), and Collared dove (*Streptopelia decaocto*). In addition, any episode of abnormal or increased mortality in other wild birds must be reported to veterinary authorities.

Entomological surveillance aims at identifying the mosquito fauna, defining the composition of vector populations and the species responsible for WNV transmission in the enzootic and epizootic cycles of the disease, investigating their ability to overwinter.

Moreover, countrywide passive surveillance on neurological cases observed in equines is coupled with the serological survey performed in sentinel horses three times per year (in May, August, and September) in AR. In addition, when the viral circulation is detected in zones not previously affected by the infection, further activities are put in place to better identify the extent of the infection.

2.2. Veterinary WND Surveillance Information System. In 2008, the Department of Veterinary Public Health Nutrition and Food safety (VPH Department) of the MoH appointed the CESME to develop an information system collecting data on animal disease outbreaks, using standard procedures and templates for data input and output [31]. The new information system, called SIMAN, was developed to provide a tool for the management of epidemic emergencies, to collect and communicate outbreak data to the MoH, the European Commission and the World Organization of Animal Health (OIE) in compliance with current national and international legislation [32, 33]. SIMAN was firstly used by the veterinary services during the large WND epidemic occurred in 2008. The data reported to SIMAN allowed the veterinary services to have the full picture of outbreaks distribution and to plan further investigations [34].

Given the complex epidemiology of the disease and the multidisciplinary approach needed for its surveillance, an integrated and comprehensive system for the management of WND outbreaks and surveillance activities was established.

In particular, new tools were developed in SIMAN for

(i) the registration of sentinel chickens and equines into the National Database of livestock and holdings (BDN);

(ii) recording and managing the laboratory results;

(iii) publishing weekly and daily reports describing the outcomes of the surveillance activities.

A web-based geographic information system (WebGIS) was also developed for displaying thematic maps and to help the veterinary services to explore the area surrounding the outbreak, to create buffers around the reported cases, and to download the list of equine farms placed within the buffers. An automatic procedure extracting every night all data inconsistencies and errors assured the necessary quality checks. In case of errors, an automatic alert email is sent to the veterinary services asking for data verification and correction.

2.3. WNND Surveillance Plan in Humans. The national plan for human surveillance defines as "affected areas" all the provinces (secondary administrative units) where laboratory-confirmed WNV infections in animals, vectors, or humans have been notified in the previous year or during the current surveillance period (between 15 June and 30 November, as considered the period with the highest vector activity). The identification of an affected area immediately triggers the "surveillance area" that represents the regional territory of the affected area [27].

In the affected area, local health authorities have to implement an active surveillance system for WNND in

workers employed in the farms where equine cases have been identified and in individuals living or working in the surrounding area (province). Moreover, the measures for vector control have to be implemented immediately. At the same time, passive surveillance on human neurological cases has to be set up in the surveillance area, requesting physicians to report all probable and confirmed WNND cases using a modified European case definition [35]: a patient with fever ≥38.5°C and neurological symptoms (encephalitis, meningitis or Guillain-Barré syndrome or acute flaccid paralysis) and at least one of the following laboratory criteria:

(i) for probable case: anti-WNV specific antibody response in blood; Polimerase Chain Reaction (PCR) positive in urine;

(ii) for confirmed case: viral isolation in blood or cerebrospinal fluid (CSF); anti-WN IgM positive in CSF; PCR positive in blood or CSF; confirmed presence of anti-WN antibodies in blood by neutralization test.

The list of regional reference laboratories is also provided in the WNND national surveillance plan. When a probable case is reported, the regional reference laboratory has to proceed for confirmation using one of the above reported laboratory methods. In case the neutralisation test is not available at the regional laboratory, patient's sera are sent to the National Reference Laboratory at Istituto Superiore di Sanità (ISS) for further confirmatory tests.

When WNND human cases are confirmed, immediate WNV nucleic acid amplification test (NAAT) screening of all blood and haematopoietic stem cells donations must be ordered in the affected areas. Additional screening of solid organ donations in surveillance areas (regions) are also introduced [36]. At the national level, all blood, tissue and solid organ donors who travelled to an affected area have to be temporarily deferred for 28 days starting with the day they left the affected area [37].

2.4. Case Reporting System of Human WNND. All human cases are notified by regional authorities to the MoH and to the Italian National Centre for Epidemiology, Surveillance and Health Promotion (CNESPS-ISS) using a specific password-protected web-based system (http://www.simi.iss.it/inserimento_dati.htm), which permits to report probable and confirmed cases, adding available epidemiological, clinical and laboratory information. The database is accessible also for the National Blood Centre and to the National Transplant Network, which implements precautionary measures on blood donation and transplant activities on the basis of data on WNND human cases.

In addition to the activities foreseen by the national WNND surveillance, an enhanced regional surveillance for WNV fever (WNF) was established in Veneto, Emilia Romagna, and Lombardy regions. The case definition for WNF was the following: a person showing fever ≥38.5°C (or history of fever in the last 24 h) for a period no longer than seven days, from 15 July to 30 November, with no recent history of travels to tropical countries and absence of other comorbidities accounting for the febrile illness.

All WNF confirmed cases are notified by the regional authorities to the MoH and to the CNESPS-ISS through the web-based system.

2.5. The Surveillance Systems Integration. During the vector activity period, a data exchange protocol is in place between SIMAN and the CNESPS-ISS to jointly define and update the map of the affected areas (provinces). The identification of new ACV and AE following the veterinary activities immediately triggers the establishment of the "affected areas" and the "surveillance areas" as foreseen by the human WNND surveillance plan. When an outbreak is confirmed in SIMAN or a laboratory result confirms WNV circulation in a given territory, measures for the prevention and control of the infection in humans are immediately applied in the affected areas. Veterinary and human surveillances are, therefore, linked each other and work as a chain reaction.

Together with the animal and entomological monitoring, the surveillance of WNND human cases allows to detect the virus circulation in a given geographical area and to obtain an estimation of its magnitude through the systematic detection of emerging clinical cases.

The data flow in the web-based integrated surveillance system is shown in Figure 2.

3. Results

3.1. Animal and Entomological Surveillance. In 2013, the CESME confirmed 50 new cases of WND in equines, 12 of which characterized by clinical signs, in Veneto, Lombardy, Emilia Romagna, Calabria, Sardinia, and Sicily regions; 146 mosquito pools and 79 birds in Veneto, Lombardy, Emilia Romagna, and Sardinia regions tested positive for WNV PCR. The affected areas identified by the veterinary monitoring activities were published on line (http://sorveglianza.izs.it/emergenze/west_nile/emergenze_en.html).

3.2. WNND Human Surveillance. From 15 June to 30 November 2013, 44 autochthonous human cases of WNND were confirmed. The majority of patients were male (61.3%) with a median age of 73 years (range: 42–89 years). The onset of cases ranged between 21 July and 21 September: 75% had the symptoms onset in August, which represented the peak month in 2013 (Figure 3). None of the cases travelled abroad during the incubation period.

The distribution of WNND confirmed cases by age and region/province of exposure is showed in Table 1: the majority of WNND cases in 2013 were reported from Emilia Romagna (20 cases) followed by Veneto (13 cases), Lombardy (10 cases), and Apulia (1 case).

The majority of cases reported symptoms of encephalitis (70.5%), followed by meningitis (38.6%), polyradiculoneuritis (9.1%), and other neurological symptoms (18.2%). None of the patients had history of vaccination against other arboviruses. Seven cases died, corresponding to a 16.3% case fatality rate.

From 15 June to 30 November, 34 confirmed cases of WNF were also reported to the MoH and CNSPS-ISS by

Figure 2: The web-based integrated surveillance system.

Table 1: WNND confirmed cases by region/province of exposure and age group, Italy 2013.

Region/province	Age					Total
	≤14	15–44	45–64	65–74	≥75	
Lombardy						
Cremona					1	1
Mantua			2	2	2	6
Lodi			1			1
Brescia					2	2
Apulia						
Foggia				1		1
Veneto						
Rovigo				1	4	5
Treviso			3		1	4
Venice					2	2
Padua					1	1
Verona					1	1
Emilia-Romagna						
Bologna					1	1
Ferrara		1	1	1	2	5
Modena			3	1	3	7
Parma					1	1
Reggio Emilia				1	5	6
Total	0	1	10	7	26	44

the public health authorities of Emilia Romagna (20 cases), Veneto (13 cases), and Lombardy (1 case) regions.

During the surveillance period the CNESPS-ISS published a weekly bulletin available in electronic format on the website of the ISS (http://www.epicentro.iss.it).

3.3. Outcomes from the Integrated Surveillance System. Data collected by both the veterinary and human surveillance systems in the previous year (2012) allowed to identify 9 regions (surveillance areas) in which the WNV human surveillance had to be performed in 2013 (Basilicata, Latium, Friuli Venetia Giulia, Sardinia, Veneto, Emilia Romagna, Lombardy, Calabria, and Sicily). Moreover, considering the geographical characteristics of Basilicata, also its neighbouring region (Apulia), although not directly affected, was included in the surveillance areas as well. Figure 3 shows the ten Italian Regions under surveillance.

During 2013 the veterinary surveillance activities confirmed the circulation of WNV in six regions (Sardinia, Veneto, Emilia Romagna, Lombardy, Calabria, and Sicily), of which three reported human cases (Veneto, Emilia Romagna, and Lombardy). One human case was reported also in Apulia, although no animals and vectors tested positive for WNV.

4. Discussion

The existence of surveillance systems able to represent an early warning tool is pivotal for preventing the spread of infectious diseases. The rapidity of the application of appropriate control measures after the detection of an emerging infectious/disease is crucial for the success of any intervention.

Figure 3: Regions under surveillance for human WNND, Italy 2013.

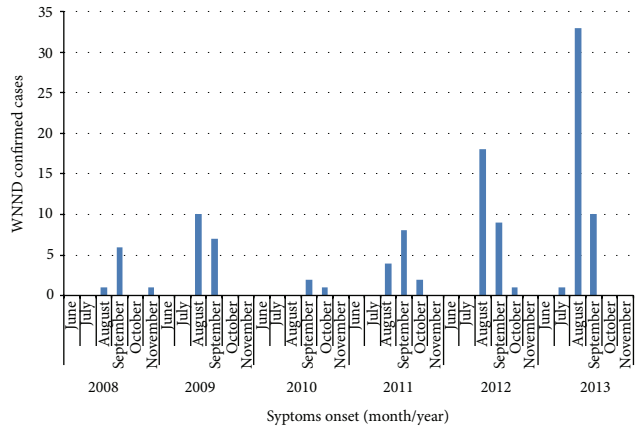

Figure 4: WNND confirmed cases by date of symptoms onset, Italy 2008–2013.

Between the first occurrence of WND in Italy in 1998 and its reemergence in 2008, WND was considered an exotic disease for the Italian territory and the main objective of the surveillance activities in place at that time was to evaluate the possible reintroduction of the virus. In this context, the data collected by the national system for the notification of animal diseases (SIMAN) were useful for a rapid epidemiological evaluation, to define areas at risk for human transmission and to facilitate the implementation of effective and prompt control measures.

In Italy the first WNND human cases were detected in 2008, when a human surveillance system was implemented in the areas where the WNV circulation was demonstrated among animals and vectors. Since then, human cases of WNND have been reported every year in Italy, with a pick of incidence in 2013 (44 confirmed cases). As shown in Figure 4, the number of reported WNND human cases has been recently increased, due to the greater attention to the disease by the national authorities, and thanks to a better integration of human and veterinary surveillance.

In fact, an early warning system for WNV detection, based on animal and entomological surveillance, can provide the basis for targeted public health interventions and risk communication activities, aiming at reducing the risk of human infection. Since the first occurrence of the virus the multispecies surveillance plan in place in Italy was capable of confirming the WNV ability of spreading to new geographical areas and infecting different host populations. Veterinary surveillance activities, therefore, were particularly useful to assess and monitor the evolution of the epidemiological situation, providing the public health authorities with precious and timely information on where and when WND prevention and control actions in humans had to be put in place. The existence of an effective double way communication system between veterinarian and human health authorities ensured a prompt implementation of the preventive measures and a more accurate assessment of the epidemiological situation of the disease as well as a more precise estimation of the extension of the infection.

Well-conducted veterinary surveillance program allowed to identify the territories at major risk for WNV circulation and to set up human surveillance activities in these geographical areas. It is noteworthy that some regions, where WNV circulation was demonstrated in animal and vectors, did not reported any human case. On the contrary, in Apulia region, one autochthonous human case of WNND was confirmed in 2013, although no WND cases in horses or in other animal species were reported, although the detection three years before of a sporadic virus circulation in poultry farms in a bordering territory highlighted the suitability of that area for virus transmission. These findings confirm the crucial role of an integrated human, animal and vector surveillance in order to timely set up preventive measures. In this context, the veterinary surveillance carried out in sentinel animals, bird and vector populations can play a crucial role for foreseeing human transmission, even considering the limits of sensitivity of a surveillance system for vector-borne diseases.

Entomological surveillance is also a central aspect, allowing at early detecting the circulation of the virus [38–40]. In some provinces of northern Italy, the detection of WNV circulation through entomological surveillance was as early as July, largely more in advance than human cases occurrence [38].

With regard to circulating viruses, in the last three years WNV lineage 2 was detected in several Italian foci, apparently showing an extension of its spread and a more important contribution played by this lineage in the overall epidemiological situation in Italy. In addition, the co-circulation

of lineages 1 and 2 in the same area [22] may create the favourable conditions for possible changes in the virulence of the viral strains, potentially leading to unexpected and adverse consequences.

5. Conclusions

The integrated human and veterinary information systems provide the Competent Authority with a large amount of data and information on WNV circulation, thus, allowing the evaluation of planned actions and, if needed, their improvement or revision. In 2013, the integrated human, entomological, and animal surveillance system was able to monitor the spread of WNV and supported the application of control measures for blood transfusions and organ donations, preventing the transmission of the disease among human population.

In conclusion, the Italian experience represents a good example of collaboration among different sectors of public health (human, veterinary, entomologists, and blood and organ donation authorities) in a "one health" perspective [41]. Vector borne diseases, in fact, need a multidisciplinary and integrated approach, that is more effective to assure animal and human health, as well as the environment protection. In case of zoonoses, such as WND, this approach is of paramount importance for a better and holistic understanding of the prevention of the diseases and the maintenance of both human and animal health.

Conflict of Interests

The authors declare no conflict of interests.

Acknowledgment

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Research Article

Sialic Acid Expression in the Mosquito *Aedes aegypti* and Its Possible Role in Dengue Virus-Vector Interactions

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Dengue fever (DF) is the most prevalent arthropod-borne viral disease which affects humans. DF is caused by the four dengue virus (DENV) serotypes, which are transmitted to the host by the mosquito *Aedes aegypti* that has key roles in DENV infection, replication, and viral transmission (vector competence). Mosquito saliva also plays an important role during DENV transmission. In this study, we detected the presence of sialic acid (Sia) in *Aedes aegypti* tissues, which may have an important role during DENV-vector competence. We also identified genome sequences encoding enzymes involved in Sia pathways. The cDNA for *Aedes aegypti* CMP-Sia synthase (CSAS) was amplified, cloned, and functionally evaluated via the complementation of LEC29.Lec32 CSAS-deficient CHO cells. *Aedes*CSAS-transfected LEC29.Lec32 cells were able to express Sia moieties on the cell surface. Sequences related to α-2,6-sialyltransferase were detected in the *Aedes aegypti* genome. Likewise, we identified Sia-α-2,6-DENV interactions in different mosquito tissues. In addition, we evaluated the possible role of sialylated molecules in a salivary gland extract during DENV internalization in mammalian cells. The knowledge of early DENV-host interactions could facilitate a better understanding of viral tropism and pathogenesis to allow the development of new strategies for controlling DENV transmission.

1. Introduction

Dengue fever (DF) is the most important and rapidly expanding arthropod-borne viral disease in tropical areas. Dengue virus (DENV) infection affects more than 100 million people worldwide each year, and 2.5 billion people live in areas of risk [1]. DF is caused by any of the four antigenically distinct dengue virus serotypes (DENV 1–4), which are transmitted to humans by the hematophagous mosquitoes *Aedes (Ae.) aegypti* and *Ae. albopictus*. The recent increase in DF and dengue hemorrhagic fever/dengue shock syndrome, now known as severe dengue, is associated with the vector's expansion to new geographic areas [2]. Severe dengue is a highly pathogenic disease, so the development of a dengue vaccine is a high priority for protecting people at risk, but no safe vaccine is available at present. Therefore, mosquito control is the primary option for preventing dengue outbreaks [3]. *Ae. aegypti* females have a key role in DENV-vector competence, which refers to the vector's permissiveness to infection, replication, and viral transmission [3, 4].

The female mosquito acquires DENV from an infected person during blood feeding. The virus undergoes its first replication cycle in the mosquito midgut, before spreading into the hemocoel and finally infecting the salivary glands (SGs). The transfer of infectious saliva into a human host (during a new blood feeding) is a key event during the DENV transmission cycle [4, 5]. Thus, it is very important to identify the molecules involved in the DENV-SG relationship because mosquito saliva is rich in glycoproteins that participate in different host responses (platelet activation, swelling, itching, and inflammation), as well as the binding and transport of vector-borne pathogens to host tissues, thereby allowing pathogens to infect and evade the host immune response [5]. In an ample range of disease models, including various hosts, mosquito species, and arthropod-borne viruses, mosquito saliva and/or mosquito feeding are associated with a potentiation of the arbovirus (arthropod-borne) infection. Host infection via vector saliva leads to an increase in viral transmission, host susceptibility, disease progression, and mortality [6]. The potential for mosquitoes to influence the course of West Nile virus (WNV) disease was investigated by assessing pathogenesis in the presence or absence of mosquito saliva [6]. Likewise, *in vitro* and *in vivo* models of saliva-mediated enhancement of DENV infectivity have been reported [7], but it is uncertain whether *Aedes* saliva glycosylated molecules contributes to DENV tissue infection. The *Aedes* sialome includes 136 putative secretory proteins, which could modify host responses [8]. During DENV-vector infection, the main genes upregulated in *Ae. aegypti* are related to carbohydrate expression [9], but the roles of glycans in vector competence are currently unknown. In addition, it is known that certain glycosidases affect the binding of DENV to mammalian (green monkey kidney and Vero) and mosquito (C636 and AP61) cell surfaces [10]. Previously, it was reported that β-glucosidase, sialidase, and heparinase reduce DENV attachment to mammalian cells but not to insect cells [10], and the inability of sialidase to affect DENV binding to insect cells is associated with a lack of mosquito sialyltransferase (ST), which is capable of transferring sialic acid (Sia) residues to mosquito glycoproteins [11]. Moreover, the occurrence of Sia in mosquito tissues is also unknown. However, the genetic and biochemical capacity for sialylation in *Drosophila melanogaster* supports a hypothesis that insect sialylation is a specialized and developmentally regulated process in insects [12–16]. This process is involved in the regulation of neural transmission in the nervous system of *D. melanogaster* [17, 18]. It is well known that sialylated glycoproteins modulate many important biological processes, including cellular and molecular recognition, subcellular and cellular trafficking, intercellular adhesion, and signaling and microbial attachment, among others [19]. In the present study, we detected the presence of a functional cytidine monophosphate- (CMP-)Sia synthase (CSAS) in *Ae. aegypti*, and we also demonstrated that DENV recognizes α-2,6-linked Sia structures on the surface of mosquito tissues, which may play key roles during early DENV-vector interactions. Furthermore, we found that DENV is capable of interacting with secretory Sia-glycoproteins, which may be involved in successful DENV-host tissue transmission. To our knowledge, these are the first demonstrations of the functional expression of an *Aedes* CSAS and the presence of Sia moieties in mosquito tissues, which may have important biological consequences for DENV-vector competence. Knowledge of specific early DENV-mosquito interactions could facilitate a better understanding of viral tropism and pathogenesis to allow the development of new effective strategies for the control of DENV transmission, as well as the improvement of antiviral agents and vaccines.

2. Materials and Methods

2.1. DENV Propagation and Titration. DENV New Guinea C strain serotype 2 (DENV-2, kindly donated by Dr. Duane Gubler, CDC Fort Collins, CO, USA) was propagated in C6/36 cells, which were grown at 28°C in supplemented minimal essential medium (MEM). Confluent monolayers were infected for 2 h at a multiplicity of infection (MOI) of 1 and incubated for 5–7 days at 28°C in a 5% CO_2 atmosphere until cytopathic effects were observed, before titrating in a lytic plaque assay using LLC-MK2 cells, as described previously [20]. The virus titer was expressed as plaque-forming units (pfu) per milliliter.

2.2. Ae. aegypti Maintenance, Salivary Glands, Midgut Isolation, and Tissue Extracts. Female *Ae. aegypti* mosquitoes were cultured in an insectarium at the Center for Infectious Disease Research (CISEI-INSP), Mexico. The SGs and midguts of female mosquitoes (at least three days old and fed only with water) were dissected using a microneedle, placed in sterile tubes in groups of 20 pairs with 20 μL of phosphate-buffered saline (PBS) and kept at −75°C. The tissues were lysed during five freeze-thaw cycles using liquid nitrogen and sonicated (ultrasonic 8849-00; Cole-Parmer, IL, USA) for 10 min before centrifugation at 3500 rpm to obtain tissue extracts. The protein concentration was determined using a micro-BCA (bicinchoninic acid) assay (Pierce, USA) at 562 nm with a spectrophotometer (Multiskan Ascent 354, Thermo Labsystem UK).

2.3. Ae. aegypti Saliva Collection. Ae. aegypti saliva was collected as described by Almeras et al. [21], with a small number of modifications. Female mosquitoes were sedated for 1 min at 4°C, and the proboscis of each mosquito was placed in a plastic pipette tip containing mineral oil. After 1 h salivation at room temperature (RT), the liquid was collected from the tip, and the saliva from 20 mosquitoes was pooled, before centrifugation at 10,000 rpm. The protein concentration was estimated using a micro-BCA assay.

2.4. Carbohydrate Determination in Ae. aegypti Salivary Glands. The salivary glands of female *Ae. aegypti* mosquitoes were dissected as described above, and the SG monosaccharides were analyzed according to Kamerling et al. [22] by GC/MS as trimethylsilyl methyl glycosides (by the Structural and Functional Glycobiology Unit of the University of Sciences and Technologies of Lille, France). Briefly, dry samples were methanolized in methanol/HCl

0.5 N, N-reacetylated, and trimethylsilylated in a mixture of N,O-Bis(trimethylsilyl)trifluoroacetamide and pyridine (1 : 1), before injection into a gas chromatograph with a BPX70 12 m × 0.22 mm diameter column (Chrompack).

2.5. Identification of Sia in Ae. aegypti Midguts by High-Performance Liquid Chromatography (HPLC). Midguts were homogenized in water, lyophilized, and incubated in 1 mL 0.1 M TFA at 80°C for 2 h. The samples were centrifuged at 5000 rpm for 15 min, and two volumes of cold ethanol were added to the supernatant. To obtain exact analytical data and to avoid false-positive results, the lyophilized Sias were dried, resuspended in 100 μL of water, and passed successively through 50 × 2 (200 × 400 mesh) and 50 × 8 (25×50 mesh) Dowex (100 μL) anion exchange columns (Bio-Rad, Marnes-la-Coquette, France). This sequential cation and anion exchange chromatography process was described in detail in a previous study [23]. The columns were eluted with three volumes of water. The total volume was dried, diluted in one volume of 0.01 M trifluoroacetic acid (TFA), and analyzed by HPLC using a Hewlett-Packard model 1100 liquid chromatography system (Palo Alto, USA), as follows. In the HPLC analysis, Sia was derivatized using 1,2-diamino-4,5-methylenedioxybenzene, according to Hara et al. [24], and separated isocratically in a C-18 reverse phase Sep-PaK HPLC column (250 × 4.6 mm, 5 μm; Vydac, Hesperia, CA, USA) using a solvent mixture of acetonitrile/methanol/water (7 : 9 : 84), followed by identification based on the elution positions of standard Neu5Ac derivatives.

2.6. Lectin Histochemistry of Ae. aegypti SGs and Midguts. *Ae. aegypti* SGs and midguts were placed on slides and fixed, and the tissues were then blocked with 2% bovine serum albumin (BSA) for 30 min at RT, washed with PBS for 5 min, and immersed in PBS-Triton X-100 (0.2%) for 10 min. Next, they were washed with PBS-Ca^{2+} (1 mM) for 10 min and incubated with different biotin-conjugated lectins, that is, *Maackia amurensis* lectin (MAA), *Sambucus nigra* agglutinin (SNA), or *Lens culinaris* hemagglutinin (LCH) (EY Laboratories, Inc, USA) at 1 : 100 dilutions for 2 h at 37°C. The slides were washed with PBS for 10 min and incubated in the dark with ExtrAvidin-fluorescein isothiocyanate (FITC; Zymed Inc., USA) at 1 : 60. The tissues were then rinsed with PBS-Ca^{2+} (1 mM) for 5 min and with deionized water for 5 min. Finally, the samples were mounted with Vectashield 4′,6-diamidino-2-phenylindole (DAPI; Vectashield, Vector Laboratories, CA, USA) and visualized using a Leica DM fluorescence microscope (DCF-300FX digital camera; Leica Microsystems Digital Imaging, Germany). To evaluate SNA-specific binding, mosquito SGs and different *D. melanogaster* tissues fixed on slides were pretreated with 0.5 IU *Clostridium perfringens* sialidase (Roche Diagnostics, Germany) for 30 min at RT. This sialidase was preincubated with casein and resorufin-labeled according to Twining [25] to prevent protease activity. Samples were incubated in the dark with biotinylated SNA lectin (1 : 100) and streptavidin-FITC (1 : 60). The fluorochromes were analyzed in two channels: green for lectins and blue for nuclei. The gut, SGs, and midgut from *D. melanogaster* were dissected, fixed (as described previously [26]), and incubated with SNA lectin or sialidase. Finally, the images were digitized with the Leica IM1000 version 1.20 program (Imagic Bildverarbeitung AG, Glattbrugg, Switzerland).

2.7. DENV-Lectin Binding Assays. SGs were fixed on slides and incubated overnight with DENV (10^7 pfu) at 4°C. The samples were washed three times each for 10 min using PBS and incubated for 2 h at 37°C with the anti-DENV protein-E antibody (dengue type-2 virus MAB8702; Chemicon International, CA, USA) at a dilution of 1 : 300. Next, the samples were washed with PBS for 10 min and incubated for 20 min at RT in the dark with rhodamine-coupled anti-IgG antibody (Zymed Laboratories, Inc., USA) at a dilution of 1 : 3000. In the competition assays, SGs were incubated with lectins before the addition of DENV. To evaluate the possible participation of Sia in DENV-SG interactions, a DENV-SG competition assay was performed where DENV was preincubated for 1 h with soluble 200 mM Sia (N-acetylneuraminic acid; Sigma-Aldrich) or 1 mM fetuin (DIG Glycan Kit; Roche), before adding it to the SG. Images were acquired in three channels: green for lectins, red for anti-DENV, and blue for nuclei.

2.8. Trypsin and Sialidase Assays of SGs and Glycoprotein Identification Using a Lectin Blot Assay. SGs were treated with 0.5 IU of *C. perfringens* sialidase (Roche Applied Science, USA) for 30 min or with 0.075% trypsin (Sigma-Aldrich, Inc, USA) for 5, 15, or 30 min, before the glands were fixed and incubated with DENV. The SGs were incubated with SNA, MAA, or LCH lectins. Finally, images were obtained, as described earlier.

2.9. SG Glycoprotein Detection by Blot Assay. Glycoproteins in the SG protein extracts were identified by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) with a polyacrylamide gradient of 4–20%, which was then stained to detect all carbohydrates using a Pro-Q Emerald 300 Glycoprotein Gel Stain kit (Molecular Probes, Invitrogen P21855), according to the supplier's protocol. The gel image was captured under a UV transilluminator (Kodak Gel Logic 1550). For the lectin blot assay, proteins were transferred to nitrocellulose membranes (Trans-Blot 162-0112, Bio-Rad), blocked with 1% BSA + 0.2% Tween-20 in PBS, and washed. The membranes were incubated with biotinylated SNA or *Canavalia ensiformis* agglutinin (ConA; EY Laboratories Inc., USA) at a dilution of 1 : 10 for 3 h at RT, followed by streptavidin-horseradish peroxidase conjugate (43-4323; Zymed Laboratories Inc., USA) at a dilution of 1 : 3000 for 1 h at RT. The membranes were then washed with PBS and visualized with luminol (Western Blotting Reagent sc-2048; Santa Cruz Biotechnology, USA). Finally, the membranes were exposed to a film (Kodak).

2.10. Virus Overlay Protein Binding Assays (VOPBA). VOPBA was performed as described by Salas-Benito and del Angel [27]. Briefly, SG protein extracts or salivary proteins were

transferred to nitrocellulose membranes, blocked (1% BSA + 0.2% Tween-20 in PBS) for 1 h at RT, washed three times with PBS, and incubated overnight (4°C) with DENV (10^7 pfu) in 1% BSA in PBS + 1 mM $CaCl_2$. The membranes were washed with PBS and incubated for 3.5 h at RT with a monoclonal antibody against DENV protein E (MAB 8702; Chemicon International, CA, USA) at a dilution of 1 : 300. Next, the membranes were washed twice with PBS + 50 mM NaCl and incubated for 1 h at RT with a secondary anti-mouse IgG antibody (1 : 5000) coupled with peroxidase (81-6520; Zymed Laboratories Inc.). Finally, the membranes were washed, treated with luminol, and exposed to film. To evaluate the role of Sia residues in interactions with DENV, the SG protein extracts and saliva were pretreated with 0.5 IU of *C. perfringens* sialidase (Roche) for 1 h before the overlay assay, as described earlier.

2.11. DENV Infection of Mammalian Cells in the Presence of Ae. aegypti SG Protein Extract. The internalization of DENV in mammalian cells (LLC-MK2 and wild-type Chinese hamster ovary cells CHO) was assessed in the presence or absence of SG extract protein, where DENV was metabolically labeled with [^{35}S]-methionine at 37°C for 1 h. Confluent monolayers of mammalian cells were infected with labeled DENV at an MOI of 1 in the presence or absence of SG proteins extracted from 80 SGs, which were pretreated (or untreated) with 0.5 IU of *C. perfringens sialidase* for 1 h at RT. After infection, the medium was removed, and the cells were washed twice with citrate buffer (10 mM citric acid, 0.05% Tween-20, pH 6.0) and PBS to remove any nonspecifically associated virus after the incubation period, thereby avoiding counting virus that was not internalized. Cells were subsequently lysed and fixed on mats filters (Skatron Instruments, UK). The [^{35}S]-methionine level was measured using an LS6500 Scintillation Counter (Beckman Coulter, USA).

2.12. LC/ESI-MS/MS Analysis. VOPBA protein bands were selected for protein identification by mass spectrometry (MS) analysis. The bands were carefully excised from Coomassie Brilliant Blue-stained gel and prepared for liquid chromatography-electrospray ionization tandem mass spectrometry (LC-MS/MS). Briefly, individual protein bands were destained, reduced, carbamidomethylated, digested with trypsin, and extracted from the gel using a standard in-gel digestion procedure [28]. The volumes of the extracts were reduced by evaporation in a vacuum centrifuge at RT, before adjusting to 20 μL with 1% formic acid. Peptide MS analysis was performed using a 3200 QTRAP System (Applied Biosystems/MDS, USA), which was equipped with a nanoelectrospray source and a nanoflow LC system (1100 Nanoflow Pump; Agilent, Waldbronn, Germany). Mass tuning of the hybrid triple quadrupole linear IT spectrometer was performed using [Glu1]-fibrinopeptide B. Sample digests were injected into a Zorbax 300SB C18 column equilibrated with 2% ACN and 0.1% formic acid and separated using a linear gradient of 2% to 7% CAN with 0.1% formic acid over an 80 min period, at a flow rate of 300 nL min^{-1}. The interface heater used for desolvation was held at 150°C, and the spray voltage was 2.4 kV. Spectra were acquired in the automated mode by information-dependent acquisition. Precursor ions were selected in Q1 using the enhanced MS mode. The scan ranges for EMS were set to 400–1500 and 4000 amu s^{-1}. Selected ions were subjected to an enhanced resolution scan at a low speed of 250 amu s^{-1} over a narrow (30 amu) mass range, followed by an enhanced product ion scan (MS/MS). The precursor ions were fragmented by collision-activated dissociation in the Q2 collision cell using rolling collision energy. The fragmented ions were captured and mass analyzed in the Q3 linear IT. Database searches (Swiss-Prot, NCBInr, or MSDB) and protein identification were performed using the MASCOT program (http://www.matrixscience.com/) with trypsin plus one missed cleavage and carboxyamidemethylation as a fixed modification and methionine oxidation as a variable modification, using a mass tolerance of 0.5 Da for the precursor MWs and 0.3 Da for the fragment MWs. The criteria used to accept a protein hit as a valid identification were two or more tryptic peptide matches with the protein sequence and at least one peptide with $P < 0.05$.

2.13. Analysis of the Protein Glycosylation Sites. The sequence obtained from the MASCOT database was analyzed with Glycomod [29], which is available at http://www.expasy.ch/tools/glycomod/. This program explores the mass values of ions obtained experimentally with MALDI-ToF and their relationships with sequences in the MASCOT database. The search parameters specified N-glycosylated and O-glycosylated proteins, with modifications of oxidized methylation and cysteine-treated iodoacetamide, using a mass tolerance of 0.1 Da.

2.14. Ae. aegypti RNA Purification. Groups of 25 female mosquitoes were homogenized and sonicated with RNAse-free water. The lysates were passed through a 0.9 mm needle. RNA extraction was performed using a Nucleospin RNA II kit (Macherey-Nagel, Germany), and the RNA quality was evaluated using Agilent RNA Nano 6000 chips (Agilent 2100 Bioanalyzer).

2.15. Ae. aegypti CSAS and ST Gene Synthesis. BLink and BLAST searches for CSAS and ST genes were performed using the NCBI tBLAST algorithm based on the CSAS (gi|24667125) and ST (gi|24762715) sequences of *D. melanogaster*. Putative CSAS (XP 001663017) and ST (XP 001649590) genes were identified in the *Ae. aegypti* genome and confirmed by VectorBase (https://www.vectorbase.org/) as AAEL012868 and AAEL014772, respectively. cDNA synthesis was performed using 200 ng of RNA template (QPCR cDNA kit; Stratagene, USA) with random primers. Five microliters of cDNA was used in a 25 μL PCR reaction, which was amplified with Taq DNA polymerase (Thermo Fisher Scientific) as follows: 95°C for 5 min; 38 cycles at 94°C for 1 min, 50°C for 1 min, 72°C for 1.5 min, and 72°C for 10 min, holding at 4°C. The following primers were used for *Ae.*CSAS gene synthesis: 5′aedsy (5′GTT GAA TTC CAT GCG GCT AGT TTT GAT 3′), 3′aedsy (5′AAT GGT ACC

TTA TTC TAC TGT GGA TCC 3′), 5′aedtr (5′CAC AAG CTT ATG TTG CGT GAC CTT TCG 3′), 3′aedetr (5′CTA GGT ACC TCA ACA TCC ACT GTT GCT 3′), 5′Act (5′TGG TTA CTC GTT CAC CA 3′), and 3′Act (5′GGC ATA CAG ATC CTT TCG GA 3′).

The forward primer 5′aedsy included an *EcoRI* site and the first six codons of *Ae*.CSAS. The 3′aedsy primer contained a *KpnI* site and the last six codons of *Ae*.CSAS. The 5′aedtr forward primer contained a *HindIII* site and the first six codons of the hypothetical *Ae. aegypti* ST sequence, and 3′aedtr included a *KpnI* site and the last six codons of the same sequence. The *Ae. aegypti* actin gene was used as a housekeeping control.

2.16. Ae. aegypti CSAS cDNA Cloning and Sequencing. The CSAS PCR product was cloned using a Topo vector (Invitrogen) and transformed into *Escherichia coli* strain DH5α. The cloned cDNA was evaluated by PCR using M13 forward (–20) and reverse primers. The CSAS cDNA was nicked at the *EcoRI* and *KpnI* sites, and subcloned using a p3XFlag-CMV-10 (Sigma-Aldrich) vector. The plasmid sequence was confirmed by PCR using the primers 5′p3 FLAG (5′-GTTGACGCAAATGGGCGGTAG-3′) and 3′p3 FLAG (5′-CTTGCCCCTTGCTCCATACCAC-3′), as follows: 96°C for 5 min; 38 cycles at 96°C for 45 s, 50°C for 45 s, 72°C for 1 min, and 72°C for 10 min, holding at 4°C. The 786 bp CSAS product was sequenced (Genoscreen, Lille, France).

2.17. Complementation of CSAS-Deficient Cells with Ae.CSAS. Wild-type CHO cells and LEC29.Lec32 cells, which were deficient in CMP-Neu5Ac synthase, were grown in MEM containing 10% FBS in 5% CO_2 at 37°C. One million LEC29.Lec32 cells were transfected with lipofectamine reagent (Invitrogen) using 5 μg of the p3XFlag-CMV-10 vector with the *Ae*.CSAS insert or the empty vector as a control. Cells were harvested at 36 h posttransfection. *Ae. aegypti* Sia expression was evaluated by FACS analysis. Cells were detached and incubated for 1 h at 4°C with biotin-conjugated MAA, washed, and incubated for 1 h on ice with Alexa Fluor 488 conjugated streptavidin (Invitrogen). Appropriate isotype and secondary antibody controls were used. In the FACS analysis, 10,000 cells were analyzed using a FACSCalibur system (Becton Dickinson, USA). *Ae*.CSAS expression was also evaluated by histochemistry; that is, WT CHO and LEC29.Lec32 cells were grown on slides and transfected as described previously. Cells were incubated with MAA lectin and Alexa Fluor conjugated antibody and stained in parallel with DAPI.

2.18. Hemagglutination Assay with DENV. The assay was performed as described by Goldsmith (see [30] and Casals and Brown [31]). DENV was propagated in C6/36 cells, purified by ultracentrifugation (see Methods in the paper), and suspended in borate solution (pH 9). Borate solution was used as the negative control. In a microtiter plate, a series of twofold dilutions of the viral stock was generated, which was followed by the addition of a suspension of chicken erythrocytes (4% in borate solution) and incubation of the samples for 1 h at 4°C. The hemagglutination activity was expressed as a titer defined as the reciprocal of the maximal dilution that gave positive hemagglutination. A parallel assay was performed using the influenza virus.

2.19. Sialidase-Treated Erythrocytes. Sialidase-treated erythrocytes were obtained according to Sano and Ogawa [32]. Briefly, native chicken erythrocytes (10%, v/v) were mixed with an equal volume of the incubation buffer (0.1 M acetate buffer containing 1 mM $CaCl_2$, pH 5.5) containing sialidase from *Clostridium perfringens* (1 U/mL), which was preincubated with casein and resorufin to prevent protease activity. The sample was incubated at 37°C for 1 h with occasional careful shaking. The cells were washed by centrifugation using cold PBS (pH 7) and stored as a 10% suspension at 4°C until use. The HA assay was carried out as previously described. A parallel assay was performed using the influenza A virus.

2.20. Statistical Analysis. Data were expressed as the mean and standard deviation and compared using a Mann-Whitney *U* test with Statistical Analysis Software version 8 (SAS Institute, USA). The significance level was set at $P < 0.05$. To identify the D7 protein in MASCOT, and the score for an MS/MS match was based on the absolute probability (P) that the observed match between the experimental data and the database sequence was a random event. We used a probability-based MOWSE score; that is, the reported score was $-10 \log (P)$, where P was the probability that the observed match was a random event, and the protein scores were significant at $P < 0.05$.

3. Results

3.1. Identification of Sia in Ae. aegypti Mosquito Tissues and Genes Involved in the Sia Synthesis Pathway. The total carbohydrate composition of the *Ae. aegypti* SG protein extract was determined by gas chromatography, which showed that the most abundant monosaccharide was N-acetylgalactosamine, with an average of 170 μg per 10 salivary glands, followed by mannose (84 μg), N-acetylglucosamine (42 μg), galactose (16 μg), and Sia (Neu5Ac with 7 μg). We also assessed the presence of Sia in midguts using HPLC by referring to the retention times of standard Sia derivatives [33]. Sia was determined at a concentration of 1.4 μg per single midgut. As a consequence of the presence of Sia in different mosquito tissues, we evaluated the possible existence of genes encoding enzymes involved in Sia synthesis pathways. The sialylation process requires the biosynthesis of glycosyl-nucleotide cytidine 5′-monophosphate-N-acetylneuraminic acid (CMP-Neu5Ac) by CSAS and enzymes from the ST family, which transfer Sia to a glycoprotein or glycolipid acceptor substrate. Therefore, using the available genome database of *D. melanogaster*, we searched for the amino acid (aa) sequences of both enzymes, that is, CSAS (gi|24667125) and D.SialT6 ST (gi|24762715), and we performed BLAST and BLink analyses of the *Ae. aegypti* genome using the NCBI genome database. We detected hypothetical sequences for both proteins, that is, CSAS (XP 001663017; *Ae*.CSAS) and ST (XP 001649590; *Ae*.ST), in the *Ae. aegypti* genome,

which were validated in the VectorBase database. The *Ae. aegypti* ST gene sequence was identified and associated with the ST6Gal, α2,6-sialyltransferase (ST6Gal) family, which is closely related to *D. melanogaster* D.ST6 and orthologous to the common ancestral gene that was present before the split of ST6Gal I and ST6Gal II [34]. We used these sequences to generate a complementary DNA (cDNA) that comprised 786 bp for *Ae.*CSAS and another of 1396 bp for *Ae.*ST (Figure 1(a)). Likewise, we obtained *Ae. aegypti* cDNAs for *Ae.*CSAS and *Ae.*ST from the SGs and midguts (Figure 1(b)). The *Ae.*CSAS cDNA was cloned into the p3XFlag-CMV vector. Two clones, that is, C4 synthase and C8 synthase, were sequenced, analyzed, and compared with previously reported CSAS sequences (See Figure S1 in Supplementary Material available online at http://dx.doi.org/10.1155/2015/504187). Both clones contained the start point of an open reading frame for a protein containing 261 aas, with a molecular mass of 29.8 kDa and a theoretical isoelectric point of 6.72. We detected a polymorphism site in the *Ae.*CSAS gene (Figure 1(c)). In clone 4, a point mutation from A (residue 183) to T changed an aspartic acid (D) residue into glutamic acid (E).

3.2. Evaluation of Ae.CSAS Complementation of CHO Sia-Deficient Cells. To determine the functional activity of *Ae.*CSAS, a p3XFlag-CMV vector containing the *Ae.*CSAS insert was transfected into CHO LEC29.Lec32 cells [35], which were deficient in CSAS expression and did not express sialoglycoconjugates. Sia expression was evaluated by a flow cytometry (FACS) assay using MAA, which recognizes Sia in α-2,3-linkages, because CHO cells mainly express α-2,3-STs [36]. We observed that *Ae.*CSAS-transfected cells expressed α-2,3-linked Sia (Figure 1(d), blue line) at a similar level to the parental CHO cells, which were used as a positive control (Figure 1(d), magenta line). The intensity of fluorescence in the nontransfected CHO LEC29.Lec32 subpopulation was similar to that in the negative control (Figure 1(d), green and black lines). In addition, nearly 30% of the LEC29.Lec32-transfected cells were able to express Sia (Figure 1(d) shows the fluorescence intensity percentages). To confirm the functional activity of *Ae*.CSAS, we tested for the presence of Sia in *Ae.*CSAS-transfected CHO LEC29.Lec32 cells using an affinocytochemical assay with MAA lectin. Sia expression was observed on the cell surface of *Ae.*CSAS-transfected CHO LEC29.Lec32 cells (Figure 1(e)), as shown by the FACS assay. These results demonstrate the functional expression of *Ae*.CSAS in *Ae. aegypti*.

3.3. DENV-Sia Interaction in Ae. aegypti Tissues. The *Ae. aegypti* ST gene is related to the ST6Gal family [37]; thus, we evaluated gene expression based on the presence of α-2,6-Neu5Ac moieties on the surface of mosquito tissues (SG, head, and midguts) using affinocytochemistry and confocal microscopy assays with the lectin SNA, which recognizes Sia in α-2,6-linkages. We observed strong SNA staining in the different mosquito samples (Figure 2(a)). *D. melanogaster* tissues were used as the positive control and are well known [15] to express α-2,6-linked Neu5Ac moieties (Figure 2(b)). No MAA binding was observed in *Ae. aegypti* tissues, which indicates that *Ae. aegypti* does not express α-2,3-ST (similar to *D. melanogaster*, Figure S2). To validate the SNA binding assay, SGs were pretreated with *C. perfringens* sialidase and incubated with SNA lectin. In the absence of sialidase treatment, strong SNA staining was observed in *Ae. aegypti* mosquito and *D. melanogaster* tissues (Figures 2(a) and 2(b)). However, the SNA binding decreased after sialidase treatment of the mosquito and *D. melanogaster* tissues (Figure 2(c)).

SG is the main tissue where DENV is replicated and amplified in the mosquito before transmission to its vertebrate host; thus, we evaluated the possible role of Sia in DENV-SG interaction. We performed a binding assay with *Ae. aegypti* SG in the presence of different lectins (SNA, LCH, or ConA). Figure 3(a) shows that there was a positive DENV-SG interaction in the absence of SNA lectin. However, DENV binding decreased when α-2,6-Sia residues were blocked with SNA (Figure 3(b)), whereas the blocking of mannose residues with ConA or LCH did not modify the DENV-SG interaction (Figure 3(b); DENV-midgut interaction Figure S3). To confirm the possible role of Sia during DENV-SG binding, SGs were pretreated with *C. perfringens* sialidase at 30 min prior to DENV addition. We observed a large decrease in the DENV-SG interaction when the SGs were pretreated with sialidase (Figure 3(c)). To evaluate the specific role of Sia in DENV-SG binding, we performed a DENV-SG competition assay using free Neu5Ac and sialylated glycoprotein fetuin. We observed that the DENV-SG interaction decreased in the presence of fetuin, and it was lost in the presence of free Neu5Ac (Figure 3(c)), thereby suggesting the involvement of Sia in DENV-SG recognition. SGs were pretreated with trypsin for 5, 15, or 30 min to determine whether the sialylated molecules related to DENV-SG were proteins (Figure 3(d)). The interaction with DENV decreased after 15 min of incubation and it was abolished completely at 30 min. These data suggest the possible participation of sialylated glycoproteins in DENV tissue attachment.

3.4. Detection of Ae. aegypti SG Glycoproteins by Blot Assays. To confirm the presence of total sugars in the SG protein extracts from *Ae. aegypti* and to characterize the putative glycoprotein(s) that may recognize DENV, we separated the SG proteins by electrophoresis and stained them to detect any carbohydrates. The SG protein extracts were transferred to nitrocellulose membranes and subjected to a western blot assay. The membrane was also incubated with ConA or SNA lectins (Figures 4(b) and 5(a), lane 9). For the control assay, we used a carbohydrate staining kit (Pro-Q Emerald 300 Glycoprotein Gel Stain Kit, Molecular Probes; Figure 4(a), lane 1), and we observed a range of glycoproteins from 29 kDa to 116 kDa, with more intense bands of 29, 45, and 66 kDa. When we incubated the SG protein extracts proteins with ConA, we observed a glycoprotein of 50–60 kDa, which has not been identified previously with the carbohydrate staining kit. We also observed an increase in the intensity of the band at 97 kDa. Therefore, these proteins could have contained mannose and glucose residues (Figure 4(b)). The interaction with SNA produced several bands that ranged from 10 to 97 kDa (Figure 5(a), lane 9), so these proteins could possess Sia motifs. In agreement, we observed no significant changes

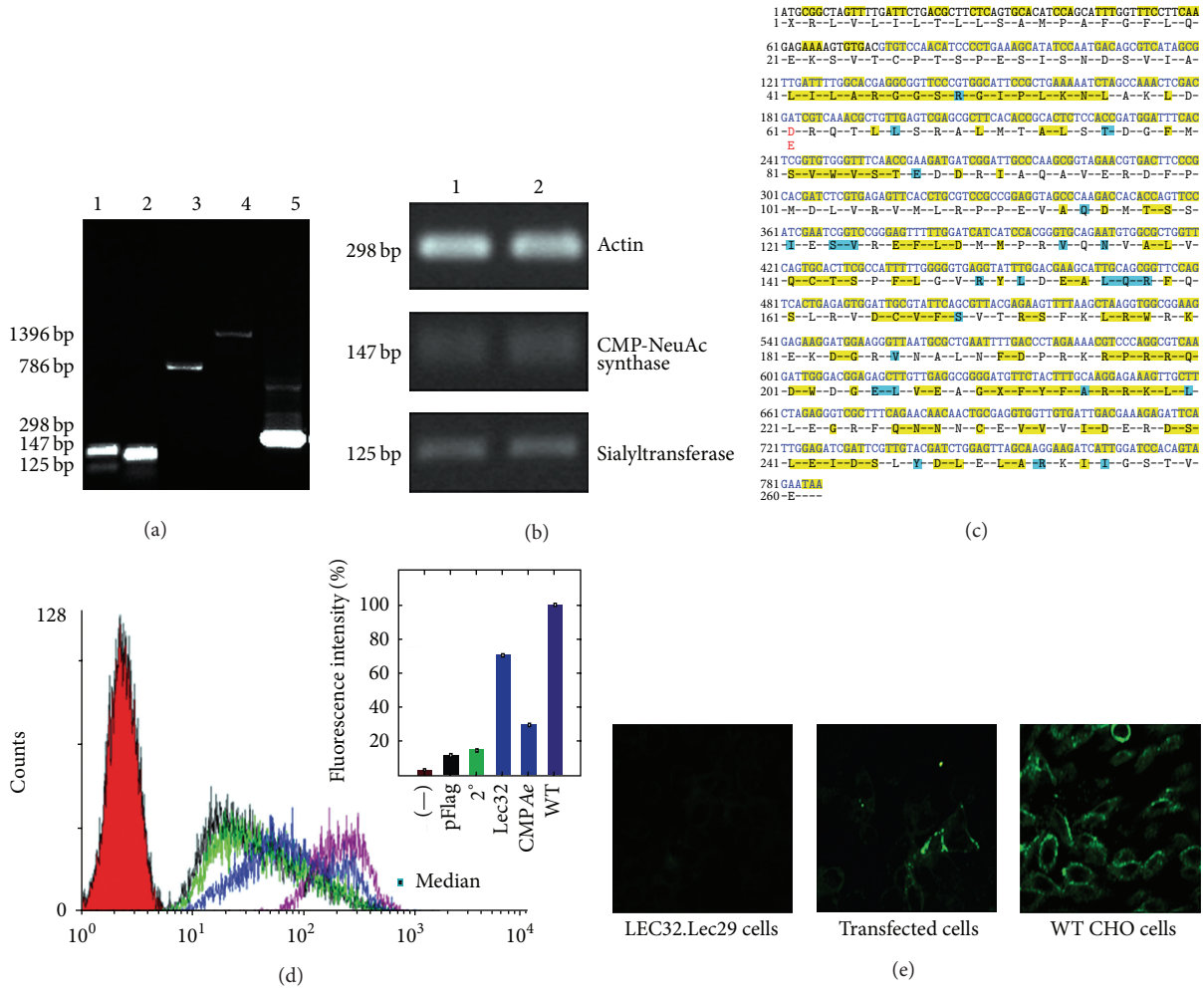

FIGURE 1: *Ae.*CSAS functional expression evaluation. (a) RT-PCR analysis of *Ae.*CSAS and *Ae.*ST. The figure shows the bands obtained with the internal and external primers of each enzyme using a whole extract of *Ae. aegypti* mosquito. Lanes 1-2: *Ae.*CSAS (147 bp) and *Ae.*ST (125 bp) sequences obtained using the internal primers. Lanes 3-4: *Ae.*CSAS (786 bp) and *Ae.*ST (1396 bp) complete sequences obtained with the external primers. Lane 5: *Ae.* actin (298 bp) was used as a housekeeping gene control. (b) RT-PCR analysis of *Ae.*CSAS and ST using total RNA from five pairs of *Ae. aegypti* SGs (lane 1) and five midguts (lane 2): *Ae.*CSAS (147 bp); *Ae.*ST (125 bp); and actin control (298 bp). (c) cDNA and aa sequences of *Ae.*CSAS. Identical residues in yellow show multiple alignments with different sequences from other organisms (Figure S1), whereas conserved residues are indicated in blue. (d) Flow cytometry analysis using LEC29.Lec32 untransfected and transfected cells with *Ae.*CSAS, which were incubated with MAA lectin to evaluate Sia expression. Red: isotype control; black: LEC29.Lec32 cells transfected with empty p3XFlag-CMV vector (negative control); green: untransfected cells in the presence of secondary antibody only; blue: LEC29.Lec32 transfected with *Ae.*CSS cDNA; and magenta: wild-type CHO cells (positive control for the expression of α-2,3Sia). The bars show the percentage of fluorescence intensity. Approximately 30% of LEC32.Lec29-transfected cells expressed Sia (blue bar) compared with 100% Sia expression in the positive control CHO cells (magenta bar). (e) Affinocytochemistry and confocal microscopy assays using MAA lectin staining to assess Sia expression. Left: LEC29.Lec32-transfected cells with an empty pFlag vector. Center: LEC29.Lec32-transfected cells with the *Ae.*CSAS pFlag vector. Right: wild-type CHO positive control transfected with an empty pFlag vector.

when we pretreated the SG protein extracts with sialidase (Figure 5(a), lanes 2 and 3).

3.5. Identification of DENV Attachment Glycoproteins in Ae. aegypti SGs and Saliva. To identify putative sialylated glycoproteins involved in DENV-SG interactions, different VOPBAs were performed using *Ae. aegypti* SGs and saliva. We observed that DENV interacted with different SG proteins with approximate molecular weights (MWs) of 115, 95, 65, 62, 51, 37, 34, 32, 17, 15, and 9-10 kDa (Figure 5(a), lane 10). The proteins with MWs from 65 to 9 kDa were also observed in the samples detected with SNA lectin (Figure 5(a), lane 9). To test the possible participation of Sia in DENV-mosquito protein interactions, we performed a parallel VOBPA assay, where we pretreated protein extracts from the SGs or saliva with sialidase. Interestingly, DENV protein binding was partially or totally abolished in both cases (Figure 5(a), lane 11; Figure 5(b), lane 2). It was also interesting that the SG proteins of 95 and 65 kDa, which did not interact with SNA lectin (Figure 5(a), lane 9), were not affected in the VOBPA

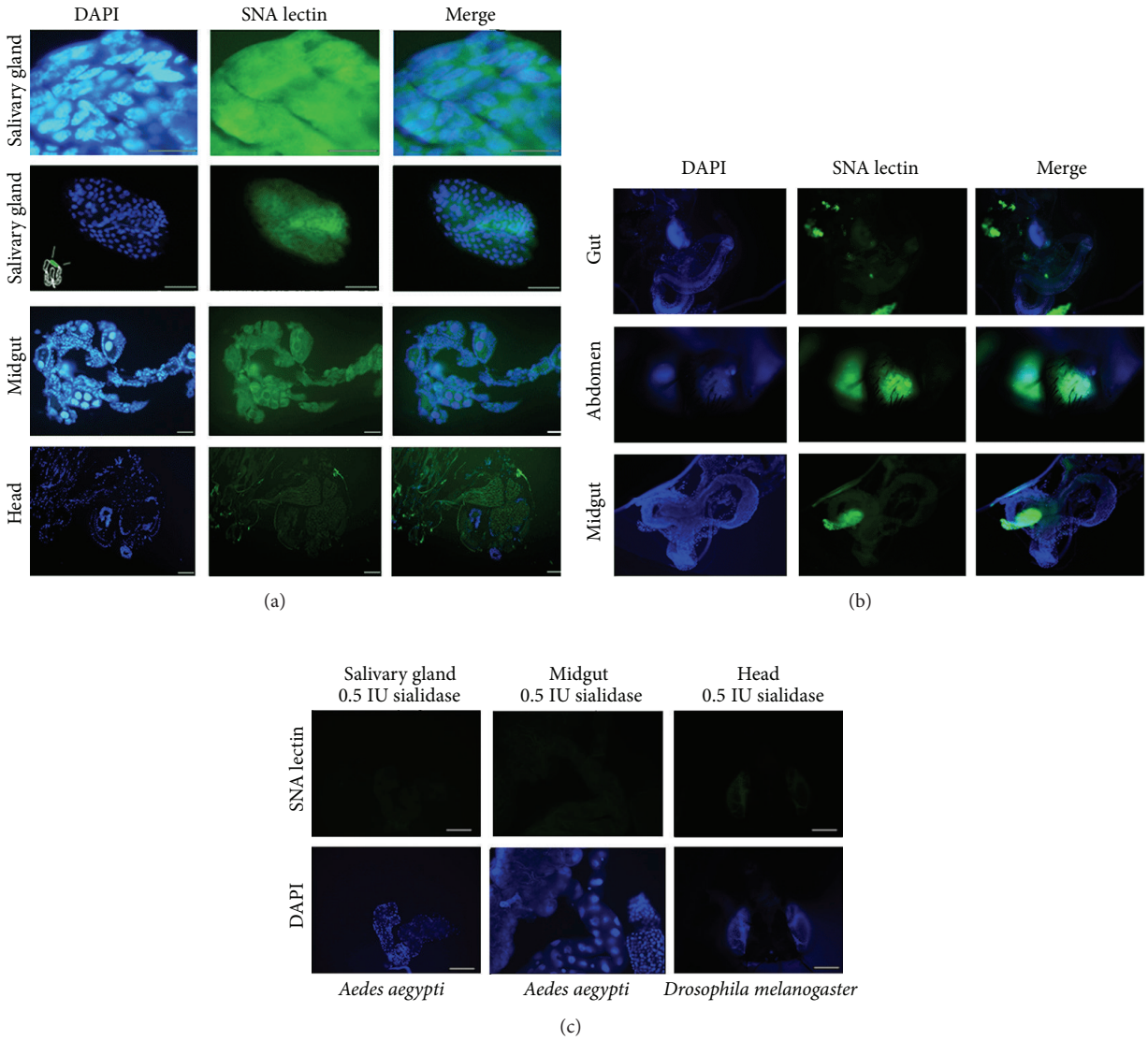

Figure 2: Lectin histochemistry of *Ae. aegypti* tissues. (a) Results of α-2,6-linked Sia detection in *Ae. aegypti* SG, midgut, and head incubated with SNA lectin (1 : 100) and stained with FITC. SG, upper panel: 60x microscopic magnification, lower panel: 40x lens. The inner box in the SG-DAPI panel shows the SG region analyzed. To identify Sia, the midgut and head transverse sections were evaluated with SNA lectin (green) (20x magnification). (b) Results for the α-2,6-linked Sia positive control in *D. melanogaster* abdomen, gut, and midgut using SNA lectin, which are similar to those for *Ae. aegypti* tissues. (c) SNA staining of mosquito SG and midgut pretreated with 0.5 IU sialidase for 30 min before SNA incubation. The control comprised *D. melanogaster* heads pretreated with sialidase. Blue: nuclei stained with DAPI. Green: (FITC) SNA lectin interaction.

pretreated with sialidase (Figure 5(a), lane 11). In the saliva-DENV binding assay, we observed a protein with a MW of 45 kDa (Figure 5(b), lane 3), which was also present in the samples with SNA lectin (Figure 5(b), lane 1), but it was eliminated when we used sialidase in the VOPBA (Figure 5(b), lane 2). Thus, we propose that the DENV-mosquito SG interaction is at least partially dependent on the presence of Sia residues. We used the sialylated glycoprotein fetuin as a positive control for SNA lectin (Figure 5(a), lanes 4 and 12), whereas asialofetuin (Figure 5(a), lanes 5 and 13) and fetuin pretreated with *C. perfringens* sialidase were used as the negative controls (Figure 5(a), lanes 6 and 14).

3.6. Identification of Ae. aegypti SG and Saliva Glycoproteins by LC/ESI-MS/MS. The different DENV-SG and DENV-saliva binding proteins observed in the VOPBAs were identified by LC/ESI-MS/MS analysis. The identities of the SG and saliva proteins are shown in Table 1. The DENV-SG binding proteins were as follows: (1) *Aedes* apyrase, which is a protein that hydrolyzes ATP and ADP to adenosine, thereby inhibiting ADP-dependent platelet aggregation; (2) *Aedes* salivary serpin, which is an anticoagulant molecule that inhibits coagulation factor Xa [38]; and (3) the *Aedes* long form of the D7 salivary protein. D7 is the most abundant subfamily of salivary proteins, and they are classified as

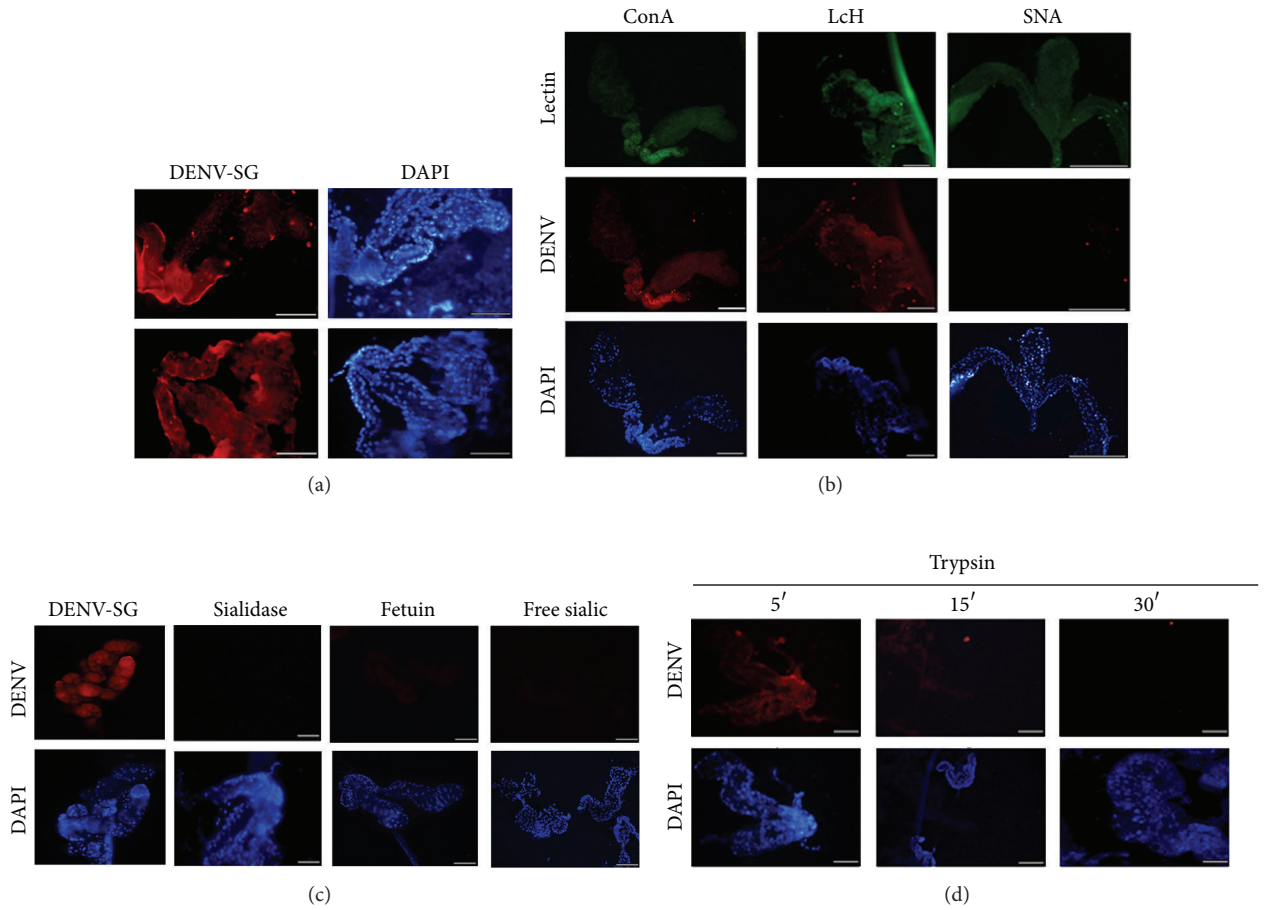

Figure 3: DENV interaction with *Ae. aegypti* SG. (a) DENV interaction with *Ae. aegypti* SGs. SGs from *Ae. aegypti* were incubated with DENV and stained with anti-DENV E antibody and rhodamine-coupled anti-IgG antibody. (b) DENV-SG competence assays using ConA, LCH, and SNA lectins, which were added to SG before incubation with DENV. The interaction with DENV was blocked when DENV was incubated in the presence of lectins that recognized Sia. With LCH and ConA lectins, the magnification = 10x and with SNA lectin = 20x. Scale bar = 10 μm. (c) DENV-SG interaction in the absence or presence of sialidase. SGs were untreated or pretreated with *C. perfringens* sialidase for 30 min before adding DENV. The DENV-SG interactions in the presence of Sia competitors, fetuin (1 mM) and free Sia (200 nM), are also shown, where the DENV-SG interaction was blocked. (d) DENV-SG interaction in SGs pretreated with trypsin for 5, 15, or 30 min before adding DENV. There was a decrease in the DENV-SG interaction after 15 min, and it was lost completely at 30 min. Scale bar = 10 μm. Blue: nuclei stained with DAPI. Red: DENV stained with an antibody against viral protein E and a secondary antibody coupled to rhodamine. Green: (FITC) SNA lectin interaction.

odorant pheromone-binding proteins, although they also function as scavengers of biogenic amines [39]. They also include (4) the *Aedes* 30-kDa SG allergen. Glycosylated proteins are associated with allergies [40]. Another one of the DENV-SG binding proteins is (5) the *Aedes* putative 34 kDa secreted salivary protein, which is distributed widely in mosquito saliva. The protein product of the 34 kDa family had significant matches with cytoskeletal proteins such as actin and myosin, mainly because of the presence of a repeated charged aa [41]. Another one of the DENV-SG binding proteins is (6) the *Aedes* 14.5 kDa salivary protein, which has an unknown function. Another one of the DENV-SG binding proteins is (7) the *Aedes* short form of the D7 salivary protein, which can bind biogenic amines such as serotonin, histamine, and epinephrine [41]. The sequestration of biogenic amines during mosquito feeding is an important function that inhibits platelet aggregation, vasoconstriction, and inflammation. Another one of the DENV-SG binding proteins is (8) the *Aedes* putative C-type lectin. In mammalian cells, two membrane C-type lectins, DC-SIGN and L-SIGN, interact with DENV via high-mannose glycans on viral glycoproteins [42], while another C-type lectin, the mannose receptor, interacts with the DENV envelope protein, which may enhance viral attachment to phagocytes [43]. It has also been demonstrated that the *Ae. aegypti* C-type lectin recognizes West Nile virus *in vivo* and *in vitro* during cell infection [44]. Another one of the DENV-SG binding proteins is (9) the *Aedes* beta subunit protein translocation complex. Silencing of the *Drosophila* and human ortholog gene (Sec61) of the beta subunit protein significantly reduces DENV infections in the S2 cell line and HuH-7 cells [45]. The ion masses and the sequences of the SG proteins involved

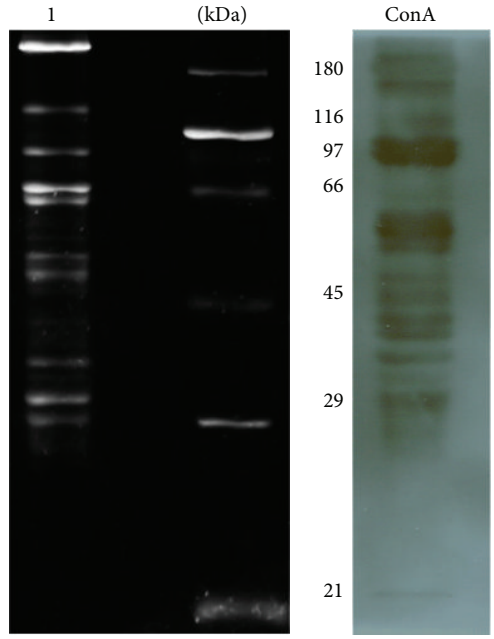

Figure 4: SDS-PAGE assay of the glycoproteins from *Ae. aegypti* SG protein extracts. (a) Total carbohydrates stained with Pro-Q Emerald, where the molecular weights are shown on the right. (b) Western blot assay using ConA lectin, which binds to glycoproteins that contain mannose or glucose residues.

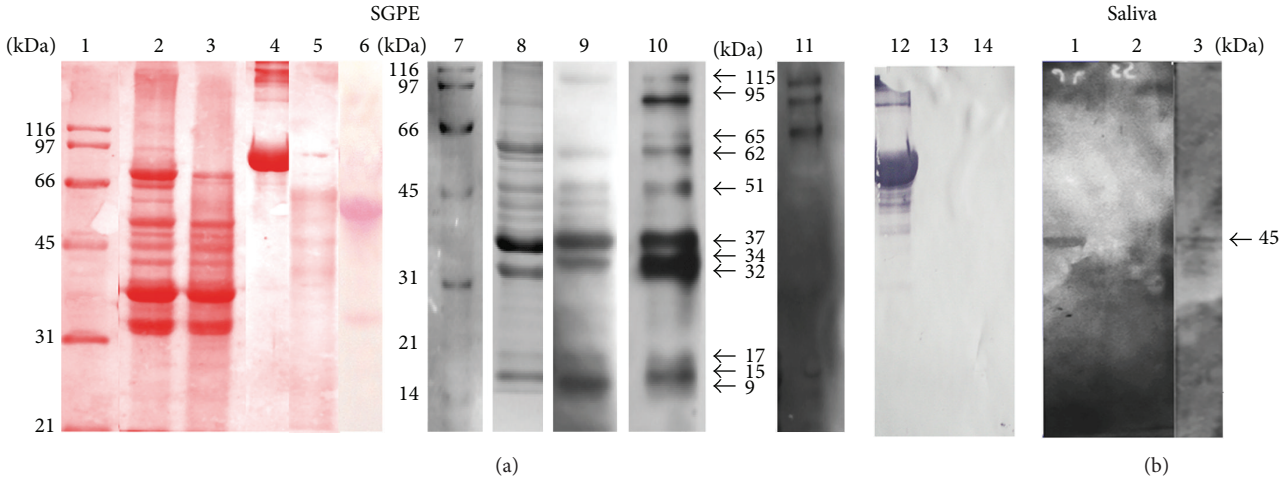

Figure 5: DENV overlay assay with *Ae. aegypti* SG protein extract (SGPE) and *Aedes* saliva in the presence or absence of *C. perfringens* sialidase. (a) DENV-SGPE interactions. Lanes 1–6 show nitrocellulose membranes stained with Ponceau red. Lane 1: MW markers; lane 2: SGPE; lane 3: SGPE pretreated with sialidase; lane 4: fetuin glycoprotein; lane 5: asialofetuin; and lane 6: fetuin pretreated with sialidase. Lanes 7–12 show the blot and overlay assays of SGPE. Lane 7: MW markers; lane 8: SGPE; lane 9: blot of SGPE with SNA lectin; lane 10: DENV overlay with SGPE; lane 11: DENV overlay with SGPE pretreated with sialidase; lane 12: blot of fetuin glycoprotein with SNA lectin; lane 13: blot of asialofetuin with SNA lectin; and lane 14: blot of SNA lectin with fetuin pretreated with sialidase. (b) DENV-saliva interactions. Lane 1: blot of mosquito saliva with SNA lectin; lane 2: DENV overlay with saliva pretreated with sialidase; and lane 3: overlay of DENV-saliva proteins.

in DENV interactions were evaluated using Glycomod to determine whether the proteins were putative glycoproteins with Sia motifs (Supplementary File 1).

The 45-kDa saliva protein that interacts with specific lectins for Sia as well as with DENV is similar to the peptide ion mass of the protein NCBI: gi|157113327 [Vectorbase: AAEL006417-RA], which is a putative molecule in the D7 family of *Ae. aegypti*. It had a 35% match in its primary sequence, with a score of 178 and an expected value of 6.4^{-13} ($P < 0.05$). Based on the analysis of the sequence

Table 1: Identification of DENV-2 binding proteins from *Ae. aegypti* SGs and saliva proteins.

Protein name	NCBI accession number	MW (kDa)		Number of matched peptides	Score	Sequence coverage (%)
		Gel	Database			
SG protein extract						
Apyrase [*Aedes aegypti*]	gi|556272	62.820	62.691	14	404	19%
Salivary serpin [*Aedes aegypti*]	gi|94469320	51.617	47.765	49	712	60%
D7 protein, putative [*Aedes aegypti*]	gi|157113327	37.200	38.603	46	862	44%
Long form D7Bclu1 salivary protein [*Aedes aegypti*]	gi|16225992	37.200	38.579	5	86	15%
D7 protein [*Aedes aegypti*]	gi|159557	37.200	37.005	3	51	9%
Putative 34 kDa secreted protein [*Aedes aegypti*]	gi|18568296	34.833	36.154	18	547	33%
Putative 34 kDa family secreted salivary protein [*Aedes aegypti*]	gi|94468336	34.833	35.698	20	533	33%
30 kDa salivary gland allergen Aed a 3 [*Aedes aegypti*]	gi|2114497	32.628	27.130	37	479	55%
Allergen, putative [*Aedes aegypti*]	gi|157133926	32.628	29.529	13	216	31%
Short form D7Cclu23 salivary protein [*Aedes aegypti*]	gi|16225995	16.947	17.676	10	150	24%
Putative salivary C-type lectin [*Aedes aegypti*]	gi|94468370	16.947	17.202	5	104	17%
Putative 14.5 kDa salivary protein [*Aedes aegypti*]	gi|94468650	14.862	17.039	6	117	40%
Protein translocation complex beta subunit, putative [*Aedes aegypti*]	gi|157138304	9.397	10.329	2	75	24%
Saliva						
D7 Protein putative [*Aedes aegypti*]	gi|157113327	45.23	39.173	18	178	35%

Proteins were identified by LC/ESI-MS/MS analysis after gel trypsin digestion. The table shows the protein name, the NCBI accession number, the theoretical (database) and observed (gel) MWs, the number of peptide sequences matched in the MASCOT database, the corresponding percentage sequence coverage, and the MASCOT score. The criteria used for accepting a protein as a valid identification were two or more tryptic peptide matches with the protein sequence and at least one peptide with $P < 0.05$.

of the putative D7 protein from *Ae. aegypti*, we identified a transmembrane region between aa residues 7 (phenylalanine) and 24 (leucine) from the amino terminus (Figure S4). Therefore, it can be considered as a membrane protein, although it has been suggested that members of this family of proteins are secreted in the salivary glands of various mosquitoes [46, 47]. We also noted that the D7 protein contains potential N-glycosylation sites, specifically in the region of aas 278–284 (Supplementary File 1). There were two possible combinations of carbohydrates involving Sia: the first was combined with hexose, and the second with N-acetylglucosamine or N-acetylgalactosamine. We evaluated the potential Sia-glycosylation sites some of which have little differences in terms of the ionic masses obtained with MALDI-ToF (experimental mass), the theoretical mass of the glycopeptides, and the carbohydrate mass. In addition, we only considered differences of <0.05 Da, and three peptide regions in the D7 protein had these characteristics. Between residues 35–39, there were two possible combinations of O-linked glycosylation via the hydroxyl groups of serine and threonine: the first combination involved the binding of Sia to two molecules of N-acetylglucosamine or N-acetylgalactosamine; and the second involved a combination with hexose, NeuAc, and ketodeoxynonulosonic acid. The second peptide with the potential to be O-glycosylated was in the region of aas 285–290, where a threonine residue could be linked to pentose, N-acetylglucosamine, or N-acetylgalactosamine, and Sia residues. Finally, there was a serine residue in the region of aas 311–316, where the difference between the experimental mass and theoretical mass was only 0.019 Da. Therefore, it is possible that a Sia residue linked to a deoxyhexose occurs in this region.

3.7. DENV Infection of Mammalian Cells in the Presence of Ae. aegypti SG Protein Extracts. It is known that *Ae. aegypti* saliva enhances West Nile and Cache Valley virus infections, but it is unknown whether *Aedes* saliva can modulate DENV infections [6]. Based on our detection of interactions between DENV and salivary glycoproteins, we evaluated the possible participation of the *Ae. aegypti* SG protein extract in the modulation of DENV infection in different mammalian cell lines (LLCMK2 and CHO WT) using a DENV internalization assay, in the presence or absence of SG extracts. We found that DENV infection was enhanced in the presence of SG extract in both mammalian cell lines (Figure 6(a)). CHO cells appeared to be more permissive (fourfold enhancement; Figure 6(a), lane 7) than LLCMK2 (twofold enhancement; Figure 6(a), lane 3). We pretreated the SG protein extract with

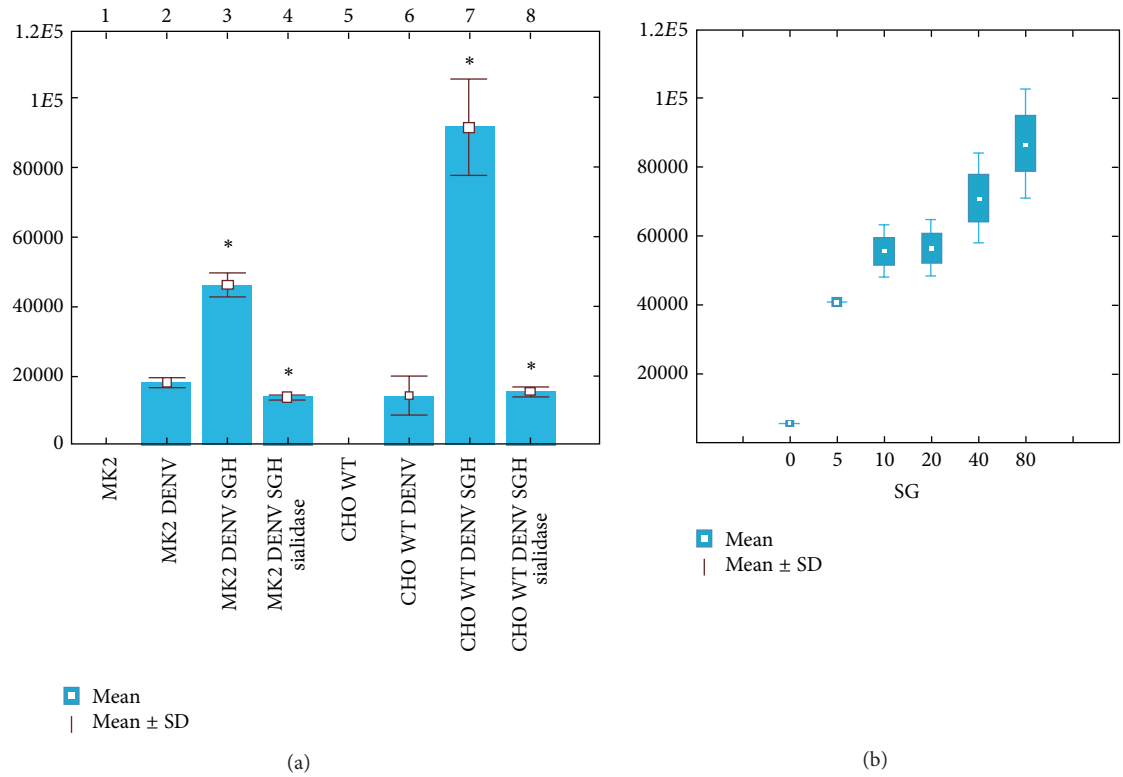

Figure 6: DENV-mammalian cells internalization assay. (a) DENV internalization by LLC-MK2 and CHO cells. The plot shows the internalization of [^{35}S]-methionine-radiolabeled DENV by LLC-MK2 and CHO cells in the absence (lanes 2 and 6) and presence (lanes 3 and 7) of *Ae. aegypti* SG protein extract (SGH) and in the presence of SGH pretreated with sialidase before DENV incubation (lanes 4 and 8). DENV was mixed with SGH (from 80 SGs), which was pretreated or untreated with sialidase, before infecting mammalian cells with the DENV-SGH mixture. In the plot, the y-axis shows the counts per min of internalized DENV, $^{*}P < 0.05$. (b) DENV internalization by CHO cells in the presence of different amounts of SGH. The plot shows that DENV internalization was enhanced by the presence of the protein extract from five SGs, which was dose dependent.

sialidase before the internalization assay to evaluate the possible participation of Sia during DENV cell internalization, and we observed the effect on DENV internalization, which was reduced in sialidase-pretreated samples (Figure 6(a), lanes 4 and 8). The internalization of DENV in CHO cells in the presence of different amounts of SG protein extract was dose dependent, as shown in Figure 5(b). These results support a general hypothesis that molecules in mosquito saliva and secretory SG proteins can potentiate pathogen-host transmission and that Sia residues play a role during DENV internalization in mammalian cells.

4. Discussion

Sialylation is a biologically important modification of glycoconjugates, which is observed mainly in the deuterostome lineage. However, the occurrence of this process in protostomes is less clear [19]. Using the available *Ae. aegypti* genome database, we identified two putative genes encoding enzymes (*Ae.*CSAS and *Ae.*ST) implicated in the *Ae. aegypti* sialylation pathway. The cDNA of *Ae.*CSAS was amplified, cloned, and functionally evaluated by the complementation of CSAS-deficient LEC29.Lec32 CHO cells. Sia moieties were present at the cell surface in *Ae.*CSAS-transfected CHO LEC29.Lec32 cells. The identification of a functional Sia synthase in *Ae. aegypti* indicates that *Aedes* mosquitoes have the biosynthetic capacity for endogenous Sia production. Our data are consistent with previous studies [12–16] of the expression of a functional *D. melanogaster* CSAS and the presence of α-2,6-linked Sia moieties in *D. melanogaster*. Sia is distributed widely in nature at the nonreducing termini of glycoproteins, glycolipids, or secreted glycoconjugates, and it may be attached to different acceptors via α-2,3, α-2,6, or α-2,8-linkages, which are determined by the specificity of different STs [48]. In this study, we demonstrated the presence of *Ae. aegypti* ST cDNAs in different *Ae. aegypti* tissues (Figures 1(a) and 1(b)) and observed the presence of α-2,6-linked Sia moieties (in a lectin binding assay) at the tissue level. These data are consistent with a report where it was shown that arthropods STs, including *Ae. aegypti* ST, are associated with the ST6Gal ST family, which is orthologous to the common ancestral gene that was present before the split of ST6Gal I and ST6Gal II in vertebrates [34].

To our knowledge, this is the first report of the presence of Sia glycans in *Ae. aegypti* tissues. The type of Sia linkage also plays a key role in the specific recognition of different viruses, because α-2,3- or α-2,6-specificity could define the cell and host tropism [49]. For example, human influenza

A virus hemagglutinin binds primarily to Neu5Acα2-6Gal structures, whereas avian influenza virus binds specifically to Neu5Acα2-3Gal [50]. This specificity limits the cell tropism and viral host range significantly. The participation of α-2,6-Sia structures during early DENV-vector interactions may have key roles in DENV infection, host tropism, and viral pathogenesis.

It was reported that *Anopheles* salivary glands contain several glycoconjugates in the surface, which are critical for recognition of different pathogens [51–53]. Perrone et al. [54] suggested that the salivary gland carbohydrate complexity reflects the functional diversity of this tissue. By lectin-binding assay, the authors detected the presence of α-D-mannose, α-D-N-acetyl-galactosamine, β-D-gal-(1,3) N-acetyl-galactosamine, β-D-galactose, N-acetyl-galactosamine, α-L-fucose, and β-N acetyl-glucosamine. Likewise, different oligosaccharide structures such as Man3GlcNAc2, Man3 (Fuc) 1-2GlcNAc2 were detected [55]. Recently, Francischetti et al. [56] demonstrated the presence of sulfated glycans in the salivary gland of *Anopheles gambiae*. Because of the glycan complexity in the vector salivary glands and in order to ensure that sialic acid detection in *Aedes aegypti* mosquito tissues was specific, the role of Sia in DENV-SG binding was evaluated by a DENV-SG competition assay using free sialic acid and also the sialylated glycoprotein fetuin. We observed that the DENV-SG interaction decreased in the presence of fetuin, and it was lost in the presence of free sialic acid (Figure 3(c) and supplementary Figure 5(B)–5(E)). In the same way, we observed in a hemagglutination assay of dengue virus with sialylated red blood cell (chicken erythrocytes) an inhibitory effect in presence of free sialic acid. Moreover, the Sia participation in DENV-sialic acid interaction was confirmed by the loss of hemmagglutination activity in the presence of desialylated erythrocytes (Supplementary Table 1).

DENV cellular infection is a multistep process that involves different molecules, some of them present in *Aedes* tissues like the laminin receptor, the tubulin like protein, HSP90 protein, unknown proteins of Mw: 35, 40–45, 48, 74, and 80 KDa and several detergent-soluble proteins of salivary glands with Mw: 35–80 KDa [57]. However, neither evaluated the possible participation of Sia glycoconjugates [58, 59], and the occurrence and participation of Sia in interactions among mosquito tissues and DENV have not been considered previously.

However, the participation of Sia in *Plasmodium gallinaceum* ookinetes-midgut interactions has been documented previously. Zieler et al. [60] reported that the chemical modification of the midguts from *Ae. aegypti* mosquito with a periodate concentration of <1 mmol inhibit the adhesion of ookinetes in the midguts, and they also found that free N-acetylneuraminic acid competed for ookinete binding to midguts. Interestingly, Barreau et al. [61] found that wheat-germ agglutinin (WGA) lectin, which binds residues of N-acetylglucosamine, blocks the interaction between *Plasmodium gallinaceum* sporozoites and the surface of *Ae. aegypti* SGs. WGA is a *Triticum vulgaris* lectin that specifically recognizes N-acetylglucosamine residues, but it also has regions that interact with Sia residues. These reports suggest the possible participation (and presence) of sialic acid in the interactions between mosquito tissue and *Plasmodium*. Colpitts et al. [9] reported that Sia residues are important for the recognition of DENV in mammalian (Vero and LLC-MK2) cells, and a large number of DENV binding molecules are known [58, 62–66]. However, there have been no evaluations of the possible role during DENV-vector-host transmission.

In the present study, we found that a sialylated saliva glycoprotein (45 kDa Figure 5(b) lane 1) of *Ae. aegypti* forms complexes with DENV. This protein belongs to the D7 proteins family and is secreted in the saliva [21]; thus, it could be implicated in DENV host transmission. The modulation of DENV infection in different mammalian cells by *Aedes* salivary extracts and the observation that desialylated salivary proteins decrease DENV internalization highlight the key roles played by sialylated molecules during DENV vector-host interactions. Several studies have demonstrated the effects of arthropod saliva on vertebrate responses in a wide range of disease models using various hosts, arboviruses, and mosquito species [5, 6, 67, 68]. In all cases, an increase in virus transmission, modification of host susceptibility, or disease progression were observed. The enhancement of infection as a result of SG extracts is attributed to the modulation of host immune response, reduction of T-lymphocytes, and antiviral activity [69]. In the current study, we detected enhanced DENV internalization in presence of *Aedes* SG extracts, but the virus internalization decreased when the salivary proteins were pretreated with sialidase. In agreement with our results, Surasombatpattana et al. [70, 71] observed enhanced DENV infection of human keratinocytes in the presence of SG extracts. Recently, Conway et al. [7] reported that the *Aedes aegypti* saliva serine protease activity enhances dissemination of DENV into the mammalian host, although the role of Sia was not considered. Identification of molecules that mediate infectivity enhancement will allow for the production of vector-based vaccines and therapeutics that will target arthropod saliva components and interfere with viral transmission, as is exemplified by antimaxadilan (MAX) and anti-SP15 vaccines [72, 73]. These data may represent a general property for other vector-borne pathogens as is the case of *Plasmodium*. The knowledge of early DENV-host interactions could lead to a better understanding of viral tropism and pathogenesis and provide information for the development of new strategies for the control of DENV transmission.

To our knowledge, this is the first report of the participation of Sia structures during early interactions between DENV and *Ae. aegypti* mosquito tissues.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Research Article

Occurrence of West Nile Virus Antibodies in Wild Birds, Horses, and Humans in Poland

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Serum samples of 474 wild birds, 378 horses, and 42 humans with meningitis and lymphocytic meningitis were collected between 2010 and 2014 from different areas of Poland. West Nile virus (WNV) antibodies were detected using competition enzyme linked immunosorbent assays: ELISA-1 ID Screen West Nile Competition, IDvet, ELISA-2 ID Screen West Nile IgM Capture, and ELISA-3 Ingezim West Nile Compac. The antibodies were found in 63 (13.29%) out of 474 wild bird serum samples and in one (0.26%) out of 378 horse serum samples. Fourteen (33.33%) out of 42 sera from patients were positive against WNV antigen and one serum was doubtful. Positive samples obtained in birds were next retested with virus microneutralisation test to confirm positive results and cross-reactions with other antigens of the Japanese encephalitis complex. We suspect that positive serological results in humans, birds, and horses indicate that WNV can be somehow closely related with the ecosystem in Poland.

1. Introduction

West Nile virus (WNV) can affect a wide range of bird species, horses, and humans. The virus is an emerging agent responsible for diseases in these animals and humans worldwide. The main vectors of WNV are several species of the blood sucking insects, which can transmit the virus to birds, horses, and humans [1].

The infections are characterised by high pyrexia, paralysis, and morbidity caused by the factors so far acknowledged as pathogenic only for the animals. More often infections are characterised by mild clinical signs [2].

WNV is on the list of the World Organization for Animal Health (OIE) as the neurotropic factor causing the disease under the obligation to notify. West Nile Fever (WNF) caused by the virus is a zoonosis, which is the major public health problem in USA [3].

WNV is an arbovirus belonging to the Flaviviridae family, genus *Flavivirus* included in Japanese encephalitis antigenic complex induced by related antigenically Japanese encephalitis virus (JEV), St. Louis encephalitis virus (SLEV), Murray Valley encephalitis virus (MVEV), and Usutu virus (USUV). The ssRNA+ genome of the virus contains a single open reading frame from 11.000 to 12.000 nucleotides. The genome of the virus consists of seven nonstructural proteins, NS1, NS2a, NS2b, NS3, NS4a, NS4b, and NS5, and three structural proteins: glycoprotein E, core protein C, and premembrane protein prM [4].

Tropical and migratory birds, which belong to different species, are the main reservoir of the virus [5]. Lineage 1 of WNV is a lineage isolated over the world, but lineage 2 was only isolated in Africa and Madagascar, until Hungarian outbreaks of WNV, which was caused by a novel lineage 2,

which was introduced to Hungary, most likely by migratory birds from Africa, in 2004 [6]. The same lineage 2 caused in summer of 2010 endemic neuroinvasive disease (WNND) in humans in Greece with 262 confirmed cases and 35 deaths. In 2011 the virus spread to central Greece, but with fewer cases, 101 diagnosed and 9 fatalities. The total number of people in Greece infected by the virus is estimated to 18000 [7].

Over 300 different bird species were infected by the WNV. Usually, the virus circulates between wild birds and mosquitoes in closed cycle and can be carried by the birds during their migrations to the different regions [8].

The virus occurs in many countries around the world and also in 20 European countries, including countries, which are close neighbours of Poland, where the presence of the virus has been confirmed [9–12]. The presence of WNV antibodies was also confirmed in serum samples from wild birds and humans in Poland [13, 14].

In 2010, human morbidity and mortality caused by WNV infection were reported in Greece, Russia, Romania, Italy, and Israel [15]. the circulation of Usutu virus has been also confirmed in Poland [13]. No other studies have been published on the circulation of other viruses responsible for Japanese encephalitis complex in Poland.

The aim of the study was the detection of WNV antibodies in serum samples from different wild birds, horses, and hospitalised patients with neurological symptoms.

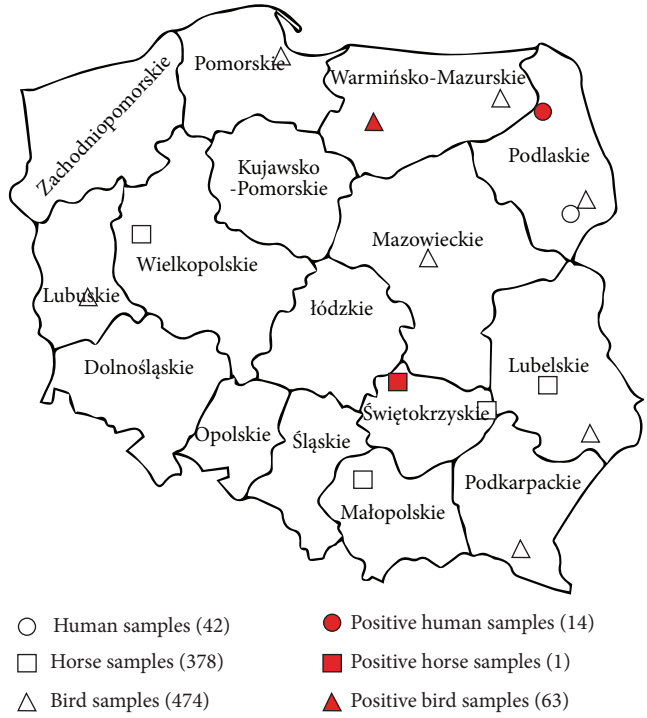

Figure 1: Map of Poland. Areas where the samples were collected.

2. Materials and Methods

Birds. Serum samples from 474 wild birds, 400 white storks (*Ciconia ciconia*), 21 common pheasants (*Phasianus colchicus*), 19 common chaffinches (*Fringilla coelebs*), nine wild ducks (*Anas platyrhynchos*), four white-tailed Eagles (*Haliaeetus albicilla*), two western capercaillies (*Tetrao urogallus*), six passenger pigeons (*Ectopistes migratorius*), three northern goshawks (*Accipiter gentilis)*, two hooded crows (*Corvus cornix*), three northern goshawks (*Accipiter gentilis)*, and one common swift (*Apus apus*), common blackbird (*Turdus merula*), common starling (*Sturnus vulgaris*), common raven (*Corvus corax*), and common buzzard (*Buteo buteo*), were collected in different locations in Poland (Zoological Gardens, Rehabilitation Centre of Protected Animals). However, most of the samples derived from Wild Birds Rehabilitation Center, Albatros Foundation. Samples have been collected during March–October, 2010–2014, when the mosquito's activity was the highest (Figure 1).

Horses. 378 serum samples have been obtained from healthy domesticated horses from few ranches located in four districts. The serum samples were taken also from racehorses (Figure 1).

Human. Serum samples derived from 42 patients (17 men and 25 women) from Department of Infectious Diseases and Neuroinfections, Medical University of Bialystok. The patients displayed neurological symptoms characteristic of meningitis, lymphocytic meningitis, and tick-borne encephalitis. They were hospitalised between May and September, 2010. All patients were tested for tick-borne encephalitis virus antibodies by Enzygnost Anti-TBE/FSME Virus, Siemens [16]. The samples were preserved at −20°C.

All examined samples were double-checked and tested under biosafety level 3+ conditions.

ELISA-1. Serological study was performed with commercially available competition, ELISA ID Screen West Nile Competition (Innovative Diagnostics, Montpelier, France), for detection of West Nile virus antibodies against the pr-E and pr-M envelope proteins containing an epitope common to Japanese encephalitis virus according to the manufacturer's protocol. All samples were examined twice. Microplates were read at 450 nm. The ELISA validated when the residual binding rations (S/N%) were calculated. Serum samples with S/N rations equal to or lower than 40% have been considered as positive; samples with rations higher than 50% were considered as negative. S/N values between 40% and 50% were doubtful.

ELISA-2 was performed using ID Screen West Nile IgM Capture (Innovative Diagnostics, Montpelier, France). Wells were coated with IgM polyclonal antibody. When the positive results were obtained, the presence of antibodies appeared as blue solution and after the addition the stop solution became yellow. In the absence of antibodies, no coloration appeared. Plates were read at 450 nm.

ELISA-3 was performed using Ingezim WNV Compac enzymatic assay (Ingenasa, Spain) based on the blocking ELISA, in which a monoclonal antibody (MAb) specific to the protein E of WNV was used. If antibodies were present in serum samples, they bound to the antigen. When MAb

specific to protein E was added, it bound to the antigen that was not blocked by antibodies from serum sample. In case of antibodies blocking the antigen, conjugate did not bind it. The assay was read by a colorimetric reaction after the substrate addition. Samples were considered as positive when the OD values were equal to or lower than the positive control sera samples. Samples were considered as negative when the OD value was equal to or higher than the negative cut-off. Samples with OD value in the range of both values were considered as doubtful.

Virus Microneutralisation Test. Virus microneutralisation test with ELISA positive samples from wild birds was conducted in the European Reference Laboratory, ANSES, France. Vero cells and West Nile virus, strain IS-98-ST1, were used in the test. The test was based on the OIE standard procedure [17].

3. Results

Districts of Poland and areas where the samples were collected are shown in Figure 1.

Most of the samples were taken from the birds during the assignation and veterinary treatments. Some of the birds were at the rehabilitation center after some accidents. Birds did not manifest any symptoms of neurological diseases and any signs of neurological infections. Every bird was supplied by the organization that send the samples with the information about the location, gender, age, and when it was possible, about travel history.

The examinations of serum samples revealed specific WNV antibodies in 63 samples from wild birds: 62 from white storks and one from common chaffinch. One positive serum sample was obtained from mare.

Concerning human serum samples, 14 out of 42 were positive and one was doubtful. All the patients had history of mosquito and tick bites. Tick-borne encephalitis was diagnosed in 16 patients [16].

To confirm the results, ELISA-2 was performed to detect serum WNV IgM specific antibodies as a first line of immunity response. The results were negative and therefore did not confirm the presence of IgM antibodies or any evidence of recent infection of the animals.

To verify results obtained by ELISA-1, ELISA-3 was performed, and the presence of specific WNV antibodies was confirmed in these cases. The results are presented in Table 1.

For confirmation of positive results, birds' positive serum samples were retested by virus microneutralisation test. All 63 serum samples from wild birds were confirmed and identified as positive for WNV antibodies with the titre 10 in 60 samples and titre 30 in three samples. Concerning examinations of other members of JECV, samples were also negative for Usutu virus. No cross-reactivity reactions were observed.

Comparison of the results obtained by different diagnostic methods (ELISAs and virus microneutralisation tests) is presented in Table 1.

Table 1: Comparison of results obtained by different methods.

	Serum		
	Wild birds	Horses	Humans
ELISA ID Screen West Nile Competition IDvet	63*/474** (13.29%)	1/378 (0.26%)	14/42 (33.33%)
ELISA ID Screen West Nile IgM Capture ID-vet	0/474	0/378	0/42
Ingezim West Nile Compac	63/474	1/378	14/42
Virus microneutralisation test (on positive samples)	63/63	—/—	—/—

*Positive sera.
**Total number of examined samples.

4. Discussion

During the last years, it has been noted that WNV spreads in the countries with moderate climate. mosquitoes from the Culicidae family are the most common vector of the virus spreading [8, 10, 18–21] and nowadays six of the mosquito species exist in Polish climate zone [13, 22]. In the nineties of the last century, Juřicová et al. [20] using haemagglutination inhibition assay confirmed WNV antibodies in 12.1% of house sparrows (*Passer domesticus*) and in 2.8% of Eurasian tree sparrows (*Passer montanus*) in Campinas forest area in Poland. In the next few years, WNV antibodies were found in three storks, one crow (*Corvus corone cornix*), and one mute swan (*Cygnus olor*) on the other part of Poland [13].

We have performed three different ELISAs to present and then confirm the obtained results. The first ELISA was ID Screen West Nile Competition, test specific for WNV, but it also gives serological cross-reactions among the JECV members. When positive results were obtained, ELISA-2 West Nile IgM Capture test was performed, which is specific only for WNV and allows excluding cross-reactions but detects only IgM. It means that we can identify only recent infection and cannot detect IgG antibodies. ELISA-3 was used to confirm the obtained results by ELISA-1 and the results were confirmed in 100% of cases.

In our study, positive results with serum samples of wild birds obtained by the ELISAs were confirmed by virus microneutralisation test. Additionally, ELISA showed WNV antibodies in 14 out of 42 serum samples from patients with symptoms of meningitis, lymphocytic meningitis, and tick-borne encephalitis, but we were unable to verify the obtained results with plaque reduction neutralisation test (PRNT). WNV infection in humans and identification of WNV in cerebrospinal fluid were usually confirmed by RT-PCR, Nested PCR [4, 5, 23–26], or in formalin-fixed, paraffin-embedded human tissues by RT-PCR. According to the study of Czupryna et al. [16], all samples of cerebrospinal fluid from 24 patients hospitalised at the Department of Infectious Diseases and Neuroinfections, Medical University of Bialystok, were negative for WNV RNA.

serum samples of humans, birds, and horses were used as proper samples to determine specific WNV antibodies. If we did confirm the specific antibodies in serum samples,

the animal or human was considered as having contact with the virus (in Poland or outside the country). It is supposed that positive wild birds could have contact with the virus outside the country. Concerning the positive samples from humans, 11 patients never left the country; that means they had to have the contact with the virus already in Poland. The same is in a case of the horses, which, according to their travel history, never left the country.

On the basis of epidemiological situation in Europe, the role of wild birds in WNV transmission and results of previous serological examinations of humans and birds [10, 20, 22, 26, 27] indicate that the presence of WNV in Poland can be possible. In Poland, WNV antibodies were detected in febrile woman [22] and in healthy forest workers from regions of Swietokrzyskie (28.85%) and Podlaskie (34.14%) [27]. Our serological results can suggest that the virus is already present in our climate zone and in our ecosystem. The possibility of its cross-reaction with viruses of tick-borne encephalitis and endemic disease should be taken into consideration.

Ethical Approval

The study was approved by the II local Ethics Commission in Lublin (number 92/2010 of November 16, 2010) and got the permission of the General Director of Environmental Protection (number DOP-OZGŁZ. 6401.03.36.2011.dł).

Conflict of Interests

The authors declare no conflict of interests.

Acknowledgments

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Research Article

Identification of the Alternative Splicing of the UL49 Locus of Human Cytomegalovirus

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The UL49 ORF of human cytomegalovirus (HCMV) is essential for viral replication; conserved among all herpes viruses; however, the function is unclear. Once the UL49 ORF was precisely deleted from the start to stop codon, the mutant did not yield infectious progeny. In this study, we find out many alternatively processed ESTs in UL49 locus in HCMV-infected cells, in which there are two novel transcription termination sites in UL49 locus. Most of these ESTs are rare transcripts that contain directed repeat sequences in the intron splicing regions. There is a typical GU-AG intron splicing site in UL49Y transcripts. The 1847 bp UL49Y cDNA spans an ORF from 335 to 1618 and encodes a putative protein of 427 amino acids with a predicted molecular mass of 47.1 kDa. All the new EST sequences and UL49Y cDNA sequence have been deposited in the GenBank database (GenBank Accession nos. GW314860-GW314900 and GU376796). This study provides us with very important clues for revealing the importance of the UL49 locus alternative splicing.

The human cytomegalovirus (HCMV), a β-herpes virus, is the most common cause of congenital infection and an important pathogen in immunocompromised individuals [1]. As the largest virus in the herpes virus family, the HCMV genome comprises ≈230 kb of double-stranded linear DNA [2]. Evaluation of the genome led to estimates of the number of protein-coding ORFs ranging from a maximum of 252 potentially functional ORFs that are conserved in different clinical isolates to a minimum of 165 ORFs that are conserved between HCMV clinical isolates [3–5]. Another significant uncertainty to the number of ORFs is our incomplete understanding of HCMV splicing. A variety of spliced mRNAs have been successfully identified [6–9], but so far there has been no exhaustive experimental search for spliced HCMV mRNAs. It is not possible to predict splice donors and acceptors with certainty.

HCMV UL49 ORF with unclear function is conserved among all herpes viruses. Once the UL49 ORF was precisely deleted from the start to stop codon, the mutant did not yield infectious progeny even after repeated transfection and extensive incubation [10]. Loss of replication due to a deletion of the whole UL49 locus may be caused by deletion of essential overlapping ORFs because the UL49 ORF overlaps with UL50 ORF and UL48.5 ORF nearby. The UL48, UL48.5, and UL50 ORFs all encode essential proteins. Since our recent study suggested a novel alternative transcript originating from the UL49 locus of the HCMV genome, which is different from the UL49 ORF and UL48.5 ORF, we still do not know whether there are new transcripts or not in UL49 ORF. For this reason we thoroughly examined HCMV-infected cells for more alternatively processed ESTs in UL49 locus.

Towne HCMV BAC containing a cassette for GFP eukaryotic expression was a gift from Professor Fenyong Liu, University of California, Berkeley [11]. Human dermal fibroblasts neonatal (HDFn) (Cascade Biologics) cells were electroporated with the Towne HCMV BAC DNA and an expression plasmid of the HCMV pp71 tegument protein, which can increase the infectivity of HCMV BAC DNA. Then we plated cells onto 75 cm^2 flasks and observed for GFP expression under fluorescence microscopy. Infected cell culture media

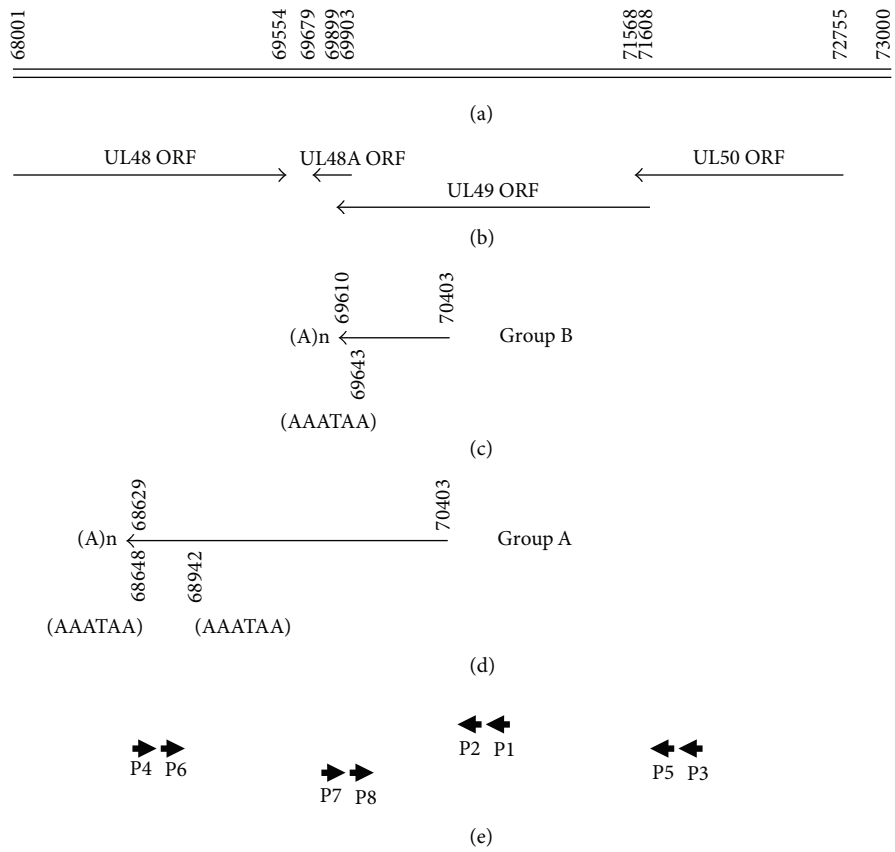

Figure 1: Organization and transcription summary of HCMV Towne UL49 locus. (a) Nucleotide positions correspond to the genome sequence under GenBank Accession no. AY315197.2. (b) ORF map summary of the UL48, UL48A, UL49, and UL50. (c) cDNAs and the location of polyadenylation signals and polyadenylation site are identified in group B. (d) cDNAs and the location of polyadenylation signals polyadenylation site are identified in group A. (e) Primers (Table 1) corresponding to the nucleotide position of HCMV Towne genome used in this paper.

were cleared of cell debris by low-speed centrifugation and collected as stocks of cell-free virus.

HDFn cells were infected with the cell-free virus in Dulbecco's modified Eagle medium supplemented with 10% fetal calf serum (GIBCO/BRL). The final mixture will contain 10 μg/mL gentamicin and 0.25 μg/mL amphotericin B. Cells were cultured at 37°C in a humidified incubator with 5% CO_2. RNA was extracted from infected HDFn cells cultured in six-well plates at various time points (2 h, 6 h, 12 h, 48 h, 72 h, and 96 h) after infection. To select for IE transcripts, cells were treated with the protein synthesis inhibitor cyclohexamide (100 μg/mL) for 1 h prior to infection and throughout the 24 h infection period, when cells were harvested for RNA isolation. To select for E (early) transcripts, viral DNA replication inhibitor phosphonoformic acid (100 μM) was added to the medium after the 24 h infection period, and cells were harvested 72 h after infection [11]. Total RNA of all samples was isolated using TRIzol (Invitrogen).

To define the 3′ end of the UL49 locus alternative mRNAs, we carried out the RACE method with 3′-Full RACE Core Sets (Takara) and all the primers have been listed in Table 1 and Figure 1. The cDNA template was synthesized with the Oligo dT-3sites Adaptor Primer of the 3′-Full RACE Core Set according to the manufacturer's instructions. The 1st PCR was performed with the 3′ RACE Outer primer (5′-TAC CGT CGT TCC ACT AGT GAT TT-3′) and Primer 1 in 50 μL of the following reaction: 1 × LA PCR Buffer, 0.4 mM dNTP mixture, 0.2 μM each primer, 2.5 U LA Taq polymerase, and 1 μL of cDNA template. PCR amplification was done at 95°C for 5 min and followed by 30 cycles at 94°C for 30 s, 55°C for 1 min, and 72°C for 3 min. The 2nd PCR reactions and conditions were the same as the 1st PCR except for using the 3′RACE Inner primer (5′-CGC GGA TCC TCC ACT AGT GAT TTC ACT ATA GG-3′) and Primer 2, 1 μL the outer PCR product as the template. Each sample was analyzed on a 1.5% agarose gel. Gel-purify of the PCR products were performed by using Gel Extraction Kit (E.Z.N.A.). Then all the isolated fragments were directly cloned into the TA-vector pMD18-T (TaKaRa) and sequenced using the sequencing Primer RV-M and Primer M13-47. Through the 3′-RACE PCR approaches, 10 novel cDNA fragments were cloned, with length from 188 bp to 1594 bp. These new EST sequences have been deposited in the GenBank database (GenBank Accession no. GW314860-GW314869) (Table 2). The various sequences comprised two groups. The group was characterized by the usage of a distinct alternative poly(A)

Table 1: List of primers used in this paper.

Primer	Nucleotide sequence (5′-3′)	Corresponding to the nucleotide position of HCMV Towne genome
Primer 1	CTGCCAGCAAAACTTTCCGCT	70442–70422
Primer 2	TCGGGACCGTCAAGAAAAGAGCG	70403–70381
Primer 3	TGGTGCTGGCTCTCCTGCTGGTGC	71653–71630
Primer 4	GCATGTAGCCGACCTGCTGAAAGGC	68717–68741
Primer 5	ATGGCCAATCGCCGTCTCCGACAC	71608–71585
Primer 6	AGACCGCGACTTCCTCCGCATCCA	68783–68806
Primer 7	GTTACAAAACAACGTATCACTTTTACGG	69612–69639
Primer 8	CTTGGGCTGTCAGCGCCGGGTG	69667–69688
3′RACE Outer primer	TACCGTCGTTCCACTAGTGATTT	3′-Full RACE Core Sets kit supply
3′RACE Inner primer	CGCGGATCCTCCACTAGTGATTTCACTATAGG	3′-Full RACE Core Sets kit supply

sites within the 3′ untranslated region (3′UTR). Group A has two polyadenylation signals (AATAAA) located at 68947-68942 and 68648-68643 with the polyadenylation site at 68629. Group B has one polyadenylation signal (AATAAA) located at 69643-69638 with the polyadenylation site at 69610 (Figure 1).

According to the 3′ end of group A/B and the UL49 ORF 5′ end, primers were designed to do the nested PCR. The first-strand cDNA was synthesized using Takara 1st Strand cDNA Synthesis Kit. Total RNA samples (3 μg) were reverse transcribed in a 10 μL volume in the presence of 5 μM Oligo dT Primer, 1 mM dNTP mixture. The reaction tube was incubated at 65°C for 5 min, followed by keeping in the ice. Then the following reagents were added to each reaction tube: 1 × PrimeScript Buffer, RNase Inhibitor 20 U, PrimeScript RTase 200 U, and RNase free DEPC H_2O 4.5 μL (Takara, Japan). Samples were incubated at 42°C for 60 min and 70°C for 15 min and then stored at 4°C. Then nested PCR was performed. For amplifying the transcripts from the UL49 ORF 5′ end to the group A, the outer PCR reactions volume is 50 μL, which contains 1 μL synthesized cDNA template, 1 × LA PCR Buffer, 0.4 mM dNTP mixture, 0.2 μM Primer 3 and Primer 4, and 2.5 U LA Taq polymerase. Amplification conditions were 94°C for 30 s, 55°C for 1 min, and 72°C for 3.5 min for 30 cycles. The inner PCR reactions and conditions were the same as the outer PCR except for using Primer 5 and Primer 6, 1 μL the outer PCR product as the template. To amplify the transcripts from the UL49 ORF 5′ end to group B, the outer PCR was performed with the Primer 3 and Primer 7, and inner PCR was performed with Primer 5 and Primer 8. The products of nested PCR amplification were inserted into pMD18-T cloning vector. The recombinant plasmid was transformed into *E. coli* DH5α. The amplicons were sequenced using Primer RV-M and Primer M13-47. A complete list of the cDNA clones and their positions relative to genomic DNA is listed in Tables 3 and 4. These new EST sequences have been deposited in the GenBank database (GenBank Accession no. GW314870-GW314900). We identified the situation of these transcripts in HCMV genome by BLAST software (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

All these transcripts in UL49 locus have not been reported before. All the cDNA clones were acquired by using RACE and nested PCR. The results show the defects of bioinformatics methods in predicting alternatively spliced transcripts on one hand. In addition, nested PCR method was sensitive enough to find more transcripts. In fact, there were a lot of rare transcripts in this locus. The alternatively spliced UL49 variants detected suggest the complexity of transcription in the UL49 locus. We summarized all the novel transcripts, which was mapped with the R package software (ver.3.1.1) (Figure 4).

Most of these novel transcripts have directed repeat sequences in intron splicing regions, rather than typical RNA splice site GU-AG. The directed repeat sequences existed in many viruses, with different lengths and functions [12–16]. Further research was required in order to clarify the importance of directed repeat sequences in UL49 locus. All these transcripts in HCMV UL49 locus had not been found in past. It might be because of low abundances, which is similar to the transcripts in HCMV UL37 locus. Alternatively UL37 spliced variants are exceedingly low abundances relative to the UL37x1 unspliced transcript, some ~100 fold less and below detection by RT-PCR and gel detection, so the alternatively UL37 spliced variants cannot be detected by either S1 or Northern blot analysis [17, 18]. Consistent with these results, some UL49 spliced variant cDNAs were too low abundances to be rarely obtained in the HCMV-infected cells (Figure 2).

The functions of all the rare UL49 transcripts were unclear. We speculated that the functions of these transcripts might be for encoding virus proteins, or that they might play roles in regulating host cells or viral genes in the form of RNA. We will further do some research on the functions of these RNAs. Nonetheless, alternative processing of known HCMV transcripts results in the production of functionally different gene products. In the best-studied locus, differential processing of the major IE pre-mRNAs leads to the production of multiple spliced and polyadenylated RNAs. Moreover, Alwine has recently identified novel IE1 RNA splice variants, whose abundances differ during HCMV infection; however,

Table 2: Sequences of novel HCMV UL49 alternatively spliced RNA fragments by 3′RACE.

Name of novel transcript	HCMV sequence (nt)[a]	Nested PCR product length (bp)	Intron length (bp)	Splice donor (**exon**/intron)[b]	Splice acceptor (intron/**extron**)[b]	GenBank accession number
UL49RA1	70403–70116; 68959–68629	619	1156	**GTCTCCTGCG**/CCTCACTTCG	GTCCTGTGCG/**TTGTAAATGA**	GW314860
UL49RA2	70403–70271; 68672–68629	177	1598	**TGGACAGCGC**/TGCGTACGCG	AGCCCAGCGC/**GTAGTCTAGG**	GW314861
UL49RA3	70403–70138; 69948–68626	1456	189	**GTACGCGGCG**/TGACGGTCCC	GCGCGCGGCG/**GTTTAGCAGC**	GW314862
UL49RA4	70403–70121; 68966–68626	624	1151	**GCGTCTCCTG**/CGCCTCACTT	TGTGGTCCTG/**TGCGTTGTAA**	GW314863
UL49RB1	70403–70146; 70001–69610	650	144	**CTGTGGTGGT**/ACGCGGCGTG	GCGCGCTGGT/**TAAACAGACC**	GW314864
UL49RB2	70403–70163; 70092–69610	724	70	**GCGGCACGCACCTGC**/GACTC	GGGCACGGCC/**TGCTGTGCGC**	GW314865
UL49RB3	70403–70207; 69777–69610	365	429	**TACGATCACC**/GGCTGGCTTT	GTTGGCCACC/**ATGCTGAGCA**	GW314866
UL49RB4	70403–70090; 69906–69610	611	183	**CGGGCACGGCCTGC**/TGTGC	CCACGGCCTGCC/**CCATGTCT**	GW314867
UL49RB5	70403–70133; 69857–69610	519	275	**CGGCGGGACG**/GTCCCGCGTC	AAGCGGGACG/**AAAAACACCG**	GW314868
UL49RB6	70403–70138; 69948–69610	605	189	**GTACGCGGCG**/TGACGGTCCC	GCGCGCGGCG/**GTTTAGCAGC**	GW314869

These RNA fragments were acquired by nested PCR with Primer 1 and 3′RACE Outer primer for outer PCR and Primer 2 and 3′RACE Inner prime for inner PCR. [a]Numbering refers to HCMV genomic sequences (GenBank Accession no. AY315197.2). [b]Underlines indicate direct repeat sequences.

Table 3: Novel sequences of HCMV UL49 alternatively spliced RNA fragments (group A).

Name of novel transcript	HCMV sequence (nt)[a]	Nested PCR product length (bp)	Intron length (bp)	Splice donor (**exon**/intron)[b]	Splice acceptor (intron/**extron**)[b]	GenBank accession number
UL49A1	71608–71124; 69003–68783	706	2120	**GTTTCGAACG**/CTACGACACC	TGTTCGAACG/**TGGAGGGCGG**	GW314870
UL49A2	71608–71530; 70080–68783	1377	1449	**CTCTTCGCGC**/CCCTCTGCGT	CTGTGCGCGC/**GCTGTCAGAC**	GW314871
UL49A3	71608–71321; 69108–68783	614	2212	**TCTGAATCGC**/GAGCTGGGCG	GGAGCGGCGC/**CAGACGCAGC**	GW314872
UL49A4	71608–71490; 70001–68783	1338	1488	**TGCAGCTGGT**/GATCGGCCGC	GCGCGCTGGT/**TAAACAGACC**	GW314873
UL49A5	71608–71288; 68963–68783	502	2324	**ACGCTTCCTG**/CGCGAATGGC	TGTGGTCCTG/**TGCGTTGTAA**	GW314874
UL49A6	71608–71457; 69101–68783	381	2355	**CGGAGGAAGC**/GGCGGTAGAA	CGCCAGACGC/**AGCGACGTTG**	GW314875
UL49X	71608–71530; 69188–68783	485	2341	**CTCTTCGCGC**/CCCTCTGCGT	GAACTCGCGC/**GATGGGTGGC**	GW314876
UL49A8	71608–71416; 69108–68783	519	2307	**TTTGCGGCGC**/AGACGTCGGC	GGAGCGGCGC/**CAGACGCAGC**	GW314877
UL49A9	71608–71565; 69157–68783	419	2407	**CCGCGACTGA**/GGAGTTCCAC	CAGAAACTGA/**ATATTGACTG**	GW314878
UL49A10	71608–71562; 69450–68783	715	2111	**CGACTGAGGA**/GTTCCACCAG	GCACTGAGGA/**CGTTCTGCGT**	GW314879
UL49A11	71608–71321; 68989–68783	495	2331	**TCTGAATCGC**/GAGCTGGGCG	GGGCGGTCGC/**AGCACGCGTA**	GW314880
UL49A12	71608–71515; 69142–68783	454	2372	**TGCGTTCACG**/AGGACCATTT	GACTGGCACG/**CGGTTGAAAG**	GW314881
UL49A13	71608–71519; 68957–68783	265	2561	**CCTCTGCGTT**/CACGAGGACC	CCTGTGCGTT/**GTAAATGACT**	GW314882
UL49A14	71608–71478; 69231–68783	580	2246	**TCGGCCGCGG**/TGCGCTGCAG	GCGGCCGCGG/**CCGCTCGATG**	GW314883
UL49A15	71608–71561; 70695–68783	1961	865	**GACTGAGGAG**/TTCCACCAGG	CGCGGAGGAG/**GCTCGACGGC**	GW314884
UL49A16	71608–71276; 71180–71114; 69028–68783	646	95[C] 2085	**CGAATGGCTG**/GTGTGTCGGC **CTACGACACC**/GACTACCTGC	ACGTCTACAG/**CATGGACTGT** GTCCAACACC/**AGCCGACTGT**	GW314885
UL49A17	71608–71276; 71180–71079; 69107–68783	760	95[C] 1971	**CGAATGGCTG**/GTGTGTCGGC **TCTACCCGCC**/CGAGCGGCTG	ACGTCTACAG/**CATGGACTGT** GAGCGGCGCC/**AGACGCAGCG**	GW314886

Table 3: Continued.

Name of novel transcript	HCMV sequence (nt)[a]	Nested PCR product length (bp)	Intron length (bp)	Splice donor (**exon**/intron)[b]	Splice acceptor (intron/**extron**)[b]	GenBank accession number
UL49A18	71608–71276; 71180–70683; 68860–68783	909	95[C] 1822	**CGAATGGCTG**/GTGTGTCGGC **CGACGGCGGC**/AGCTGCGGCG	ACGTCTACAG/**CATGGACTGT** CTCCAGCGGC/**GTTTCGGTCC**	GW314887
UL49A19	71608–71276; 71180–71055; 69115–68783	792	95[C] 1939	**CGAATGGCTG**/GTGTGTCGGC **CGTTGTTGGA**/CGGTGTCACC	ACGTCTACAG/**CATGGACTGT** CGGTGTTGGA/**GCGGCGCCAG**	GW314888

These RNA fragments were amplified by nested PCR with Primer 3 and Primer 4 for outer PCR and Primer 5 and Primer 6 for inner PCR. [a]Numbering refers to HCMV genomic sequences (GenBank Accession no. AY315197.2). [b]Underlines indicate direct repeat sequences. [C]Introns conform to the GU-AG rule.

Table 4: Sequences of novel HCMV UL49 alternatively spliced RNA fragments (group B).

Name of novel transcript	HCMV sequence (nt)[a]	Nested PCR product length (bp)	Intron length (bp)	Splice donor (**exon**/intron)[b]	Splice acceptor (intron/**extron**)[b]	GenBank accession number
UL49B1	71608–71179; 70097–69667	861	1081	**GTCTACAGCA**/TGGACTGTCT	CGCACGGGCA/**CGGCCTGCTG**	GW314889
UL49B2	71608–71548; 70691–69667	1086	856	**CACCAGGCTC**/TGCGCCGTCT	GAGGAGGCTC/**GACGGCGGCA**	GW314890
UL49Y	71608–71276; 71180–69667	1847	95[C]	**CGAATGGCTG**/GTGTGTCGGC	ACGTCTACAG/**CATGGACTGT**	GU376796
UL49B4	71608–71282; 70097–69667	758	1184	**CCTGCGCGAA**/TGGCTGGTGT	CGCACGGGCA/**CGGCCTGCTG**	GW314891
UL49B5	71608–71527; 70021–69667	437	1505	**TTCGCGCCCC**/TCTGCGTTCA	GCGCCCCGCTG/**TGTCGGGGCT**	GW314892
UL49B6	71608–71284; 69951–69667	610	1332	**TTCCTGCGCG**/AATGGCTGGT	CAGGCGCGCG/**GCGGTTTAGC**	GW314893
UL49B7	71608–71576; 70166–69667	533	1409	**CGCTCCGCAC**/ACCGCGACTG	CGGCACGCAC/**CTGCGACTCC**	GW314894
UL49B8	71608–71501; 69968–69667	410	1532	**CCATTTCCAT**/GTGCAGCTGG	GCGGCCACAT/**TGTGCAGCAG**	GW314895
UL49B9	71608–71543; 70007–69667	407	1535	**GGCTCTGCGC**/CGTCTCTTCG	GGGGCTGCGC/**GCTGGTTAAA**	GW314896
UL49B10	71608–71533; 69925–69667	335	1607	**CGTCTCTTCG**/CGCCCCTCTG	TTCCTCTTCG/**TCCTCCCCCC**	GW314897
UL49B11	71608–71341; 70131–69667	733	1209	**TTCGTGACGG**/ATAAGCGCTT	GGCGTGACGG/**TCCCGCGTCT**	GW314898
UL49B12	71608–71276; 71180–70822; 69882–69667	908	95[C] 939	**CGAATGGCTG**/GTGTGTCGGC **CACGCCGGGA**/AGGTACCCTG	ACGTCTACAG/**CATGGACTGT** CGCGCCGGGA/**CCCACGGTGG**	GW314899
UL49B13	71608–69667	1942	—	—	—	GW314900

[a]Numbering refers to HCMV genomic sequences (GenBank Accession no. AY315197.2).

[b]Underlines indicate direct repeat sequences. [C]Introns conform to the GT-AG rule.

—: There is no intron in the transcript of UL49B13.

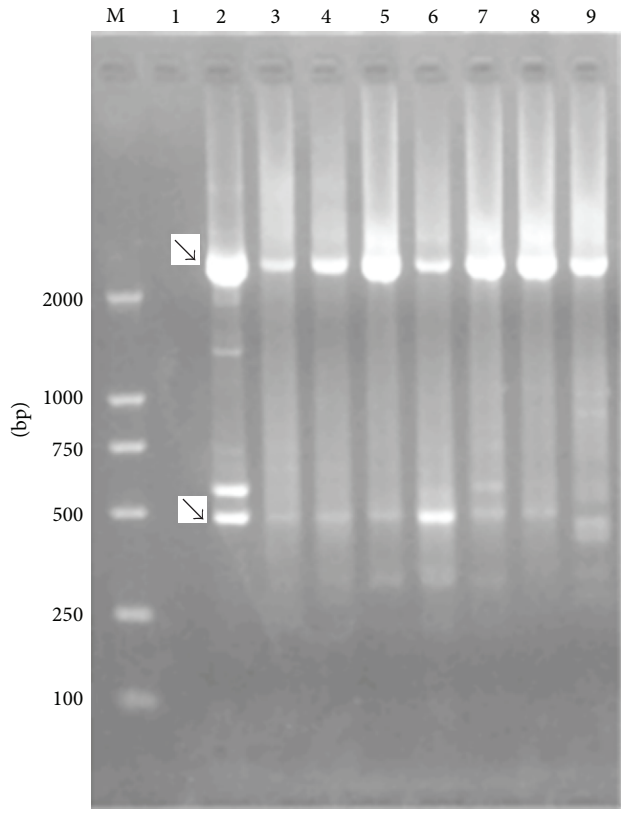

Figure 2: Agarose gel electrophoresis of differential transcripts of alternatively spliced UL49 cDNAs. Lane M: DNA Marker; Lane 1: cells uninfected by virus; Lanes 2 to 7: RT-PCR from HCMV-infected HDFn poly(A) RNA at 2, 6, 12, 48, 72, and 96 h postinfection, respectively; Lane 8: poly(A) RNA harvested at 72 h postinfection in the presence of inhibitor of DNA replication phosphonoformic acid (100 μM); and Lane 9: poly(A) RNA harvested at 24 h postinfection in the presence of inhibitor of protein synthesis cyclohexamide (100 μg/mL) 1 h prior to infection. UL49X and UL49Y were indicated by arrows.

their temporal expression is similar to that of IE1 mRNA. The products of the differentially spliced IE1 and IE2 transcripts differ in functions [17, 19].

Only the UL49X and UL49Y were detected from 2 h to 96 h after HCMV-infected cell and the other transcripts founded expressed temporally (Figure 2). UL49X and UL49Y can always be acquired, which might result from the fact that the UL49X and UL49Y have higher abundance and other transcripts have lower abundance. Although accurate splicing of the UL49X and UL49Y spliced junctions has been verified, we just obtained the EST fragments of UL49X and UL49Y, and the full-length UL49X and UL49Y cDNAs have not been cloned yet. Our next work is to obtain the UL49X and UL49Y full length cDNA.

The open reading frame was predicted by ORF finder program (http://www.ncbi.nlm.nih.gov/gorf/gorf.html). The full-length cDNA of UL49 began at 73134 bp and ended at 71047 bp, in which ORF began at 73043 bp and ended at 71331 bp. UL49X had the same 5′ end with UL49, but its 3′ end terminated at 68783 bp. A 2341 bp intron was deleted when UL49X cDNA transcript from the HCMV genome. So, it encoded a completely different protein from the UL49. The UL49X cDNA sequence has been deposited in the GenBank database (GenBank Accession no. GW314876). Because we could not search a complete ORF in UL49X by bioinformatics methods, we only analyzed the protein sequences of UL49Y with the complete ORF. The UL49Y cDNA sequence has been deposited in the GenBank database (GenBank Accession no. GU376796).

The 1847 bp UL49Y cDNA spanned an ORF from 335 to 1618 and encoded a putative protein of 427 amino acids with a predicted molecular mass of 47.1 kDa and an isoelectric point (pI) 9.35. There is an inframe stop codon at −72 prior to the first initiation codon. UL49Y cDNA contained a 95 bp intron and the intron conformed to the GU-AG rule (GGCTGgtgtg. . .tacagCATGGA). UL49Y is the truncated form of UL49, which lacks 143 aa in the 5′ end of UL49 (Figure 3).

We identified that UL49Y could encode a complete ORF by bioinformatics methods. But we need further experiments to prove whether it encodes a protein or not. UL49Y and UL49 have a common 3′ end, and current studies suggest that UL49 protein is an E (early) viral protein. In our experiment, we found that UL49Y began to express two hours after viral infection, which indicates that it is an IE (immediate early) protein. While UL49Y protein can continue to express till 96 hours, the different expression phase suggested that UL49Y may have distinct biological significance which is different from UL49. It is important for us to determine the functions of UL49Y.

Previous data reported that virus could not replicate if UL49 ORF was deleted [10]. Through bioinformatics analysis, we found that the deletion of UL49 also destroyed the UL50 ORF and UL49A ORF (Figure 1). And it may destroy the 3′UTR of UL48. We also found that the ORF of UL49Y was completely missing. Although so far we have not received full-length form of UL49X, but it is sure that the ORF of UL49X has also suffered damage when the UL49 ORF is deleted. We will examine the functions of different transcripts of this locus and reveal the molecular mechanisms why this locus is critical for virus replication. At meantime, we hope to learn the transcription situation of UL49 locus in other low passage CMV strains such as Merlin, TB40, and patient-derived clinical isolates.

Overall, we found two novel transcription termination sites in UL49 locus. In these two transcription termination sites, we found a large number of new transcripts, most of which are rare transcripts and contain directed repeat sequences in intron splicing regions. UL49X gene can express stably and has directed repeat sequences in intron splicing regions. There are typical GU-AG intron splicing sites in UL49Y transcripts. UL49Y might encode a full-length ORF. The above studies provide us with important clues for revealing the importance of the UL49 locus alternative splicing. This surprising transcript complexity makes the UL49 locus be the most complex of any known HCMV transcript [20].

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1     ATGGCCAATCGCCGTCTCCGACACGCTCCGCACACCGCGACTGAGGAGTTCCACCAGGCTCTGCGCCGTCTC
73    TTCGCGCCCCTCTGCGTTCACGAGGACCATTTCCATGTGCAGCTGGTGATCGGCCGCGGTGCGCTGCAGCCG
145   GAGGAAGCGGCGGTAGAAACGTCGCAGCCACCGGCGCAGTTTGCGGCGCAGACGTCGGCGGTCCTCCAGCAG
217   CAGCTGGTGCATCACGTGCCACGTTCTTGCGTCCTTCATCTCTTCGTGACGGATAAGCGCTTTCTGGATCGC
289   GAGCTGGGCGACCGTCTCTACCAACGCTTCCTGCGCGAATGGCTGCATGGACTGTCTGCACACCGTGGCCGT
                                                    M  D  C  L  H  T  V  A  V
361   CCGCACGATGGCCTTTCTGCGTTTCGAACGCTACGACACCGACTACCTGCTGCGCCGTCTGCGGCTCTACCC
       R  T  M  A  F  L  R  F  E  R  Y  D  T  D  Y  L  L  R  R  L  R  L  Y  P
433   GCCCGAGCGGCTGCACGCGTTGTTGGACGGTGTCACCTCCTCGCTGCTAGGTGACCTTCACCGTTTCCTTTT
       P  E  R  L  H  A  L  L  D  G  V  T  S  S  L  L  G  D  L  H  R  F  L  F
505   CGGGGTGGACCTTCGGCTGCCCGTGCTTCACCCCACCAGCTCGCCGTGCCTAGCACTACTACGCGCCAAGCG
       G  V  D  L  R  L  P  V  L  H  P  T  S  S  P  C  L  A  L  L  R  A  K  R
577   CTTCGACGCCCGCGCTGATTTGGCCGTATACCACCGTAACCAGTGGTGTCACCAACGGCAGCCGCGGTCGCC
       F  D  A  R  A  D  L  A  V  Y  H  R  N  Q  W  C  H  Q  R  Q  P  R  S  P
649   GCAGCTGCGGGGCCTCATCGCAGCCTTGCGCCGGCACGCCGGGAAGGTACCCTGCGGAAACCCGCTCTACGT
       Q  L  R  G  L  I  A  A  L  R  R  H  A  G  K  V  P  C  G  N  P  L  Y  V
721   ACTGGCCCGACAAGCGGTGCAGACGTTTTGCGACACGTGTCCGCGATACCTGGTGCCGTTGCGGGCACTGGG
       L  A  R  Q  A  V  Q  T  F  C  D  T  C  P  R  Y  L  V  P  L  R  A  L  G
793   TCTGCACGACGAGACGCGCGGAGGAGGCTCGACGGCGGCAGCTGCGGCGGTAGGACACGCGGGCGCCGGACA
       L  H  D  E  T  R  G  G  G  S  T  A  A  A  A  A  V  G  H  A  G  A  G  Q
865   GCAGGCGCGTCACGTAGAGCCCACCAAGATCGTCCTCTTTGCACTCAGCGCCGCGCTGCGCGGCGGCCTCAT
       Q  A  R  H  V  E  P  T  K  I  V  L  F  A  L  S  A  A  L  R  G  G  L  I
937   CGGCAGCGTCATCGACCTGCCGCTCTGGTGTCTGTGTCGACTCAAGTGCGAGCGCCATCTGGACGCGCGCTC
       G  S  V  I  D  L  P  L  W  C  L  C  R  L  K  C  E  R  H  L  D  A  R  S
1009  GCTGGTGGCCGTGGTCTGTCGGCAGTGCGGACACTGCCTTAACCTGGGTAAGGAAAAGCTACACTGCCAGCA
       L  V  A  V  V  C  R  Q  C  G  H  C  L  N  L  G  K  E  K  L  H  C  Q  Q
1081  AAACTTTCCGCTCAACTCGATGTTCTATTATCGGGACCGTCAAGAAAAGAGCGTCATCTTCAACACGCACGC
       N  F  P  L  N  S  M  F  Y  Y  R  D  R  Q  E  K  S  V  I  F  N  T  H  A
1153  CGAGCTCGTGCACTGCTCGCTCTGCGGCAGTCAGCGGGTGGTGCGGCAGCGCGTCTACGAGCTCGTCTCCGA
       E  L  V  H  C  S  L  C  G  S  Q  R  V  V  R  Q  R  V  Y  E  L  V  S  E
1225  GACGCTCTTTGGACAGCGCTGCGTACGCGTGGGCTGGAAAGCCGTGCTGGGCCTCAACGCGGCTTGTGCCGT
       T  L  F  G  Q  R  C  V  R  V  G  W  K  A  V  L  G  L  N  A  A  C  A  V
1297  GTACGATCACCGGCTGGCTTTCGACGTCATTCTGCCCTGCGCGGCACGCACCTGCGACTCCACTGTGGTGGT
       Y  D  H  R  L  A  F  D  V  I  L  P  C  A  A  R  T  C  D  S  T  V  V  V
1369  ACGCGGCGTGACGGTCCCGCGTCTCCTGCGCCTCACTTCGCACGGGCACGGCCTGCTGTGCGCGCGCTGTCA
       R  G  V  T  V  P  R  L  L  R  L  T  S  H  G  H  G  L  L  C  A  R  C  Q
1441  GACGGGCGAGTACCGCGACAGCTGTTTGGAAAGCGAAGACGGCGCCCCGCTGTGTCGGGGCTGCGCGCTGGT
       T  G  E  Y  R  D  S  C  L  E  S  E  D  G  A  P  L  C  R  G  C  A  L  V
1513  TAAACAGACCGCCTGCCACGTCGGCGGCCACATTGTGCAGCAGGCGCGCGGCGGTTTAGCAGCGGCTTCCTC
       K  Q  T  A  C  H  V  G  G  H  I  V  Q  Q  A  R  G  G  L  A  A  A  S  S
1585  TTCGTCCTCCCCCCACGGCCTGCCCCATGTCTAACACCGCGCCGGGACCCACGGTGGCCAACAAGCGGGACG
       S  S  S  P  H  G  L  P  H  V  *
1657  AAAAACACCGTCACGTCGTCAACGTCGTTTTGGAGCTGCCGACCGAGATATCAGAGGCCACTCACCCGGTGT
1729  TGGCCACCATGCTGAGCAAGTACACGCGCATGTCCAGCCTGTTTAATGACAAGTGCGCCTTTAAGCTGGACC
1801  TGTTGCGCATGGTAGCCGTGTCGCGCACCCGGCGCTGACAGCCCAAG
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Figure 3: Nucleotide and deduced amino acid sequence of the UL49Y gene. Numbers on the left refer to the first nucleotide in each corresponding line. An upstream inframe stop codon (TGA) at 5′ to the start codon is shaded in gray. An asterisk indicates the stop codon. The DNA sequence has been deposited in the GenBank database (GenBank Accession no. GU376796).

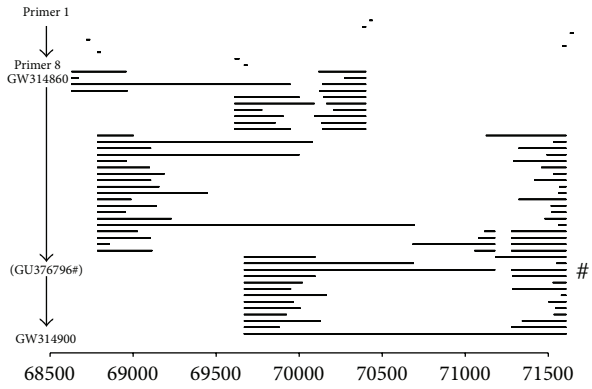

Figure 4: The summarize of all the alternative splicing transcripts founded in UL49 Locus. Primer 1–Primer 8 are the primers used in the research. The GW314850-GW314900 is the GenBank Accession number and the situations are the same in Tables 2, 3, and 4. The GU376796 and the pound symbol are the UL49Y. The numbers in x-axis mean the HCMV genome situation (AY315197.2).

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Authors' Contribution

Guang Yang and Wei Li contributed equally to this work.

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Review Article

The Global Ecology and Epidemiology of West Nile Virus

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Since its initial isolation in Uganda in 1937 through the present, West Nile virus (WNV) has become an important cause of human and animal disease worldwide. WNV, an enveloped virus of the genus *Flavivirus*, is naturally maintained in an enzootic cycle between birds and mosquitoes, with occasional epizootic spillover causing disease in humans and horses. The mosquito vectors for WNV are widely distributed worldwide, and the known geographic range of WNV transmission and disease has continued to increase over the past 77 years. While most human infections with WNV are asymptomatic, severe neurological disease may develop resulting in long-term sequelae or death. Surveillance and preventive measures are an ongoing need to reduce the public health impact of WNV in areas with the potential for transmission.

1. Introduction

First described in 1937 from a febrile illness case in Uganda, West Nile virus (WNV) caused infrequent outbreaks typically associated with mild febrile illnesses from the 1950s through the 1980s in Israel, Egypt, India, France, and South Africa [1–11]. The first outbreak of neuroinvasive disease caused by WNV (WNND) was reported among the elderly in Israel in 1957 [6, 11]. Subsequent outbreaks included adult and pediatric WNND cases [4, 5, 9, 12, 13].

Starting in the mid-1990s, the frequency, severity, and geographic range of WNV outbreaks increased, and outbreaks of WNV meningitis and encephalitis affecting primarily adults struck Bucharest, Romania, in 1996, Volgograd, Russia, in 1999, and Israel, in 2000 [14–16]. WNV crossed the Atlantic and reached the western hemisphere in the summer of 1999 when a cluster of patients with encephalitis was reported in the metropolitan area of New York City, New York, in the United States, and within 3 years the virus had spread to most of the contiguous U.S. and the neighboring countries of Canada and Mexico. In addition, although few human cases have been reported, WNV has also been found in Central and South America through surveillance studies in field specimens, suggesting a potential risk for an outbreak in humans [17, 18]. In the 77 years since its discovery, the virus has propagated to a vast region of the globe and is now considered the most important causative agent of viral encephalitis worldwide (Figure 1).

2. Viral Genome and Structure

WNV belongs to the genus *Flavivirus*, family Flaviviridae, and is a member of the Japanese encephalitis serocomplex, which also includes Japanese encephalitis virus, St. Louis encephalitis virus, Rocio virus, and Murray Valley encephalitis virus [19, 20]. Like other flaviviruses, WNV has a single-stranded positive-polarity RNA genome of approximately 11 kb, containing 10 genes flanked by 5′ and 3′ noncoding regions (NCR) with no polyadenylation tail at the 3′ end [21–25]. The NCRs of the WNV genome form stem-loop structures essential for viral replication [26, 27]. The viral genome encodes a single polyprotein that is co- and posttranslationally cleaved into 3 structural proteins: Capsid (C); Pre-M/Membrane (prM/M); and Envelope (E); and 7 nonstructural (NS) proteins: NS1; NS2A; NS2B; NS3; NS4A; NS4B; and NS5 [24, 28] (Figure 2).

Structurally the WNV virion is a ~50 nm icosahedral particle, surrounded by a lipid bilayer (reviewed in [29]). The nucleocapsid is composed of C protein, which associates with the RNA genome and mediates viral assembly [30, 31]. Heterodimers of prM and E protein become embedded in

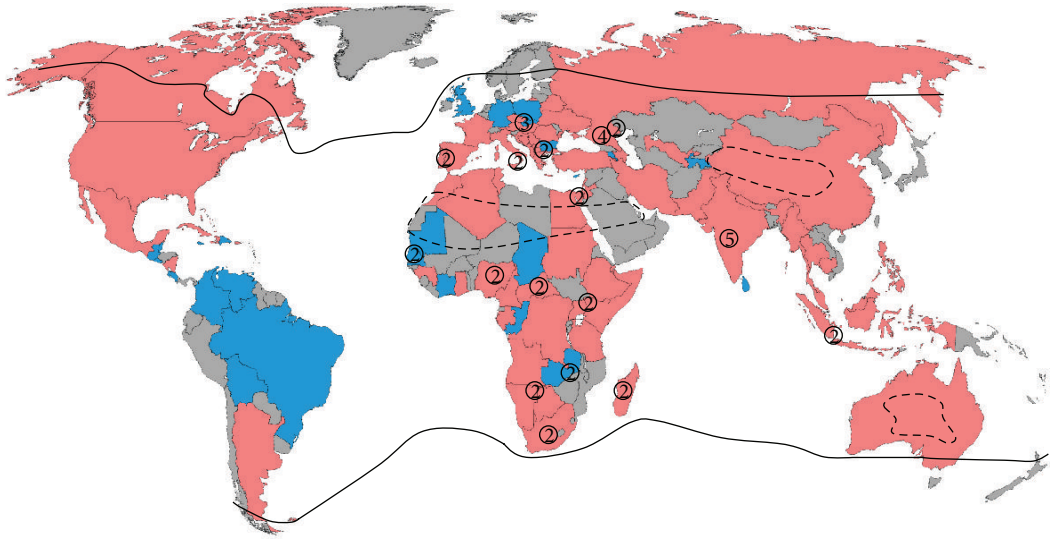

Figure 1: Global distribution of WNV by country: Red—human cases or human seropositivity; Blue—nonhuman/mosquito cases or seropositivity; Gray—no data or no positives reported. Black lines represent worldwide distribution of the main WNV mosquito vectors, excluding areas of extreme climate denoted by dashed lines. Circled numbers indicate the reported presence of WNV lineages other than lineage 1 in that specific area. For Japan, South Korea, Finland, and Sweden, seropositivity for WNV has been detected only in nonresident birds, which was not considered indicative of local transmission. Kading et al. [182] reported infections in gorillas living near the border of the Democratic Republic of the Congo and Rwanda, which were sampled in the D.R.C., but may have been infected in Rwanda.

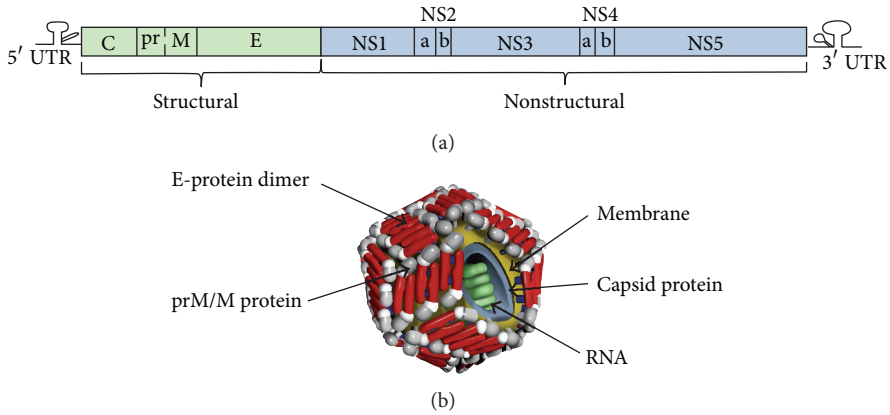

Figure 2: WNV genome organization and virion composition: (a) the viral genome is represented with one ORF encoding 3 structural and 7 nonstructural proteins. The 5′ and 3′ UTRs are indicated. Structural proteins are colored green, whereas nonstructural proteins are blue. (b) Structure of WNV virion.

the lipid bilayer of the virus during assembly and are exposed on the virion surface [32]. The prM protein is thought to protect the immature virion from undergoing premature fusion prior to viral budding from the cell surface by blocking the fusion loop of E and is cleaved off during the viral maturation process [32–36]. During infection, mature, immature, and partially mature virus particles are produced, containing a varying number of immature prM protein molecules on the surface [37]. The E protein mediates both binding of the receptor on the cell surface for viral entry and fusion with the membrane of the host cell [38–40].

The seven nonstructural proteins are multifunctional, playing critical roles in viral RNA synthesis and/or assembly. NS1 is believed to play an early role in regulation of viral replication [41–43]. NS3 has multiple enzymatic functions, serving as a viral serine protease which cleaves the other nonstructural proteins from the viral polyprotein, in association with NS2B; an RNA helicase in association with NS4a; and an NTPase in association with NS5 [44–49]. The NS5 protein is necessary for viral replication, containing RNA-dependent RNA polymerase (RdRp) activity in the C-terminal region and methyltransferase activity in the N-terminal region [41, 50–53]. NS2A, NS2B, NS4A, and NS4B are small, hydrophobic proteins that have no known enzymatic functions, but are believed to act as cofactors for viral replication complex assembly and localization [54–57].

The WNV NS proteins can also modulate cell signaling and immune responses [58–67]. In particular, the WNV NS1 protein antagonizes the host's antiviral defenses through inhibition of TLR3 signal transduction and STAT1/STAT2 activation [64, 67]. It has also been shown that NS1 inhibits complement activation through fH and C4b binding, contributing to flavivirus immune evasion [58–60]. Alternatively, cell surface-associated NS1 represents a major target for host antibodies which contributes to clearance of WNV-infected cells through Fc-gamma receptor-mediated phagocytosis [61].

3. Genetic Classification

WNV is a genetically and geographically diverse virus. Four or five distinct WNV genetic lineages have been proposed based on phylogenetic analyses of published isolates [3, 68–73]. Their genomes differ from each other by more than 20–25% and correlate well with the geographical point of isolation (Figure 3). Lineages 1, 2, and 5 of WNV have been associated with significant outbreaks in humans [3, 68, 72, 74, 75]. Lineage 1 is distributed widely throughout the world and consists of two clades: 1a and 1b [76, 77]. Clade 1a includes isolates from Africa, Europe, the Middle East, Asia, and the Americas. Clade 1b is represented by the Australian Kunjin virus (KUNV).

Phylogeographic analysis has shown that the most probable origin of WNV lineage 1a was sub-Saharan or Northern Africa [78]. This clade emerged in the beginning of the 20th century and then spread northwards in the 1970s–80s, mainly following the eastern bird migratory route connecting Northern Africa and Israel with Russia and Central Europe. Later, in the 1990s, a strain of WNV genotype 1a appeared in Morocco and Western Europe, where the virus became endemic, causing small sporadic outbreaks. In 1999; this WNV lineage 1a virus was exported, most likely from the Middle East, to the Americas, where it spread over North America and then to South America, making WNV a global public health problem [78]. Zehender et al. further divided clade 1a into A and B subclades, with most isolates from Western Europe and some from Eastern Europe belonging to subclade A and the remaining Eastern European isolates belonging to subclade B [78].

WNV lineage 2 isolates are historically endemic in sub-Saharan Africa and Madagascar and have caused sporadic zoonotic outbreaks in South Africa [71, 76, 79]. More recently, WNV lineage 2 strains have been associated with bird and human outbreaks in southern and eastern Europe [80]. Lineage 2 WNV was also sequenced from a 2004 Indonesian clinical specimen [81]. It has been suggested that WNV lineage 2 originated in Africa and was introduced into Europe, where it became endemic, on at least two separate occasions during the last two decades [82].

Lineage 3 of WNV is represented by a pair of isolates from mosquitoes collected in the Czech Republic border region near Rabensburg, Austria, in 1997 and 1999, which have been shown experimentally to infect only mosquitoes and mosquito cells [68, 83]. Lineage 4 comprises viruses circulating in Russia since 1988, including a tick isolate from the south-west Caucasus and a number of isolates from mosquitoes and reptiles in the delta of the Volga river [84, 85]. Lineage 5, formerly considered clade 1c of lineage 1, includes isolates from India from 1955 to the present [3, 72, 86].

Other potential lineages of WNV have been described, including Koutango virus from Africa, a group of isolates from Spain, a variant of Kunjin virus isolated from Sarawak, Malaysia, and a Senegalese isolate [73, 75, 87, 88].

4. Hosts and Vectors

4.1. Hosts. Maintained in nature in an enzootic transmission cycle between birds and mosquitoes, WNV can also infect humans and other vertebrates and cause serious disease and death (Figure 4). Birds are considered the most important hosts for the WNV life cycle because they can develop viremia sufficiently high to infect mosquitoes (reviewed in [89]). Birds in the family Corvidae such as American crows (*Corvus brachyrhynchos*) and blue jays (*Cyanocitta cristata*) become ill or die from WNV, but other birds such as common grackles (*Quiscalus quiscula*) and house sparrows (*Passer domesticus*) develop high viremia with lower mortality rates [90]. American robins (*Turdus migratorius*) and house finches (*Carpodacus mexicanus*) are considered important amplifying hosts in different regions of the U.S. [91]. In addition to birds, at least 30 other vertebrate species, including reptiles, amphibians, and mammals, are susceptible to WNV infection. However, only a few nonavian vertebrates, including brown lemurs (*Lemur fulvus*), lake frogs (*Rana rinibunda*), hamsters, fox squirrels (*Sciurus niger*), eastern gray squirrels (*Sciurus carolinensis*), eastern cottontail rabbits (*Sylvilagus floridanus*), and eastern chipmunks (*Tamias striatus*) have been reported to develop viremia levels expected to support vector transmission [89, 92–98]. Humans and horses may suffer serious disease or death from WNV infection but are considered incidental hosts which do not participate in the WNV lifecycle because they do not develop sufficient viremia to infect mosquito vectors (reviewed in [89]).

Although transmission between hosts by mosquitoes is by far the most common route of transmission, WNV can also be transmitted directly if infected animals or mosquitoes are consumed by susceptible hosts or if susceptible birds come in close contact with cloacal or oral fluids from other birds with high WNV viremia [89].

WNV can also be transmitted between humans by blood transfusion, organ transplantation, transplacental transmission, and via breast milk [99–103]. Although blood donations in the United States have been screened for WNV by nucleic acid testing since 2003, thirteen instances of transfusion-associated transmission have occurred, most recently in 2012 [104–107].

4.2. Vectors. Mosquitoes are the vector for natural transmission of WNV. After a mosquito feeds on an infected competent host, the arbovirus replicates within the mosquito and can then be transmitted to a susceptible host through salivary gland secretions (Figure 4). Compared to related

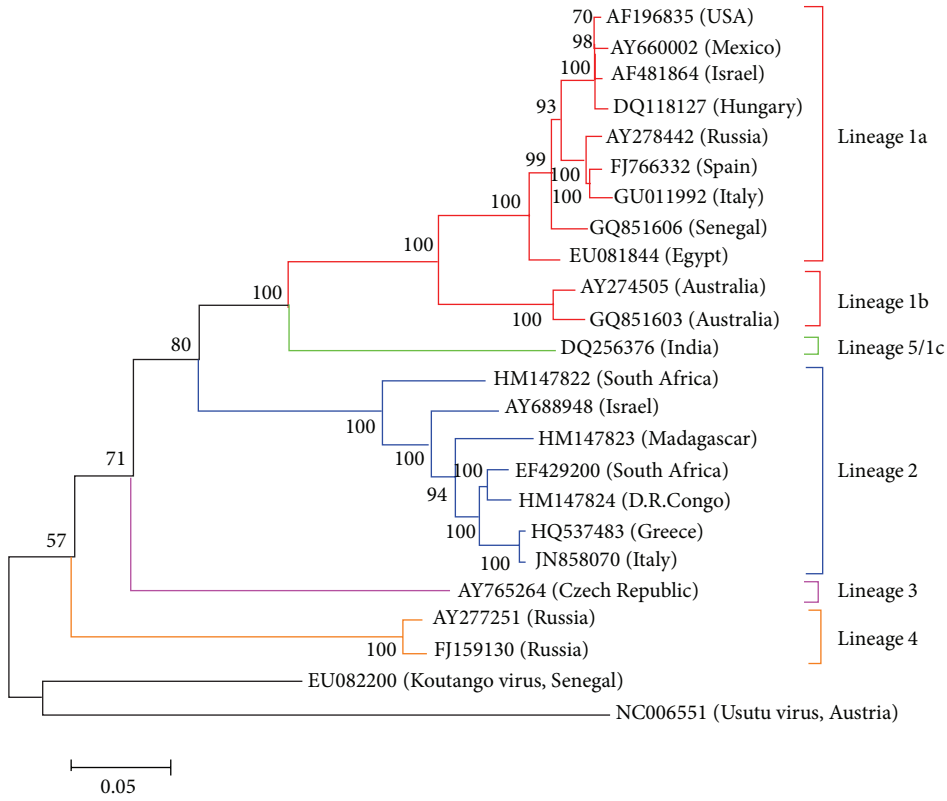

Figure 3: Major WNV lineages. Maximum-likelihood phylogenetic tree is based on complete genome sequences and Nearest-Neighbor-Interchange as heuristic search method. The tree was constructed using MEGA 6 with 1000 bootstrap replications. The tree was rooted using Koutango and Usutu viruses.

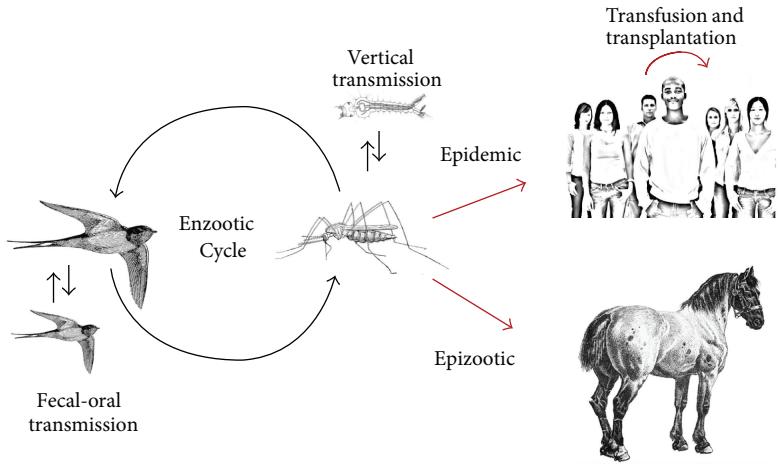

Figure 4: WNV transmission cycle: enzootic amplification of WNV by birds and mosquitoes supplemented by bird-to-bird transmission and transmission between cofeeding mosquitoes. Vertical transmission by mosquitoes provides the mechanism of virus overwintering. Humans and horses are counted as incidental dead-end hosts. Human-to-human transmission may come through blood transfusion, organ transplantation, and breast feeding and in utero.

arboviruses such as dengue virus and yellow fever virus, WNV can be transmitted by a variety of mosquitoes with different host-feeding preferences with up to 45 species and 8 genera reported positive in the U.S. between 2004 and 2008 [108]. However, not all mosquito species reported as WNV-positive are competent vectors of WNV, and not all species that are transmission-competent in the laboratory will play a role in natural transmission [109].

Mosquitoes that feed on both birds and mammals are referred to as bridge vectors for WNV because they act as a "bridge" between an infected reservoir (birds) and mammalian incidental hosts which do not develop sufficiently

high viremia to support transmission to mosquitoes [110, 111]. Ornithophilic mosquitoes play an important role in maintaining and amplifying transmission among birds but typically do not play a role in transmission to humans [109, 111]. Mosquitoes of the genus *Culex* have been reported as the most important bridge vectors in the United States, with *Cx. pipiens* as the dominant bridge vector in the northeastern, north-central, and mid-Atlantic United States, *Cx. quinquefasciatus* in the south and southwest, and *Cx. tarsalis* in the west [110, 112]. *Culex* spp. mosquitoes have also been implicated in transmission in Europe, Australia, and South Africa [113–116]. Mosquitoes of the genus *Aedes*, the transmission vector for related flaviviruses, may also serve as important bridge vectors [108, 109]. While experimental transmission of WNV by ticks has been demonstrated, a role for ticks in natural transmission and maintenance of WNV has not been determined [117–121].

5. Epidemiology and Clinical Outcomes of Human Infections

Most human infections with WNV (~80%) are asymptomatic, and symptomatic infections may vary from flu-like malaise to serious neuroinvasive diseases, for which there is no specific treatment. Fewer than 1% of human infections progress to severe disease, for which the most frequently reported risk factors include advanced age, immune suppression, and chronic medical conditions including, but are not limited to, hypertension, diabetes, and chronic renal failure [122–131]. In 2002, out of more than 4000 cases reported to the CDC, 150 cases were in patients of age 19 or younger. The youngest fatality was a 19-year-old patient, and the median age among fatal cases was 78 years [132]. In the outbreak of 2003, at least 31 cases of WNV encephalitis and 79 cases of WNV meningitis occurred among children and adolescents; however, there were no fatalities caused by WNV disease in children or adolescents [132].

Seroepidemiological studies suggested that one in four to one in five (20–25%) WNV-infected individuals develops mild illness [133, 134] and one person in 150 (0.67%) develops WNND [135]. Subsequent epidemiological studies using asymptomatic infection data obtained from nucleic acid testing to screen blood donations combined with the reported cases to the CDC concluded that one in 244 to one in 353 infections will progress to WNND [123, 136]. These findings suggested that more asymptomatic WNV infections could be identified when prospective studies focused on healthy populations such as blood donors [123, 136]. A serosurvey following lineage 2 WNV infections in Greece in 2010 yielded estimates of one in 124 to one in 141 infections leading to WNND, with approximately 18% of infected individuals showing symptoms [137].

Among WNND patients, 50–71% develop WN encephalitis, 15–35% develop meningitis, and 3–19% develop acute flaccid paralysis [126, 128, 138–145]. Severe cases have fatality rates ranging from 3% to 19% in encephalitis cases [126, 128, 130, 139, 140, 142–144, 146, 147]. Loeb et al. reported that physical and mental impairment resolve in about a year, but patients with preexisting comorbid conditions take longer to recover [148]. However, other studies of patients infected with WNV have noted physical symptoms and/or cognitive deficits persisting over a year after infection in more than half of WNND cases [141, 149]. Persistence of WNV symptoms >6 months was reported most often in patients with WNND, hypertension, and diabetes [150]. WNND has also been reported as a risk factor for development of chronic kidney disease in a long-term follow-up study of WNV patients [151].

Although fewer cases are available from which to conduct a detailed study, it is thought that both the risk of illness and the risk of neuroinvasive infection are lower from the WNV Kunjin subtype (lineage 1b), which circulates in Australia [152]. Until recently, viruses in lineage 2 were not believed to cause WNND in humans. However, outbreaks of lineage 2 WNV strains in the past 10 years in Russia and Greece have caused WNND and death, with case fatality rates similar to those observed previously for lineage 1 WNV [124, 147, 153]. WNND caused by WNV lineage 2 in horses and humans in South Africa has also been reported [79, 154–156].

6. WNV in Africa and the Middle East

West Nile virus was first observed in Africa, in the West Nile district of Uganda, 1937 [10], and thus had been known in the Old World for over 60 years before it crossed the Atlantic. Though it was first isolated from a febrile human case, WNV was observed to cause relatively mild disease in humans and no deaths were reported from the early epidemics studied [10]. While the introduction and progress of WNV through the New World could be studied as it occurred, epidemics of WNV were believed to have occurred throughout much of Africa, the Middle East, and south Asia before clinical WNV was observed in humans in those areas. A 1939-1940 serosurvey found widespread human seropositivity for WNV, determined by comparison of neutralization titers for WNV, SLEV, and JEV, in Uganda, Sudan, the current Democratic Republic of the Congo, and Kenya, with seropositivity over 50% in some localities [157]. Seropositivity was also found in western Nigeria, in samples collected in 1951 and 1955 [158]. In South Africa, seropositivity in humans who had not traveled, monkeys, domestic animals, and juvenile birds was demonstrated in samples collected in 1954 [159]. Therefore, the past presence of WNV had been demonstrated over a wide geographic range in Africa before clinical infections were observed in most locations.

Following its first isolation in 1937, WNV was not isolated again until 1950. During a serosurvey conducted of 251 individuals, mostly children, living in Cairo, isolates were generated from the serum of three children, only one of whom had been diagnosed with a fever [160]. The same serosurvey noted that more than 70% of the study participants aged 4 and above carried neutralizing and complement-fixing antibodies to WNV and that over 50% of infants carried maternal antibodies against WNV, indicating that WNV infection was widespread among the population and that most individuals were infected as young children [160]. A subsequent serosurvey in northeastern Egypt demonstrated

widespread seropositivity of adults and children at multiple locations in the Nile Valley excluding one coastal location, indicating that WNV was not only endemic in Egypt but frequently transmitted [161]. WNV was also isolated from *Culex* spp. mosquitoes in Egypt in 1952 [162].

The first known isolation of West Nile virus in Israel was from a febrile child in 1951, as part of an outbreak that occurred on an agricultural settlement near Haifa [2]. Morbidity in children in this outbreak was substantially higher than in adults, and subsequent outbreaks in Israel in 1952 and 1953 occurring primarily in adolescents and adults were also identified as WNV, on the basis of isolation of the virus from human cases and serology from human cases and chickens [2, 6, 7]. However, WNV is believed to have been present in Israel prior to these isolations, because prior outbreaks between 1942 and 1950 were observed to have been similar clinically and epidemiologically to the ones in 1951 and 1952 [6]. Illnesses in these cases were generally self-limiting with recovery slower in adults than children [2, 6]. WNV fever was described as a "benign specific short-term fever occurring in epidemic form" and was believed to cause only mild neuroinvasive cases [7]. The first fatalities due to WNND were reported in a cluster of elderly patients in 1957; however, overall, neurological involvement in WNV cases was considered unusual [4, 10, 11, 16]. In 2000, the first WNV outbreak in Israel since 1980 was reported, with 417 serologically confirmed cases and 35 deaths [16]. Viral isolates from this outbreak were most closely related to isolates from the 1996 Romanian and 1999 Russian outbreaks [163, 164]. Since then, Israel has experienced regular annual summertime outbreaks of varying size, similar to those observed in the United States [126, 165–167].

Human seropositivity for WNV in Turkey was documented in the 1970s, and again beginning in the mid-2000s [168–172]. An outbreak of WNV occurred in Turkey in 2010-11, concurrent with other outbreaks in the Mediterranean region, causing 47 cases including 40 WNND cases and 10 fatalities [173].

Seropositivity for WNV was also reported in Iran in the 1970s [174]. A 2008-2009 survey of patients with fever and loss of consciousness identified 3 cases which were positive by RT-PCR and 6 more that were positive by IgG [175]. A study of horses conducted from 2008-2009 identified IgM-positive animals and seroprevalences up to 88% in some regions, with the highest activity in western and southern provinces [176]. Serologic evidence for infection has also been found in Jordan and Lebanon although no human cases have been reported from those countries [177–180].

WNV continued to circulate in northern and sub-Saharan Africa throughout the late 20th and early 21st century, causing outbreaks in Algeria, Morocco, Tunisia, the Democratic Republic of the Congo, and South Africa, along with sporadic cases and seropositivity in humans and/or horses distributed throughout the continent [165, 181–184]. Active transmission has continued in northern Africa, with outbreaks reported in Morocco in 2010 and Tunisia in 2012 and ongoing sporadic transmissions in Egypt and Algeria [165, 183, 185–189].

The regular pattern of infection in South Africa prior to 1974 was sporadic, relatively mild human infections and epizootics, with epidemics in humans occurring in 1974 and 1984 (reviewed in [8]). The relative nonpathogenicity of human and equine infections in South Africa had been attributed to reduced pathogenicity of lineage 2 WNV strains; however, later reports of WNND caused by lineage 2 WNV infections in South Africa suggested that the full clinical extent of WNV infection in earlier epidemics may not have been recognized [79, 156]. In 2010, the first case of lineage 1 WNV occurring in South Africa caused the death of a pregnant mare [190]. Infections caused by lineage 2 in Madagascar have also generally been considered mild to inapparent; one fatal case of WNND originated in Madagascar in 2011, although it was speculated that the patient had a deficient antibody response [191].

Recent reports have indicated ongoing transmission in other regions of sub-Saharan Africa. Eleven cases of acute febrile illness were caused by WNV in Guinea in 2006 [192]. A 2009 seroprevalence study in Ghana indicated that WNV is endemic, with most WNV cases occurring in childhood [193]. A fatality due to WNND was reported in Gabon in 2009 [194]. A study in Nigeria demonstrated that 25% of tested febrile patients, many of whom were infected with *Plasmodium falciparum* or *Salmonella* Typhi, were seropositive for WNV, suggesting that WNV infection in this region may be mistaken for these diagnoses or for other cocirculating arboviruses [195]. Seroconversion of sentinel chickens was observed in Senegal in 2009 [196]. In eastern Africa, human infections and mosquitoes positive for WNV lineage 2 were reported in Djibouti from 2010-2011 [197]. Recent positivity for WNV in Kenya has also been reported in ticks collected from 2010–2012 and mosquitoes from 2007–2011 [121, 198].

7. WNV in Southern and Eastern Asia, Australia, and Oceania

In the early 1950s and 1960s, seropositivity for WNV was demonstrated throughout India and as far east as Myanmar [199, 200]. Sporadic cases were documented in India throughout the 1970s–2000s [3]. Most sequenced isolates from India separate into a distinct lineage referred to as lineage 5 or lineage 1C, although lineage 1A isolates have also been reported [3, 72, 86, 201]. Although WNV had previously been shown to cause neurological disease, the first pediatric fatalities from WNND were reported in India, where three children died of WNV encephalitis in 1981 [5]. Pediatric WNND cases have been frequently reported in Indian WNV outbreaks, in contrast to North American and European WNV outbreaks, in which pediatric cases are relatively infrequent [3, 5, 202–204].

Recent reports on WNV in India have included ongoing isolation and sequencing of both WNV lineages 1A and 5, as well as cocirculation and possibly coinfection with JEV in 2006 in areas of northeastern India where both WNV and JEV are endemic [86, 201, 202, 204, 205]. The 2010 outbreak of lineage 1 WNV in Tamil Nadu state was associated with ocular disease, an infrequently reported WNV complication [201].

The recent lineage 5 isolates reported from northeastern India were more neuroinvasive and pathogenic in mice than Indian isolates from 1982 and earlier [86]. Cases of WNND have also been reported from Pakistan along with human seroprevalences ranging from 12 to 54% [206–209].

Isolations of WNV have been reported in Malaysia and Cambodia, and seropositivity for WNV was also noted in Myanmar, Thailand, and the Philippines [88, 200, 210–212]. Lineage 2 WNV was sequenced from an acute febrile specimen collected in 2004 in Indonesia [81]. Recently, WNV has also been isolated from clinical specimens collected in Nepal from 2009-2010; sequenced fragments of both isolates showed homology primarily to lineage 1 viruses, but one fragment of each was more similar to lineage 2 than to lineage 1 [213].

In China, seropositivity for WNV was first reported in birds from Yunnan province in 1988 [214, 215]. The first confirmed human cases of WNV in China were reported in 2013 but occurred during a 2004 outbreak of fever and neurological disease in Xinjiang province in northwestern China, in which diagnosis was delayed due to antibody cross-reactivity with Japanese encephalitis virus [214]. Seropositivity for WNV in Shanghai was reported in 14.9% of cats and 4.9% of dogs tested in 2010, as well as in captive resident birds from 2009-2010, but no human cases have been reported from southern or eastern China [216, 217]. In South Korea, antibody against WNV was detected in 5/1531 bird specimens in a 2009 study, but no resident birds were seropositive [218].

Kunjin virus, which was originally considered to be a closely related virus but is now considered a subspecies of WNV lineage I, was first isolated in Australia in 1960 [219, 220]. Symptoms of WNV/Kunjin in Australia are considered relatively mild, with infrequent WNND and no deaths reported [115, 221]. WNV/Kunjin has continued to cause intermittent cases of equine and human disease in Australia, primarily in the northwest, where it frequently cocirculates with the related flavivirus Murray Valley encephalitis virus (MVEV) [221]. In 2011, an outbreak of WNV (cocirculating with MVEV) in horses in southeastern Australia was attributed to a strain of WNV designated $WNV_{NSW2011}$, which was closely related to Kunjin virus, but carried two amino acid changes previously associated with increased virulence in North American WNV NY99 strains [222–224]. These changes rendered $WNV_{NSW2011}$ significantly more neuroinvasive than previously observed Australian strains. However, no human cases were reported from this outbreak, and a serosurvey in Victoria showed little evidence of recent human WNV infection [225].

8. WNV in Europe

The presence of WNV was first discovered in Europe in 1958 in Albania with detection of neutralizing antibodies in human sera [226, 227]. The first documented outbreak of WNV occurred in southern France in 1962-1963, causing WNND in both humans and horses [1, 9]. Following that outbreak, no further WNND cases in humans were reported until 1985, although virus activity in the region has been confirmed on multiple occasions. The virus was isolated from mosquitoes in Portugal and the Czech Republic, migrating birds in Slovakia, and western Ukraine, and ticks in Hungary and the Moldavia region [227–231]. WNV was also sporadically detected in serological surveys of humans, migratory birds, and domestic animals in the countries throughout southern and eastern Europe and the Mediterranean basin, although the virus was not considered a public health concern during that time due to the absence of reported WNND [227, 232, 233].

However, the situation has changed dramatically in the last three decades with a series of symptomatic WNV outbreaks in several European countries. Human WNND cases were first observed in western Ukraine in 1985, followed by a period of relative silence and two major epidemics: in Romania in 1996 and in Russia in 1999 [14, 15, 232]. During the outbreak in Romania, 835 patients were hospitalized with neurological symptoms and 343 were confirmed to be WNV-positive. The epidemic caused 17 deaths [15]. The mortality rate was even higher in Russia: out of 826 patients who presented with symptoms, 183 tested positive in serological essays, and 40 died of acute aseptic meningoencephalitis [14].

Other notable outbreaks with human cases from 2000–2009 include the reemergence of WNV in France in 2000–2003, Italy in 2008-2009, and Hungary in 2008 [232, 234, 235]. In France in 2000, WNND cases were confirmed in 76 horses, and 21 of them died; interestingly, the same region of the country was affected as in the outbreak of 1962 [236]. WNND cases described in 2003 involved horses and humans [237]. A subsequent serologic survey in horses suggested the possibility of persistent WNV circulation in the area [238]. The 2008 outbreak in Italy was preceded by a 1998 event with 14 encephalitic equine cases and 4 asymptomatic cases in humans; a retrospective study revealed a 38% seroprevalence rate in horses in the region [239, 240]. In 2008-2009, both equine and human WNND cases were reported [241]. In Hungary, the sudden spread of the virus in 2008 caused 12 equine and 22 human neuroinvasive cases [234]. Following the large outbreak in 1999, Russia experienced annual summer transmissions with sporadic outbreaks primarily in the south [242]. The three most affected regions were Astrakhan, Rostov, and Volgograd provinces with outbreaks in 2007, 2010, and 2012, although recently the range of the virus has apparently expanded, with cases reported further north- and eastward in several provinces including southern parts of Siberia [166].

With the outbreaks becoming more frequent and sporadic cases surfacing all over Europe, enhanced surveillance programs were established in many European countries [232, 243]. One such program was instrumental in promptly identifying WNV cases during the largest recorded outbreak in Italy in 2012, where simultaneous circulation of both WNV lineage 1 and lineage 2 was documented [244–247]. From 2010–2013, human WNV cases were reported in Austria, Bosnia and Herzegovina, Croatia, Greece, Hungary, Italy, Kosovo, the Former Yugoslav Republic of Macedonia, Montenegro, the Russian Federation, Serbia, Spain, and Ukraine [165, 248–253]. Greece and Russia experienced high WNV activity each year from 2010–2013, and 302 cases were

reported from Serbia in 2013 [165, 166]. Additionally, the presence of WNV was confirmed in the Czech Republic, Portugal, and other countries where it had not previously been identified, with most of the reports suggesting seasonal introduction by multiple routes and continuous low-level WNV circulation in Europe [254, 255].

Notably, prior to 2008, lineage 1 viruses were responsible for severe WNND cases in humans in Europe, and lineage 2 viruses were only reported in sub-Saharan Africa and Madagascar until 2004. However, the cases in Hungary were caused by lineage 2 WNV, with subsequent spread into Austria, Italy, Russia, Greece, Serbia, and Croatia [85, 248, 255–260]. The strain isolated from the 2010 Greek outbreak, WNV Nea-Santa-Greece 2010, was shown to carry a mutation previously associated with increased virulence in corvids in lineage 1 WNV strains [261, 262].

Countries with established surveillance programs and no reported clinical cases so far include the U.K., Germany, and Switzerland [263–265]. One serologic study in the U.K. identified WNV-seropositive wild birds, although subsequent studies have not found evidence of WNV circulation in birds [266–269]. In Germany and Poland, seroprevalence in birds was relatively low [264, 270].

9. WNV in the United States

WNV infection is a major public health concern in the United States, where the virus has become endemic causing recurring outbreaks for 14 consecutive years. The initial outbreak in the U.S. resulted in 62 reported cases, including 59 WNND cases and 7 deaths; however, estimates based on serosurveillance suggest that 2.6% of the population near the outbreak epicenter in New York City was affected in that outbreak [133]. The WNV strain associated with the U.S. outbreak, designated WNV NY99, was a lineage 1 strain closely related to an isolate from the outbreak in Israel in 1998, and both the U.S. and Israeli strains were related to a Tunisian isolate from 1997 [70, 71, 77, 271]. By the summer of 2000, the virus had also caused human disease in the states of New Jersey and Connecticut with a total of 21 cases reported including 19 WNND cases that resulted in 2 deaths [272].

In contrast to the historically observed pattern of outbreaks in Europe and Africa between the 50s and the 90s, in which epidemics were followed by years of inactivity, WNV continued to spread in the U.S. following its introduction. In the summer of 2001, the virus was found in 10 states with 66 total reported cases (64 WNND) and 9 deaths. In 2002, 40 states reported a total of 4,156 WNV human cases to the CDC, with 70.9% (2,946) classified as WNND resulting in 284 fatalities. Possibly due to increased awareness in the medical community through an outreach program by the HHS, in 2003 the total number of reported cases increased 42% to 9,862; however the number of WNND cases declined to 2,866, which represented 29% of reported cases. By 2004, WNV had been detected in all of the contiguous 48 states and was considered endemic. Another large outbreak occurred in 2006, with 177 deaths and 1,495 WNND cases, out of a total of 4,260 cases. Coincident with the 2002-2003 outbreaks, a new viral genotype known as WN02 replaced the original viral genotype NY99; the new genotype was observed to disseminate more efficiently in North American *Culex pipiens* and *Culex tarsalis* mosquitoes than the NY99 genotype [273–276].

The intensity of WNV activity in the U.S. was very high between 2002 and 2007, with over 1,000 WNND cases per year. A decline in the number of cases began in 2008, and comparatively low activity continued through 2011 when only 712 total WNV cases were reported. In 2012, however, another large outbreak of WNV occurred in the U.S., causing 2,873 WNND cases and the most deaths [277] ever reported in a single WNV season. Activity in the U.S. in 2013 was moderate, with 1,267 WNND cases and 119 deaths in 2013 reported as of May 9, 2014. From 1999–2013, there have been a total of 39,557 reported cases of WNV in the U.S. of which 17,381 were WNND, resulting in 1,667 deaths, an average of 111.1 deaths/year [278] (Figure 5).

Based on epidemiological estimates that for each case of WNND there are 150 to 350 human infections, 2.6 to 6.1 million people in the United States have been infected with WNV over the past 14 years. Through 2010, it was estimated that 1.1% of the U.S. population had been infected by WNV, with the highest incidence in the state of South Dakota (13.3%) [123, 279]. Because most WNV infections are asymptomatic or mild, many human infections may not be recognized, and there may be significant underreporting of milder symptomatic cases [133, 279].

10. WNV in Other Parts of North America

In 2001, WNV was first detected in 128 dead birds and 9 mosquito pools in Ontario, Canada [280]. Human cases in Canada were first reported in 2002, with 394 in Ontario and 20 in Quebec [281]. In 2003, WNV spread westward to Manitoba, Saskatchewan, and Alberta, but did not reach British Columbia until 2009 [281, 282]. Since 2002, Canada has experienced annual summer outbreaks similar to those in the United States, with the largest outbreaks occurring in 2003 (1,481 cases), 2007 (2,215 cases), and 2012 (428 cases) [281, 283] (Figure 6).

The first WNV activity in the Caribbean was a human WNV encephalitis case reported at the end of 2001 in the Cayman Islands [284]. Most Caribbean WNV activity for the next few years was limited to observations of seropositivity in birds and horses. In 2002, WNV activity was observed in migratory and resident birds in Jamaica and the Dominican Republic, and in horses in Guadeloupe. An avian serosurvey in Jamaica, Puerto Rico, and Mexico in spring 2002 reported detection of specific neutralizing antibodies for WNV in 11 resident species from Jamaica only [285]. In the Dominican Republic, a seroepidemiological study performed in birds sampled in November 2002 on the eastern side of the country showed anti-WNV antibodies in 15% (5/33) of resident birds [286]. By 2003, WNV seropositivity had also been detected in resident birds on the northwestern side of the Dominican Republic [277]. A serosurvey of 360 healthy horses in Guadeloupe showed an increase in prevalence of IgG antibodies to WNV

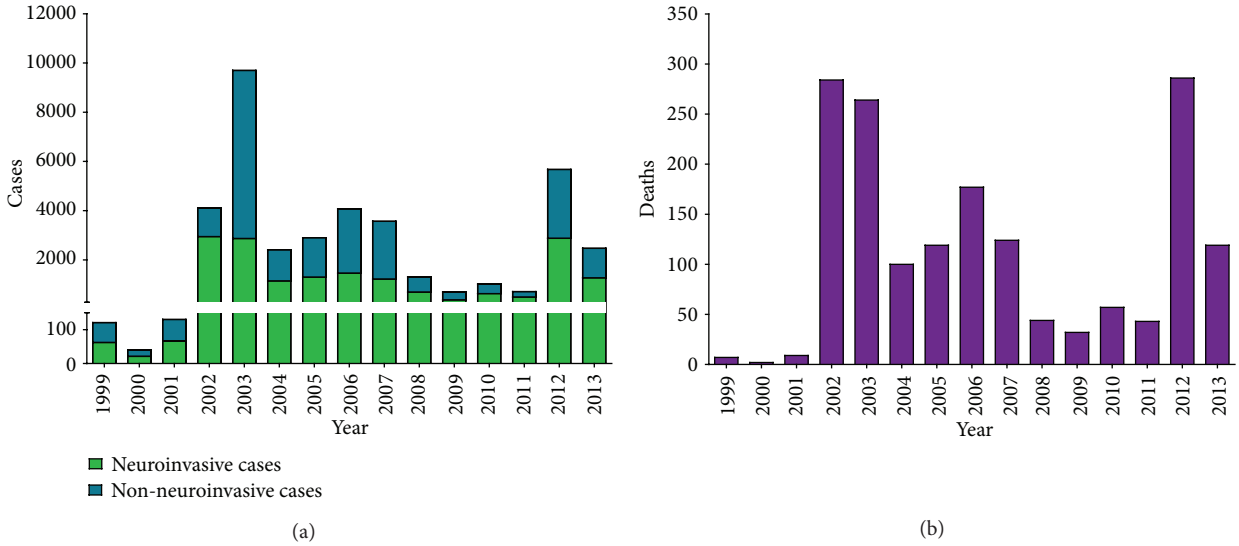

Figure 5: (a) Neuroinvasive and nonneuroinvasive cases of WNV in the United States reported to the CDC, 1999–2013. (b) Deaths from WNV infection in the United States reported to the CDC, 1999–2013.

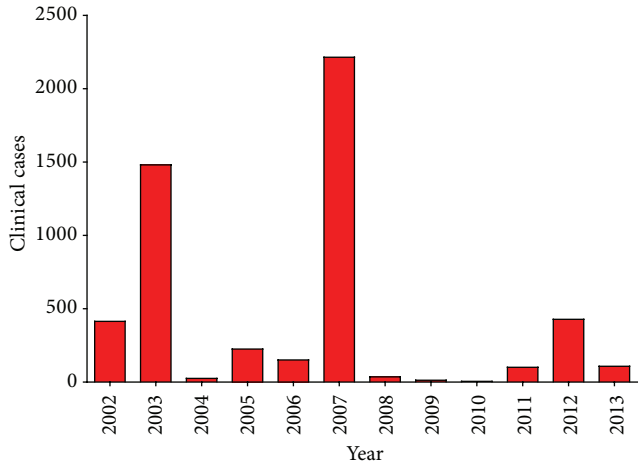

Figure 6: Total clinical WNV cases in Canada reported to the Public Health Agency of Canada, 2002–2013.

from 8.8% in June 2002 to 50% in January 2003, indicating a high incidence of WNV infections in horses within that 6-month period [287].

The second Caribbean human WNV encephalitis case occurred in the Bahamas in July 2003 [288]. WNV was detected in Trinidad in October, 2004, in a serosurvey of 60 horses and 40 birds, with reported seropositivity of 3% and 5%, respectively [288, 289]. In Cuba, WNV infection was confirmed by serologic assays in 4 asymptomatic horses and 3 humans with encephalitis in 2003 and 2004 [290]. A 2004 serosurvey of over 1900 resident and migrant birds in Puerto Rico and Cuba found 10 WNV-positive birds (9 migrant, 1 resident) in Puerto Rico and 3 birds (1 migrant, 2 resident) in Cuba [291]. Three seropositive horses were observed in 2004-2005 in Puerto Rico, followed by detection of WNV in three blood donors in 2007 [292]. A 2007 isolate from Puerto Rico carried the mutation V159A in the envelope gene, which is characteristic of the WN02 genotype which replaced the original NY99 genotype in the U.S. [293]. Two further WNV human cases were detected in Haiti during surveillance of febrile patients following Hurricane Jeanne in 2004 [294].

In 2002, WNV appeared in Mexico, with reports of encephalitis-like illness in horses in different areas, concurrent with reports of WNV encephalitis outbreaks in horses along the Texas border in the states of Coahuila, Tamaulipas, and Chihuahua [295]. Mexico has reported low numbers of cases in humans, horses, and birds, primarily from the northern border with the United States. The first confirmed autochthonous human case of WNV in Mexico was reported in 2004 [296]. A fatal human case in 2009 was reported in a 40-year-old man who had mild symptoms for several weeks then progressed to neurological disease, coma, and death [297].

A surveillance study in Mexico found relatively low levels of WNV transmission and disease, which were attributed to multiple factors including the interactions of amplifying hosts, vectors, and circulating virus strains, in combination with climate, habitat, and circulation of interfering flaviviruses [298]. A Mexican isolate from 2003 was shown to have reduced pathogenicity for mice, crows, and sparrows, which may also have contributed to a reduced presence of WNV in Mexico [299, 300].

In El Salvador, an investigation of outbreaks from 2003 revealed that 25% (18/73) of equine specimens tested had antibodies to WNV and were confirmed by plaque reduction neutralization tests (PRNT) [301]. In October 2003, WNV was also identified in horses in Belize [289].

A 2003-2004 serosurvey conducted at multiple locations in Guatemala detected 9 horses positive for WNV [302]. Seropositivity in horses in Costa Rica was also reported from

a 2004 serosurvey [303]. The only human case of WNV from Nicaragua was reported in a Spanish missionary who became ill in the summer of 2006 while living in Nicaragua and was subsequently transferred to Spain for treatment, where he was diagnosed [18].

11. WNV in South America

The first detection of WNV in South America was in an autumn 2004 epidemiological survey of horses which had not been vaccinated against WNV or traveled outside of Córdoba and Sucre in the Caribbean region of Colombia [304, 305].

WNV was next reported in northern Argentina, where WNV was isolated from the brains of 3 horses that died from encephalitis in February 2006 [306]. A later report showed that resident birds in Córdoba, Chaco, and Tucumán provinces had antibodies to WNV as early as January 2005, and seroconversions were observed in three birds between January and March 2005 [307]. In December 2006, health authorities reported 4 human cases, 1 case in the city of Marcos Juarez in Córdoba province and 3 additional cases in Chaco province [17]. The case in Córdoba occurred in March of that year in a 58-year-old man who had not traveled outside the country in recent years, suggesting that the disease was contracted locally [17].

In 2006, WNV was also reported in birds and horses in Venezuela, in a seroepidemiological study with PRNT confirmation [308]. WNV was detected in Brazil in a study performed on samples collected in 2009 from mosquitoes, horses, and caimans from the Pantanal region of Central-West Brazil, in which a total of 5 out of 168 horse specimens tested positive for WNV, using a flavivirus-specific epitope-blocking enzyme-linked immunosorbent assay with confirmation of reactive specimens by PRNT [309]. Further studies performed with specimens from the Pantanal region, where WNV cocirculates with multiple other flaviviruses, have found WNV seropositivity confirmed by PRNT in horse and chicken samples collected in 2009 and later [310–312]. WNV seropositivity was also reported in an equine sample collected in 2009 in Paraiba state in northeastern Brazil [313]. However, WNV has not yet been associated with human or equine illness in Brazil [314].

A study conducted on a subset of 20,880 samples from individuals with acute febrile illnesses from Bolivia, Paraguay, Ecuador, and Peru from 2000–2007 identified at least one patient with PRNT-confirmed seropositivity for WNV; however, no virus was isolated, and the number and location of WNV-positive patients were not given [315]. A 2011 serosurvey of horses in Bolivia found PRNT-confirmed seropositivity for WNV, although no horses were IgM-positive, indicating that WNV had circulated in the area prior to 2011 [316].

Interestingly, sequences obtained from Colombian viral isolates in 2008 were most closely related to 2001 Louisiana, U.S. sequences of the NY99 genotype, suggesting that the WN02 genotype which replaced NY99 in the U.S. had not progressed southward over that time period [317].

12. Conclusion

Since its discovery in 1937, West Nile virus has spread beyond its original known geographic range and caused human disease on every continent except Antarctica. It is now the most widespread cause of arboviral neurological disease in the world. With no vaccine available to date and limited treatment options, transmission via organ donation and blood transfusion also poses a risk.

While expansion of WNV into Central America, South America, and the Caribbean has been marked by relatively few human WNND cases and recovery of attenuated viruses, characterization of strains isolated from recent outbreaks in Greece, Australia, and India shows increased virulence in tissue culture and/or animal models [86, 222, 262, 300]. However, none of the observed changes has been directly correlated to virulence in human infections.

Recent large outbreaks of human WNND in Europe and North America, as well as ongoing transmission in the Middle East, Africa, and Asia, illustrate the need for continued surveillance and preventative measures. The risk for transmission and outbreaks remains high in the many parts of the world with suitable mosquito vectors.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Review Article

Epidemiology of West Nile Disease in Europe and in the Mediterranean Basin from 2009 to 2013

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West Nile virus (WNV) transmission has been confirmed in the last four years in Europe and in the Mediterranean Basin. An increasing concern towards West Nile disease (WND) has been observed due to the high number of human and animal cases reported in these areas confirming the importance of this zoonosis. A new epidemiological scenario is currently emerging: although new introductions of the virus from abroad are always possible, confirming the epidemiological role played by migratory birds, the infection endemisation in some European territories today is a reality supported by the constant reoccurrence of the same strains across years in the same geographical areas. Despite the WND reoccurrence in the Old World, the overwintering mechanisms are not well known, and the role of local resident birds or mosquitoes in this context is poorly understood. A recent new epidemiological scenario is the spread of lineage 2 strain across European and Mediterranean countries in regions where lineage 1 strain is still circulating creating favourable conditions for genetic reassortments and emergence of new strains. This paper summarizes the main epidemiological findings on WNV occurrence in Europe and in the Mediterranean Basin from 2009 to 2013, considering potential future spread patterns.

1. Introduction

West Nile virus (WNV) is RNA virus belonging to the genus *Flavivirus*, Flaviviridae family. Primarily transmitted by the bite of the *Culex* spp. and *Aedes* spp. mosquitoes, WNV is the most widespread member of the Japanese encephalitis virus (JEV) complex [1]. WNV is maintained in nature by a primary transmission cycle between mosquitoes and several bird species, which play the role of amplifying hosts [2, 3]. In the Old World, birds mortality has been sporadically associated with WNV infection [4], as in Israel [5], Hungary [6], and Italy [7]. Humans, horses, and other mammals may be infected by the bite of infected mosquitoes, but they are incidental and dead-end hosts, given the low levels of viraemia that they may develop [8]. Human-to-human transmission may occur only through blood transfusion [9] or organ transplants [10]. Infection in humans is generally asymptomatic, but mild influenza-like symptoms may be observed. In some human categories at risk, like elderly, chronically ill, and immunocompromised people, WNV infection can lead to severe encephalitis and to the death of the patient. In horses the disease course is usually subclinical, although some animals may show neurological symptoms and develop fatal encephalitis.

In temperate countries viral infection in humans and in equines generally occurs in warmer months, from July to October, in accordance with the hypothesis of virus introduction during bird's spring migration followed by the virus amplification in the early summer, contemporaneously with the increase of vector density and influenced by bird population dynamics in nesting geographical areas [11]. The global presence of mosquitoes belonging to the *Culex* genus and the geographical dissemination of WNV by migratory birds underlying the global spread of the infection, especially in tropical and temperate zones.

To date, WNV has been detected both in the Old World (Europe, Middle East, Africa, India, and Asia) and in the New World (North America, Central America, and the Caribbean)

and also in Australia (Kunjin virus, a subtype of WNV) [12–14].

Seven distinct genetic lineages of WNV have been described [15], but those two lineages are more frequently recognised: lineage 1, which includes WNV strains circulating in Europe, North America, North Africa, and Australia, and lineage 2, which is historically present in sub-Saharan Africa and Madagascar and more recently observed in some European countries (Albania, Austria, Greece, Hungary, Italy, Romania, Russia, and Serbia). Lineage 2 was considered in the past nonpathogenic for humans and horses [1, 16], but more recently in Europe this strain demonstrated its capacity to cause severe clinical symptoms in both humans and equines [17, 18]. The majority of viruses belonging to lineage 1 are grouped into a cluster called "European Mediterranean/Kenyan cluster," whereas those responsible for outbreaks in Israel and in the New World are grouped into the "Israeli/American cluster" [19].

Given the rising awareness towards West Nile disease (WND), an increase in the notification of human and equine cases has been observed in the last decades in Europe and in the Mediterranean Basin [20].

Several Mediterranean countries reported WND cases in animals and in humans from 2009 to 2013. During this period, WND cases were reported in Algeria, Bulgaria, Croatia, Former Yugoslav Republic of Macedonia (FYROM), Greece, Hungary, Israel, Italy, Kosovo, Montenegro, Morocco, Occupied Palestinian Territories, Portugal, Romania, Russia, Serbia, Spain, Tunisia, Turkey, and Ukraine.

The reoccurrence of WND cases in the same geographical areas was explained by both the WNV reintroduction by migratory birds and the establishment of overwintering cycles, possibly supported by local bird populations or infected adult mosquitoes surviving during the winter season [21–23]. The aim of this paper is to present the main findings on WNV occurrence in Europe and in the Mediterranean Basin from 2009 to 2013, considering possible trends and potential future further spread patterns.

The data are presented according to lineage strains, although in some cases a presumptive attribution to the most probable lineage has been followed, given the lack of available information on the viral strains involved.

2. Occurrence of Lineage 1 WNV Strains from 2009 to 2013

2.1. European Mediterranean/Kenyan Cluster

2.1.1. Northern Africa. In 2003, 9 equine WND cases were reported in *Morocco* to be caused by lineage 1 of the virus [19]. No human cases were reported at that time. In August 2010 in the Rabat-Sale-Zemmour-Zaer and Tadla-Azilal regions, respectively, in the north-western and central parts of Morocco, a total of 25 neuroinvasive cases, with 8 deaths, were confirmed in equines in Ben Slimane, Khemisset, Mohammedia, and Casablanca provinces [24, 25]. In 2010, a serological survey in humans identified 11 IgM positive people living near Meknes, Rabat, and Kenitra cities, confirming a recent WNV circulation in the country [26].

After the epidemic occurred in 1994, in October 2012 one WND human case was observed in a 74-year-old man living in France who had travelled in the Jijel province of *Algeria*. He was hospitalized in September with fever and cognitive disorders and died in the same month [27].

Three human cases were reported in *Tunisia* in 2010, in Jendouba and Tataouine regions [28]. WND human cases were also confirmed one year later in three women in Kebili governorate, close to an oasis populated by migratory birds [29]. From August to November 2012, a total of 63 suspected human cases, of which 33 were confirmed, were reported from different governorates: Kebili, Jendouba, Mahdia, Monastir, Bizerte, Sousse, Tozeur, Sfax, and Gabes [30]. In October 2013, 6 human cases were detected in 5 governorates: Gabes, Mahdia, Monastir, Nabeul, and Sousse [31].

2.1.2. Eastern Europe. Clinical cases were never officially reported in *Turkey* up to 2010, although previous serological surveys revealed the human exposure to WNV in various regions [26]. In 2010, 12 laboratory-confirmed cases were detected in humans in 15 provinces located in western Turkey. In 2011, three further laboratory-confirmed cases were detected in the same part of the country. The detection of WNV infections in humans in the same area during two consecutive years may indicate the establishment of a local endemic transmission cycle with virus overwintering [32]. The first isolation of WNV lineage 1 was reported in 2011 in 2 horses in Eskisehir province and in a man in Ankara province [33]. Lineage 1 of WNV was also isolated in 2012 in an 87-year-old woman in Ankara province [34].

In southern *Romania* the first large outbreak of West Nile neuroinvasive disease (WNND) was reported in 1996 [35, 36], albeit the virus circulation was firstly detected in 1955 in central Transylvania and in 1964 in Banat country [37]. After this first epidemic, further investigations confirmed the virus circulation in the country [38]. Lineage 1 strain circulated in Romania between 1997 and 2009 [39]. Serological studies undertaken in 2007 in horses demonstrated the presence of WNV infection in Braila county, in the southeastern part of Romania [40]. From 2008 to 2009, viral circulation was detected in Braila and Dolj counties, where 4 human cases were reported [36]. WNV was detected in *Culex pipiens* females (including overwintering females) collected in Bucharest and Tulcea County from 2007 to 2009 and also in *Coquillettidia richiardii*, *Aedes caspius*, and *Anopheles maculipennis* s.l., collected in the same period in Tulcea County [36].

In October 2010, eight cases were notified for the first time in *Bulgaria*: 5 donkeys and 3 horses bred in the northeastern part of the country were found to be positive to serological tests [41]. No confirmed human cases were detected at that time [28], but in 2012 two human cases in the Burgas oblast region were reported to ECDC [30].

In *Ukraine* 20 human cases of WND were reported between 2011 and 2012 [26]. These cases belonged probably to the same cluster involving Romania and Bulgaria. In August

2013 a further human case was reported in Zhytomyrs'ka oblast [31].

2.1.3. Southern Europe. In *Spain* several WND cases in horses and humans were reported in 2010. The first clinical case was detected in a horse in September 2010 in Andalusia (southern Spain). After the first clinical case a control program for WNV was established and other clinical cases in horses were reported [42]. Forty-four WND cases in horses from Andalusia (in Cádiz, Seville, and Málaga provinces) were notified (Anonymous, 2013). In a study conducted from September to December 2010, fragment of viral RNA belonging to lineage 1 WNV was detected from the blood and the cerebrospinal fluid of a lethally infected horse [43]. In September 2010, the first human case was confirmed in a 60-year-old man and the following month a 77-year-old patient case was reported. Both cases, showing symptoms of encephalitis, were detected in Cádiz in concomitance with WND cases in horses [42]. In 2011 a total of 12 cases in horses in Málaga, Seville, and Cádiz provinces (Andalusia) were notified (Anonymous, 2013), while in 2012 four cases in horses in Cádiz province were documented by the Andalusian authority (Anonymous, 2013). In 2013, a new epidemic involved Seville and Huelva provinces: between August and November 40 cases in horses were confirmed (Anonymous, 2013).

In *Portugal* two WND cases were reported in 2010 in equines, in Lisboa e Vale do Tejo region, showing neurological clinical signs [44].

In *Italy*, in the late summer of 1998, WNV infection was detected for the first time in horses in Toscana region [45]. In August 2008, after 10 years of silence, a large epidemic affected three regions in the northeast of Italy (Emilia Romagna, Veneto, and Lombardy) [46, 47]. In 2009, WND occurred again in the same regions of the previous year and in other regions of central Italy which have never been involved before. A total of 223 cases in equines were confirmed, 37 of which with clinical signs in Emilia Romagna, Friuli-Venezia Giulia, Latium, Lombardy, Tuscany, and Veneto regions. Virus circulation was detected also in birds (the species which was more involved was magpie, *Pica pica*) in Emilia Romagna and Veneto regions, in mosquito pools in Emilia Romagna, and in poultry in Molise region [21]. The phylogenetic analysis of the isolates indicates that the virus circulating in 2009 belonged to lineage 1, with a high identity between 2008 and 2009 WNV Italian strains [21]. This finding strongly supported the hypothesis of virus overwintering and possibly the endemisation in local host populations [21]. In 2010 WNV continued to circulate in the already affected geographical areas, but spreading to new regions, such as Sicily and Apulia regions [48]. A total of 128 equine cases were reported, with 11 of which showing clinical signs. Seroconverted animals were observed in poultry in Molise and Apulia regions. In 2011, additional 197 cases in equines (58 with clinical signs and 14 deaths) were confirmed in the same regions of the previous years, but with the involvement of new areas in southern Italy (Calabria and Basilicata regions), and for the first time Sardinia island. Surveillance in wild bird species in Sardinia allowed the isolation of WNV lineage 1 in a little owl (*Athene noctua*), a jay (*Garrulus glandarius*), and a mallard (*Anas platyrhynchos*). Lineage 1 was detected in Sicily (one mosquito pool and a horse) and in Friuli-Venezia region (one mosquito pool). In 2012, WND was confirmed in 30 horse stables in the same region affected by the virus circulation in the previous years: a total of 63 cases in Veneto, Sardinia, Friuli-Venezia Giulia, and Latium regions were reported, with 15 of which showing clinical signs. Seroconverted sentinel chickens were detected in Basilicata region. Lineage 1 was identified in wild birds and in mosquito pools in Veneto and Friuli-Venezia Giulia regions. No human cases were recorded until 2008. But from 2008 to 2011, 43 WNND cases were reported in five Italian regions (Emilia Romagna, Veneto, Lombardy, Friuli-Venezia Giulia, and Sardinia) with a 16% of case fatality rate [49]. In 2012, 28 WNND cases were identified in the same areas previously affected by WNV infection and in Basilicata region [50]. WNV lineage 1 was identified in blood donors in 2010 and in 2012 in Veneto region. Partial sequencing of the WNV RNA demonstrated an almost perfect identity with the virus isolated in the same area in 2011 in horses and a divergence from the strain responsible for the outbreak in the north of Italy in 2008-2009 [49, 51]. Four human cases occurred in Sardinia at the end of the summer of 2011. The genomic sequences of isolates from three patients revealed a strain strictly related to the WNV strains circulating in Italy in the years 2008 and 2009 and to the strains circulating in Europe and Israel from late 2004 to 2011 [52]. In 2013 WNV circulated in Emilia Romagna, Lombardy, Veneto, Sardinia, and Sicily regions. In Veneto region WNV lineage 1 was detected in an organ donor and in a blood donor [53]. In 2012 *Balkan* countries, such as Croatia, Serbia, Montenegro, Kosovo, and the Former Yugoslav Republic of Macedonia (FYROM), reported WNV human cases. For Croatia, Kosovo, Serbia, and Montenegro that was the first notification of WNV infection in humans.

Croatia reported 5 cases in humans and 12 cases in equines without apparent clinical signs from July to August 2012. Both equine and human cases occurred in the eastern part of Croatia [30, 54]. In 2013, 16 human cases, of which one was confirmed, in Medimurska, Zagreb, and Zagrebacka areas [31] were identified.

In 2012, *Kosovo* and *Montenegro* reported, respectively, 6 and 1 human cases [26]. In 2013 in Montenegro four additional human cases were notified [31]. In 2011 *FYROM* reported 4 confirmed human cases in Skopje, occurring from August the 25th to October the 6th, and additional 10 confirmed cases in horses and 36 in birds [55]. In 2012, six further human cases were reported in FYROM [30]. In 2013 a human case was identified in July [31].

For the first time, WNV human cases were reported in *Bosnia-Herzegovina* in 2012 [26]. In 2013, 3 human cases were confirmed in Modrica and Tuzlansko-Podrinjski cantons [31]. Between late August and early September 2013 WNV infection has been detected in 2 hooded crows (*Corvus cornix*) [56].

2.1.4. Western Europe. In *France*, after the cases reported in 2003 and 2004 in humans and horses, 4 distinct foci of

WND were reported: WNV was responsible for neurological syndromes in horses of Camargue region between 2000 and 2004 and between 2003 and 2006 in the Var and Eastern Pyrenees Departments [57].

2.2. Israeli/American Cluster

2.2.1. Middle East. In 2000, *Israel* experienced its largest WNF epidemic with 429 reported human cases. After this epidemic, 68 neuroinvasive human cases were reported in 2010, 36 in 2011, and 63 in 2012 [58]. In 2011 WNV lineage 1 was isolated from a mosquito pool [59]. In 2013, 63 human cases were documented in Central, Haifa, Southern, and Tel Aviv districts [31].

In Figure 1 a comprehensive map of WNV lineage 1 occurrence in Europe and in the Mediterranean Basin from 2009 to 2013 is represented.

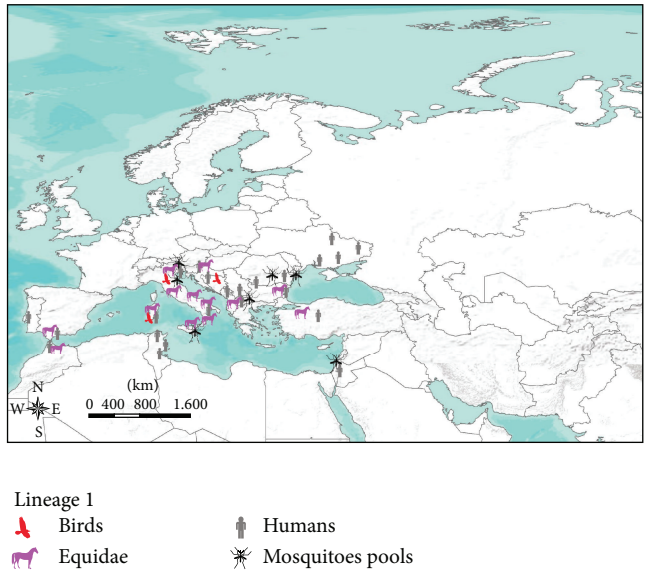

Figure 1: Lineage 1 strain occurrence in Europe and in the Mediterranean Basin from 2009 to 2013.

3. Occurrence of Lineage 2 WNV Strains from 2009 to 2013

3.1. Eastern Europe. Until 2004 lineage 2 WNV was not detected outside of Africa, but from this year lineage 2 was repeatedly identified in several parts of Europe. In 2004, a WNV strain correlated to the Central Africa lineage 2 viruses was isolated from goshawks (*Accipiter gentilis*) in southeast *Hungary*. Sporadic cases of infection were observed in this country between 2004 and 2007 in wild birds, sheep, horses, and humans. In 2008 and 2009 lineage 2 WNV strain was detected in Hungary and *Austria*, where the virus was isolated from wild hawks (*Accipiter* spp.) and one captive kea (*Nestor notabilis*) [18, 60]. After 2008, human cases were notified in Hungary from 2010 to 2012: 3 cases were reported in 2010, 1 of which in a man living close to the Romanian border, 3 cases in 2011, and 17 in 2012 [30]. In 2013, 31 human cases were reported between September and October [31].

In *Russia*, large epidemics of WND in humans were observed in the area of Volgograd since 2007, when RNA of WNV belonging to lineage 2 was detected in human brain and blood samples. In 2010 the same viral strain was responsible for a total of 552 human cases in Russia [61, 62], which represents the largest number of WND human cases that has never been registered in that country before. In addition to Volgograd province, other regions were involved: Rostov, Voronezh, Krasnodar, Astrakhan, Kalmoukia, Tatarstan, and Chelyabinsk oblasts [26]. Volgograd province was the most affected area by the viral circulation also in 2011, with 61 cases out of a total of 153 [26]. In 2012, 447 human cases (210 of them in the Volgograd province) were notified in Russia [30].

In 2013, 177 human cases were confirmed in Russia (Astrakhanskaya oblast: 69 cases; Volgogradskaya oblast: 49 cases; Saratovskaya oblast: 30 cases; Adygea Republic: 1 case; Belgorodskaya: 2 cases; Kaluzhskaya: 1 case; Lipetskaya: 2 cases; Omskaya: 1 case; Orenburgskaya: 1 case; Rostovskaya: 8 cases; Samarskaya: 9 cases; and Voronezhskaya oblasts: 4 cases [31].

In *Romania*, an apparent change in the epidemiological situation was observed in 2010: for the first time in more than ten years, several human confirmed cases were detected also in the central and northern provinces of the country, not reached before by the infection [36]. In 2010 molecular investigations revealed that these episodes of WNV infection were due to lineage 2, genetically related to the 2007 Russian strain [35]. A total of 83 human cases were reported between 2010 and 2012 [26]. In 2010 human cases were distributed in 19 districts all over the country, with clusters of infection in the southeastern district of Constanta and in the urban areas of Blaj (in the western Romania) and Bucharest. Many cases ($n = 35$) were recorded in the southern part of the country, which is an area known as having been endemic for WNV during previous years. However, WNV infection was reported in humans in previously unaffected areas, such as districts in central Transylvania and in the Moldavian Plateau [35]. WNV circulation was observed also in horses. In 2010, 6 cases of equine infection were notified to OIE: 5 cases in Braila and one in Constanta County, in southeast Romania [63]. In 2011 and 2012, most of the cases were recorded in Bucharest urban area [26].

From August to October 2013, 24 human cases were reported from different municipalities of Romania: Bacau, Braila, Bucharest, Constanta, Galati, Ialomita, Iasi, Ilfov, Mures, Sibiu, and Tulcea [31].

3.2. Middle East. In *Israel* lineage 2 was detected between 2009 and 2010 in mosquito pools collected in the northern part of the country [59].

3.3. Southern Europe. In the summer of 2010, 261 human WNV infections were diagnosed for the first time in *Greece*, including 197 neuroinvasive cases and 34 deaths. Most cases occurred in the northeastern part of the country [64]. Lineage 2 WNV strain was detected in *Culex pipiens* mosquitoes collected in two locations where human cases were reported,

in one blood donor living in the same area and in resident birds (*Eurasian magpie*) [65–68]. WNV lineage 2 genomic sequences obtained from viruses isolated from one affected person [68] and from mosquito pools [64, 65, 69] showed a high genetic identity to the Hungarian WNV strain isolated from birds in 2004 [70]. In 2010, additional 30 cases in equines were confirmed [71]. One year later, in 2011, 101 human cases (with 8 deaths) were reported in Greece. The infection spread to new areas and 17 further cases occurred in districts that had not been previously affected [72]. Twenty-three equine cases (with 1 death) were confirmed in 2011 [73]. Genomic sequences of the virus were obtained from a seroconverted chicken in July 2011 in the city of Agios Athanasios [74]. This isolate showed a close genetic relationship with lineage 2 strain which emerged in Hungary in 2004 as well as a high homology with the Nea Santa strain detected in *Culex pipiens* in 2010 in Greece [74]. In 2012, a total of 161 human cases were reported, but only 47 were confirmed by laboratory investigations [75]. In the same year, 15 equine cases were notified and confirmed [76]. The molecular characterization of two isolates from chickens suggested that the virus responsible for the epidemic in Greece in 2012 was again the Nea Santa-Greece-2010 strain [77]. In 2013, for the fourth consecutive year, Greece reported WNV infection in humans: 86 cases were confirmed from the regional units of East Attica, Athens, Thessaloniki, Imathia, Xanthi, Kavala, Serres, Corfu, and Pella, already affected by the virus circulation in the previous years, and the newly infected region of Ileia [78]. In addition, 15 horse cases were reported in Xanthi, Attiki, Achaia, Kavala, Evros, Serres, and Lasithi [79].

During 2011 in *Italy* the WNV caused several outbreaks among horses and birds. Lineage 2 strain was found in two pools of *Culex pipiens* collected in Friuli-Venezia Giulia region and in the tissues of a resident collared dove (*Streptopelia decaocto*) found dead in Veneto region, in northeast Italy [80]. During the summer of 2011, WNV lineage 2 was also detected in urine samples of a febrile patient in Marche region [81] and in a patient coming from northeastern Sardinia. These strains were closely related to each other and to those responsible for the outbreaks that occurred in Greece and Hungary in 2010 and 2005, respectively [52, 69].

Two mosquito pools (*Culex pipiens*) collected in 2012 were found positive to WND lineage 2 in Veneto and Sardinia [82] and in the same year WNV strains belonging to lineage 2 were detected and isolated from the tissues of goshawk (*Accipiter gentilis*) and carrion crows (*Corvus corone*) in Sardinia [7].

In 2013 the presence of 50 cases was confirmed in horses, 12 of which were clinical, in Veneto, Lombardy, Emilia Romagna, Calabria, Sardinia, and Sicily regions. The analysis, of one dead horse in Emilia Romagna, confirmed the circulation of WNV lineage 2. Lineage 2 circulation has been confirmed in mosquitoes and in wild birds in Veneto, Lombardy, Emilia Romagna, and Sardinia regions [82].

In 2013 40 neuroinvasive cases of WND (WNND) have been reported in humans in Veneto, Emilia Romagna, Lombardy, and Apulia regions and 30 people with West Nile fever tested positive to WNV in Veneto, Emilia Romagna, and Lombardy regions. Lineage 2 was identified in Veneto region in plasma and/or urine of seven patients with WNND or WNF and in a blood donor, while WNV lineage 1 was, respectively, detected in an organ donor and in a blood donor [53]. Therefore in 2013 the cocirculation of lineages 1 and 2 has been confirmed in Veneto region in mosquitoes and human [53, 82].

In *Albania* a human case was confirmed in 2010 in a 14-year-old child in the southeast prefecture of Korce (bordering Greece). In 2011, 49 human cases (15 confirmed) of WNV infections were detected in the coastal and central parts of Albania. Lineage 2 was confirmed to be the causative agent of human cases reported in 2011 [26].

In 2012, WNV infection was described in animals in Albania. In a study performed in 2012, 37 out of 167 collected equine sera were positive to serological tests, while no WNV-specific antibodies were detected in 95 samples from domestic birds [83].

In *Serbia*, in 2009 and 2010, 349 horses were randomly collected in Belgrade (in Sabac and in Vojvodina regions) and analysed for WNV-specific neutralising antibodies. This study reported the first serological evidence of WNV infection in Serbia: 42 (12%) seropositive horses were detected [84].

In 2012, 71 human cases were notified, with 53 of which in Belgrade. This was the first reported episode of WNV infection in humans in Serbia [26]. Antibodies against WNV were detected in 7 samples collected from wild bird species (four from mute swans (*Cygnus olor*), two from white-tailed eagles (*Haliaeetus albicilla*), and one from a common pheasant (*Phasianus colchicus*) in 2012 in Vojvodina [85]. Nine WNV RNA positive birds, three northern goshawks (*Accipiter gentilis*), two white-tailed eagles, one legged gull (*Larus michahellis*), one hooded crow (*Corvus cornix*), one bearded parrot-bill (*Panurus biarmicus*), and one common pheasant, were detected. The phylogenetic analysis showed two distinct clusters of lineage 2 closely related to those circulating in neighbouring countries (Greece and Hungary) [85]. This was the first report of the occurrence of WNV in wild birds in Serbia [85]. Entomological investigations performed in Belgrade in August 2012 revealed the presence of WNV lineage 2 nucleic acid in 10 mosquito pools [70, 86]. In 2013, 302 human cases were reported between July and October, with the majority of them concentrated in Grad Beograd area [31].

Figure 2 shows the geographical distribution of WNV lineage 2 strain in Europe and in the Mediterranean Basin from 2009 to 2013.

4. Discussion and Conclusions

The presence of WNV in the Old World is well known since decades. WNV was first identified in 1937 from a native woman of the West Nile province of Uganda [87]. Since then, both sporadic cases and major outbreaks of WND were reported in Africa, Middle East, Europe, and Asia. Epidemiological aspects of WNV transmission were well documented in the early 1950s in Egypt and in Israel, in the 1960s in France, and in the 1970s in South Africa [4, 13, 88].

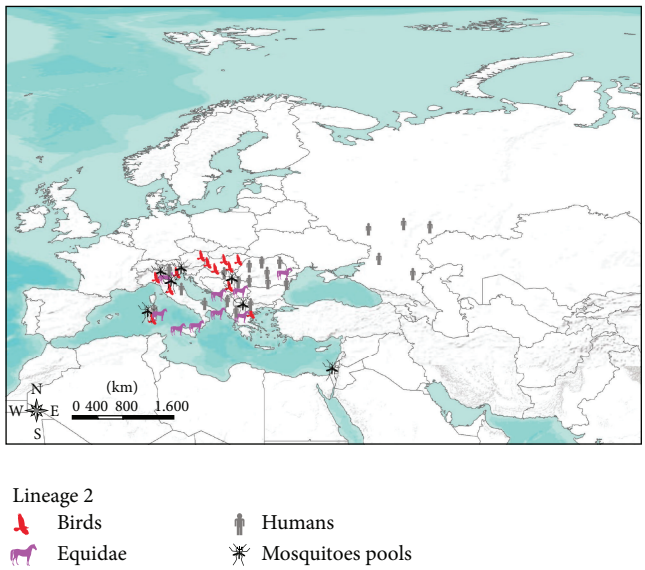

Figure 2: Lineage 2 strain occurrence in Europe and in the Mediterranean Basin from 2009 to 2013.

In Europe the first significant urban epidemic occurred in Bucharest (Romania) in 1996 [37]. Since then the WNV sporadically occurred around the Mediterranean and Eastern European countries. During the last two decades, however, the disease reemerged in Europe with an increasing frequency in humans, where severe cases of neuroinvasive disease were observed. The WNV detection in geographical areas apparently not previously affected by virus transmission, the severity of the infection in humans and horses, the absence of an effective vaccine to protect people, and the mosquito-borne transmission give to this disease all the characteristics required to be considered a major threat for public health in many countries.

The increased number of cases of WNV infection notified in Europe and in the Mediterranean Basin in the last years may be partially due to the rising awareness about this infection, but an actual spread of the virus across the Mediterranean and European countries cannot be excluded, and the possible consequences of this increased exposure to the virus for human populations should not be underestimated.

The transmission of WNV across Europe and Mediterranean Basin currently depicts new scenarios. The WNV circulation in Europe is probably greatly influenced by the flyways of migratory bird species. It is noteworthy that some territories of northwestern Europe were never affected by virus circulation. The United Kingdom, the northwest of France, The Netherlands, Belgium, Denmark, Germany, and Scandinavian and Baltic countries apparently did not experience any case of WNV infection in the past (Figures 1 and 2). This apparent difference of WNV occurrence in Europe cannot be explained simply by a different mosquito fauna composition or abundance. The known European spatial distribution of some vector species, like *Ochlerotatus caspius* or *Culex pipiens*, does not support a different susceptibility of northwestern Europe to WNV infection [89]. The actual knowledge may reasonably suggest a more decisive role of migratory birds in the spread of WNV across the European continent [90]. However, a better evaluation of the influence of bird migratory routes on the spatial spread of WNV in Europe would be fundamental for a more accurate assessment of the risk of WNV introduction into new areas.

On the other hand, the WNV is constantly detected in several territories of the Mediterranean Basin and in southeastern Europe. This constant WNV occurrence in the Mediterranean region cannot be caused only by the virus reintroduction from the sub-Saharan Africa. Probably endemic cycles are established in the Mediterranean area, although it is not clear which bird species might play a role in the local persistence of the infection or the contribution of overwintering mosquitoes. In addition, the role of persistent infection in organs of infected birds cannot be excluded, which may represent a further infection overwintering mechanism when coupled with the predation by other bird species, as, for example, those belonging to Corvidae or Falconidae families [91].

A relevant epidemiological finding in the recent years is represented by the spread of lineage 2 across Europe and Mediterranean Basin, with zones where the circulation of the two lineages coexists. To date the copresence of the two lineages has been proven in Italy and in Romania (Figures 1 and 2).

Lineage 2, which was endemic in sub-Saharan countries of Africa, was firstly identified in Hungary in 2004, then in Russia in 2007, in Romania in 2010, and in Italy in 2011 [1]. The genetic homology of lineage 2 detected in Hungary in 2004 with the Greek isolates in 2010 [69], with the Italian strains circulating in 2011 and 2012 [7], and with isolates in Serbia 2012 [85] suggests the involvement of wild birds species able to spread the virus in wide areas of the Mediterranean and Balkan areas.

In Italy lineage 1 seems to be more linked to large epidemics, especially in areas surrounding wetlands with a significant population of migratory birds (e.g., the delta of Po River or wetlands in Sardinia island), whereas lineage 2 sporadically occurs in scattered locations across the country, without causing apparent large epidemics. Similarly, WNV lineage 2 has been sporadically detected in birds in Austria, whereas a considerable number of human cases were associated with the circulation of this lineage in Romania, Hungary, Greece, and Russia.

Possible differences between lineage 1 and lineage 2 viruses in their pathogenicity for birds have been poorly investigated [91]. In 2013, through an experimental study, Ziegler et al. proved the high virulence of WNV lineage viruses 1 (isolated in New York in 1999) and 2 (strain isolated in Austria in 2009) in falcons showing no significant differences in mortality rates or viraemia levels [92].

Similarly little information is available on possible different characteristics of lineages 1 and 2 viruses in relation to the vector competence of the main mosquito species. The unique available study compares the vector competence of African vectors (*Culex neavei* and *Culex quinquefasciatus*) for different African WNV lineages [93] and, therefore, its results cannot be extrapolated outside the African continent.

WNV lineage 2 isolated in Italy is genetically related to those detected in Hungary [7, 94] and, therefore, the apparent dissimilar capacity of spreading of this lineage between Italy and other countries cannot rely on virus diversity but probably is due to local ecological and epidemiological conditions. In particular, the sequence analysis of WNV lineage 2 isolated in Sardinia Island from a northern goshawk (*Accipiter gentilis*) suggests a common origin with Hungarian isolates, thereby supporting a role of short-range migratory birds in the spread of virus in Italy from central Europe. According to this hypothesis, the differences observed in Italy between the spatial distribution and occurrence of the two lineages would be linked exclusively to a more recent introduction of lineage 2 from central and eastern European countries, where this virus is endemic [7, 94]. In addition, the observed differences in the amino acids composition of viral NS3 protein between Greek lineage 2 isolates and the Italian ones could explain the higher virulence of Greek strain for humans [7, 94].

The cocirculation of lineages 1 and 2 in some countries and the genetic variation between strains isolated in different years in the same country [21, 95] may create the favourable conditions for genetic reassortments with possible variations in the virulence of the viral strains, which would lead to consequences presently difficult to assess.

In addition, the influence of possible changes of climatic and environmental conditions should not be underestimated in the observed spread of WNV in the European and Mediterranean countries. These factors, in fact, may influence the seasonality of disease transmission [96], due to increased number of mosquito replication cycles (consequently also a higher rate of overwintering virus-carrying mosquitoes) and increased virus transmission rates [97].

Given the absence of evidence for vertical transmission in mosquitoes (although it cannot be excluded), the persistence of the infection due to the survival of infected adult mosquitoes during winters or a so-far unidentified vertebrate reservoir host is hypothesized for being responsible for the maintenance of the virus. Resident birds seem to be particularly suitable for this role, given their density and the ability for some species to fly for relatively long distances, independently of seasonal migratory pattern.

Since 2010, WNV showed a clear capacity both to spread into areas not previously affected by the viral circulation and to persist in areas where the ecological and climatic conditions are favourable to its circulation.

A further aspect to be considered is the cocirculation in the Old World of WNV and other flaviviruses, sharing the same hosts and ecological niches, as for the Usutu virus. In Italy the circulation of Usutu virus has been detected simultaneously with WNV in several geographical areas [98], with the possibility of recombination, which may influence the transmission capacity and the occurrence of these viruses in vertebrate hosts.

In the last decades WNV continued to evolve, changing its transmission rate and geographical patterns. The adaptability showed by this multihost virus should induce all researchers to continuously and carefully monitor the evolution of the epidemiological situation of WND in Europe and in the Mediterranean Basin.

Further studies would be useful also to fill some existing gaps in our current knowledge on WNV epidemiology. For such reasons, public health and veterinary officials should strictly cooperate to establish effective early warning systems across the region, useful to prevent and reduce the impact of this emerging disease on human and animal health.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Authors' Contribution

Daria Di Sabatino and Rossana Bruno contributed equally to this work.

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Review Article

Predictive Symptoms and Signs of Severe Dengue Disease for Patients with Dengue Fever: A Meta-Analysis

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The aim of the meta-analysis was to provide more solid evidence for the reliability of the new classification. A systematic literature search was performed using PubMed, Armed Forces Pest Management Board Literature Retrieval System, and Google Scholar up to August 2012. A pooled odds ratio (OR) was calculated using either a random-effect or a fixed-effect model. A total of 16 papers were identified. Among the 11 factors studied, five symptoms demonstrated an increased risk for SDD, including bleeding [OR: 13.617; 95% confidence interval (CI): 3.281, 56.508], vomiting/nausea (OR: 1.692; 95% CI: 1.256, 2.280), abdominal pain (OR: 2.278; 95% CI: 1.631, 3.182), skin rashes (OR: 2.031; 95% CI: 1.269, 3.250), and hepatomegaly (OR: 4.751; 95% CI: 1.769, 12.570). Among the four bleeding-related symptoms including hematemesis, melena, gum bleeding, and epistaxis, only hematemesis (OR: 6.174; 95% CI: 2.66, 14.334; $P < 0.001$) and melena (OR: 10.351; 95% CI: 3.065, 34.956; $P < 0.001$) were significantly associated with SDD. No significant associations with SDD were found for gender, lethargy, retroorbital pain, diarrhea, or tourniquet test, whereas headache appeared protective (OR: 0.555; 95% CI: 0.455, 0.676). The meta-analysis suggests that bleeding (hematemesis/melena), vomiting/nausea, abdominal pain, skin rashes, and hepatomegaly may predict the development of SDD in patients with DF, while headache may predict otherwise.

1. Introduction

Dengue is an infectious disease caused by dengue virus (DENV). It is endemic in many tropical and subtropical areas. Patients infected with DENV have a wide spectrum of clinical manifestation, ranging from silent infections with no symptoms to a mild flu-like syndrome, dengue fever (DF), or severe dengue disease (SDD), including dengue haemorrhagic fever (DHF) and dengue shock syndrome (DSS) [1–3]. Recently, DF has become one of the most challenging public health problems in affected regions, as the DF incidence increases rapidly worldwide [4]. There are approximately 2.5 billion people at risk for DF worldwide. Fifty million people would acquire DENV annually, and half a million among them would develop dengue hemorrhagic fever, including 22,000 deaths [5].

Several methods have been used for the diagnoses of DF. However, there lacks an accurate means to predict the severity of disease at early stages of the infection. Since patients with mild or classical DF can develop SDD later [2], it is important to look for symptoms/signs to facilitate the early prediction of the progression into SDD. The establishment of predictive symptoms/signs is essential for preventing unnecessary hospitalization, reducing disease burden, and controlling potential SDD. Based on the dengue guidelines (2009), the warning symptoms/signs for SDD include abdominal pain or tenderness, persistent vomiting, mucosal bleed, lethargy, and restlessness.

Published studies about symptoms/signs that are associated with SDD have been inconclusive. For instance, Khan et al. found that male DF patients were more likely to progress into DHF (OR: 2.3, 95% CI: 1.1–4.5, P value 0.021) [6], while

there was no association with SDD [7, 8]. The frequencies of symptoms/signs of vomiting/nausea, abdominal pain, skin rashes, and bleeding were also found to be correlated with SDD [6–11]. However, the published studies were not able to conclude that these symptoms/signs are associated with SDD. In addition, although some findings such as viral factors, varying host immune conditions, host immune reactions, and laboratory tests can predict SDD [12], the clinical manifestations might always offer the earliest markers in predicting SDD. For example, patients with nonsevere dengue could be clustered into two groups: one with warning signs, such as abdominal pain, mucosal bleeding, and liver enlargement, and the other without those signs [2], as most of the warning signs were associated with an indication for ICU admission and were severe, even for the relationship of death [13].

Because of these inconsistent reports, more accurate methods to predict SDD are needed. We conducted the meta-analysis to identify which clinical symptoms/signs are associated with SDD and to help find better methods to predict the development of SDD in patients with DF.

2. Materials and Methods

2.1. Literature Searches. Our study was performed according to the recommendations of the PRISMA Statement [14], which is available in supporting information (see Table S1 available online at http://dx.doi.org/10.1155/2014/359308). Computerized searches were conducted on NCBI PubMed, Armed Forces Pest Management Board Literature Retrieval System, and Google Scholar. As few studies before 2000 met the criteria of WHO guidelines (1997), the search time window was set between January 1, 2000, and August 1, 2012, with no language limit. Because severe dengue disease (SDD) is classified as DHF and DSS, we used the following key words for searching: dengue fever, DF, dengue haemorrhagic fever, DHF, dengue shock syndrome, DSS, and clinical diagnosis. We also manually searched the reference lists of the retrieved articles to identify more qualified studies.

2.2. Inclusion and Exclusion Criteria. Studies were eligible for inclusion if they met the following criteria: (1) retrospective, prospective, or cross-sectional studies providing the details of symptoms/signs as well as any information regarding gender, vomiting/nausea, abdominal pain, skin rashes, bleeding, headache, lethargy, retroorbital pain, diarrhea, hepatomegaly, or tourniquet test; (2) the symptoms/signs of DF and SDD were distinguished; (3) cases with DF in the study were confirmed by laboratory tests; cases with SDD were defined by one or more of the following: plasma leakage that may lead to shock (dengue shock) and/or fluid accumulation, with or without respiratory distress, and/or severe bleeding, and/or severe organ impairment. When two or more publications reported the same study, we chose the most recent one. Reports providing inadequate information were excluded.

2.3. Quality Assessment. The quality of the selected studies was assessed independently by two authors using the Newcastle-Ottawa Scale (NOS) [15]. The NOS uses different tools for case-control and cohort studies and consists of 3 parameters of quality: selection, comparability, and exposure/outcome assessment. The NOS assigns a maximum of 4 points for selection, 2 for comparability, and 3 for exposure or outcome. We assigned NOS scores of 1–3, 4–6, and 7–9 for low, intermediate, and high-quality studies, respectively. Discrepancies were settled by consensus after joint reevaluation of the original studies.

2.4. Data Extraction. For each eligible manuscript, the following information was extracted: (1) first author's name and year of publication; (2) study design (prospective, retrospective, or cross-sectional); (3) study populations (children, adults, or both); (4) distinctive numbers of patients with specific symptoms in DF and SDD groups.

2.5. Statistical Analysis. The prevalence rates of specific symptoms/signs in DF and SDD groups were compared by calculating an odds ratio (OR) with a 95% confidence interval (CI) using either a fixed-effect model or a random-effect model. Predictive factors of interest included gender, vomiting/nausea, abdominal pain, skin rashes, bleeding (hematemesis, melena, gum bleeding, and epistaxis), headache, lethargy, retroorbital pain, diarrhea, hepatomegaly, and tourniquet test.

Heterogeneity between studies was assessed using both the Chi-square test with a P value ≤ 0.10 and the inconsistency index (I^2) with a cut-off of 50% [16]. To explore the potential sources of heterogeneity among studies, subgroup analyses and metaregression were performed on the strata of study design, study population, and publication year.

Potential publication bias was comprehensively assessed by Begg's funnel plot and Egger's rank correlation test of asymmetry. Publication bias was determined present when the P value ≤ 0.10 by Egger's or Begg's test. All statistical analyses were performed using STATA version 11.0 (STATA Corporation, College Station, TX, USA).

3. Results

3.1. Study Characteristics and Quality. The search strategy identified 446 citations. Sixteen articles published between 2000 and 2012 were ultimately included in this meta-analysis based on the inclusion and exclusion criteria (Figure 1). The final collection consists of 10 prospective [9–11, 17–23], four retrospective [7, 8, 24, 25], and two cross-sectional studies [26, 27]. As listed in Table 1, eight of the 16 studies reported on a population study of children, two on adults and six on both. The factor of gender was included in five studies. The clinical symptoms/signs of vomiting/nausea were included in 13 studies, abdominal pain in 13, skin rashes in 10, bleeding in 13, headache in 13, lethargy in 6, retroorbital pain in 9, diarrhea in 7, hepatomegaly in 8, and tourniquet test in 4 studies. Four common kinds of bleeding symptoms were present in these studies, including hematemesis in five, melena in four, gum bleeding in seven, and epistaxis in five. Based on the NOS scores, 12 studies (75%) were of high quality and the other four (25%) were acceptable.

Table 1: Basic features of the eligible studies.

Author (publication year)	Study design	Population	Gender		Vomiting/nausea		Bleeding		Headache		Abdominal pain		Retroorbital pain		Rashes		Diarrhea		Hepatomegaly		Lethargy		Tourniquet test	
			DF (M/F)	SD (M/F)	DF (Y/N)	SD (Y/N)	DF (Y/N)	SD (Y/N)	DF (Y/N)	SD (Y/N)	DF (Y/N)	SD (Y/N)	DF (Y/N)	SD (Y/N)	DF (Y/N)	SD (Y/N)	DF (Y/N)	SD (Y/N)	DF (Y/N)	SD (Y/N)	DF (Y/N)	SD (Y/N)	DF (Y/N)	SD (Y/N)
Ahmed et al. (2001) [17]	Prospective	Children	—	—	4/22	5/41	16/10	46/0	22/4	34/12	—	—	7/19	10/36	3/23	24/22	6/20	8/38	0/26	36/10	—	—	—	—
Narayanan et al. (2002) [21]	Prospective	Children	22/21	9/7	34/9	15/1	23/20	16/0	13/30	4/12	10/33	4/12	3/40	4/12	4/39	1/15	—	—	19/24	12/4	11/32	3/13	5/38	9/7
Endy et al. (2002) [19]	Prospective	Children	—	—	44/89	14/5	2/130	1/18	82/50	14/5	20/111	6/13	—	—	6/125	1/18	5/127	1/18	—	—	43/89	6/13	—	—
Phuong et al. (2004) [22]	Prospective	Children	—	—	178/134	234/85	56/256	36/283	140/167	88/230	156/156	234/84	—	—	—	—	—	—	—	—	—	—	106/166	128/145
Carlos et al. (2005) [18]	Prospective	Children	143/96	72/48	—	—	11/221	6/105	—	—	69/168	51/68	—	—	—	—	—	—	—	—	0/238	4/115	—	—
Shah et al. (2006) [9]	Prospective	Children	—	—	11/0	87/2	0/11	68/21	11/0	87/2	9/2	87/2	11/0	87/2	—	—	—	—	0/11	77/12	11/0	87/2	10/1	15/15
Malavige et al. (2006) [23]	Prospective	Adult	—	—	18/15	51/24	5/28	37/38	26/7	45/30	3/30	14/61	—	—	—	—	7/26	24/51	10/23	39/36	—	—	—	—
Lee et al. (2006) [24]	Retrospective	Both	177/235	119/113	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
Riaz et al. (2009) [27]	Cross-sectional	Both	—	—	134/35	83/26	6/163	89/20	23/146	13/96	85/84	61/48	0/169	1/108	55/114	37/72	10/159	2/107	1/168	1/108	—	—	—	—
Lee et al. (2009) [25]	Retrospective	Adult	1183/672	53/29	604/1251	29/53	—	—	606/1249	16/66	252/1603	14/68	5/1850	0/82	887/968	49/33	—	—	—	—	—	—	—	—
Priyadarshini et al. (2010) [10]	Prospective	Both	—	—	90/69	44/18	—	—	101/58	33/29	29/130	31/31	34/125	2/60	31/128	26/36	—	—	—	—	—	—	—	—
Khan et al. (2010) [8]	Retrospective	Both	99/62	30/10	93/68	27/13	6/155	29/11	21/140	2/38	6/80	1/10	—	—	44/117	31/9	26/135	5/35	—	—	—	—	—	—
Giraldo et al. (2011) [7]	Retrospective	Children	77/74	16/14	96/55	23/7	51/100	14/16	92/59	17/13	78/73	22/8	18/133	4/26	63/88	16/14	29/122	5/25	27/124	7/23	54/97	20/10	—	—
Falconar et al. (2012) [20]	Prospective	Both	—	—	13/15	4/1	13/28	5/0	—	—	0/28	5/0	22/6	4/1	—	—	—	—	0/28	2/3	—	—	8/20	5/0
Karoli et al. (2012) [26]	Cross-sectional	Both	—	—	—	—	13/83	42/0	77/19	28/14	—	—	—	—	26/70	12/30	—	—	47/49	25/17	—	—	—	—
Sirivichayakul et al. (2012) [11]	Prospective	Children	—	—	95/28	32/2	—	—	107/16	25/9	59/64	25/9	35/88	13/21	53/70	18/16	21/102	17/17	—	—	23/100	6/28	—	—

Note: "M": male; "F": female; "Y": yes, the group has the symptom; "N": no, the group has no symptom. "—": no statistics.

Table 2: Results of meta-analysis for the clinical manifestations between DF and SDD.

Clinical manifestation	Number of studies	Odds ratio (95% CI)	Test for OR	Test of heterogeneity		Publication bias	
			P	I^2 (%)	P	Egger's test	Begg's test
Gender	6	1.230 (0.999, 1.513)	0.051	0	0.686	0.991	0.707
Vomiting/nausea*	13	1.692 (1.256, 2.280)	0.001	39.7	0.069	0.455	0.428
Abdominal pain*	13	2.278 (1.631, 3.182)	<0.001	55.5	0.008	0.343	0.669
Skin rashes*	10	2.031 (1.269, 3.250)	0.003	69.7	<0.001	0.581	0.592
Bleeding*	12	13.617 (3.281, 56.508)	0.001	94.0	<0.001	0.033	0.373
*Hematemesis**	5	6.174 (2.66, 14.334)	<0.001	0	0.476	0.137	0.211
*Melena**	4	10.351 (3.065, 34.956)	<0.001	0	0.955	0.36	0.734
Gum bleeding	7	2.518 (0.463, 13.685)	0.285	90.5	<0.001	0.058	0.548
Epistaxis	5	2.319 (0.599, 8.976)	0.562	86.9	<0.001	0.981	0.462
*Headache**	13	0.555 (0.455, 0.676)	<0.001	0	0.594	0.177	0.583
Lethargy	6	1.552 (0.714, 3.370)	0.267	49.6	0.078	0.864	1
Retroorbital pain	9	1.096 (0.531, 2.261)	0.804	45.2	0.067	0.810	0.602
Diarrhea	7	1.149 (0.555, 2.380)	0.708	64.1	0.010	0.185	0.230
Hepatomegaly*	8	4.751 (1.769, 12.570)	0.002	72.5	0.001	0.014	0.063
Tourniquet	4	2.194 (0.395, 12.206)	0.369	82.4	0.001	0.715	1

Note: "Italics": using the fixed-effect model, the other symptoms/signs: using the random-effect model; * significantly different between DF and SDD.

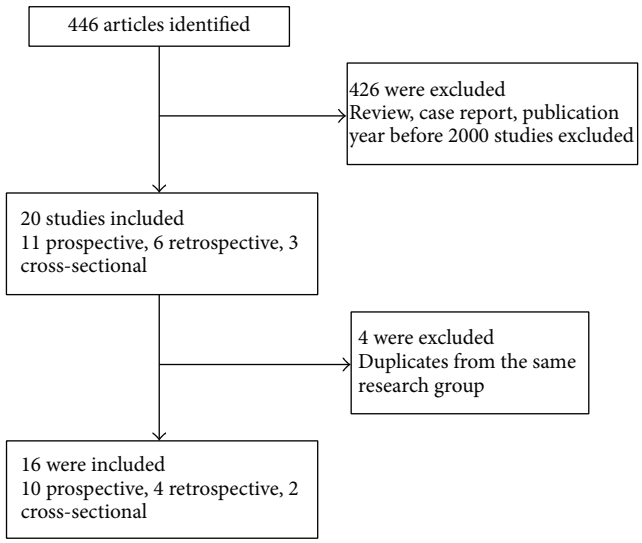
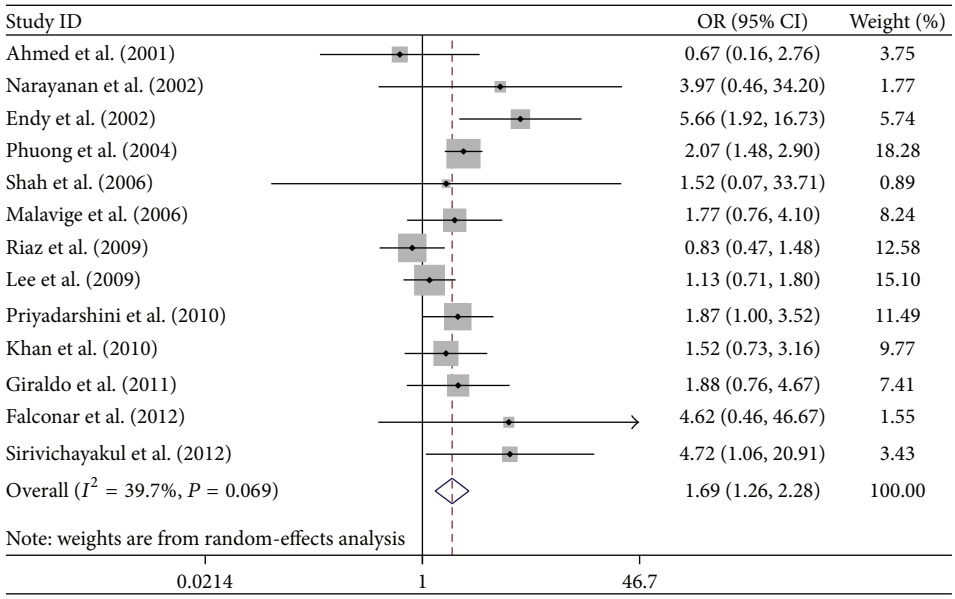


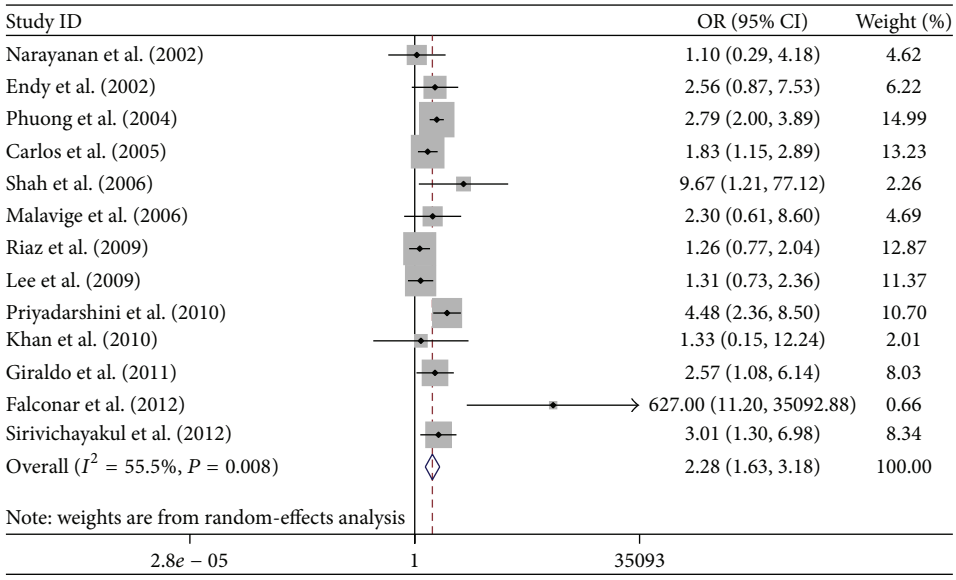
Figure 1: Flow diagram of selection and disposition of studies.

3.2. Potential Predictive Indicators of SDD. In this meta-analysis, the fix-effect model was analyzed in gender, hematemesis, melena, and headache, while the random-effect model was used in vomiting/nausea, abdominal pain, skin rashes, bleeding (gum bleeding and epistaxis), lethargy, retroorbital pain, diarrhea, hepatomegaly, and tourniquet. According to the meta-analysis results, there were no significant differences between the DF and SDD groups in association with the following factors ($P > 0.05$, Table 2): gender of patients, clinical symptoms/signs of lethargy, retroorbital, diarrhea, and tourniquet test. There were significant differences in association between the two groups with the following factors: symptoms/signs of vomiting/nausea, abdominal pain, skin rashes, bleeding, and hepatomegaly ($P < 0.05$, Figures 2(a)–2(e)). In particular, bleeding and hepatomegaly were highly correlated with the progression of DF into SDD, with the ORs at 13.617 (95% CI: 3.281, 56.508) and 4.751 (95% CI: 1.769, 12.570). The results indicate that these two factors strongly predict greater risk of the development of SDD. The other factors such as vomiting/nausea, abdominal pain, and skin rashes were also associated with SDD, while the strength of association was not as strong. In the bleeding symptoms, the ORs for predicting SDD of hematemesis and melena were 6.174 (95% CI: 2.66, 14.334; $P < 0.001$) and 10.351 (95% CI: 3.065, 34.956; $P < 0.001$), respectively, demonstrating significant differences between the DF and SDD groups, while the frequencies of the other two kinds of bleeding, gum bleeding and epistaxis, were not significantly different between the two groups. The details were shown in Table 2, Figures 2 and 3. Interestingly, headache was not associated with the low risk of SDD (OR: 0.555; 95% CI: 0.455, 0.676; Figure 2(f)).

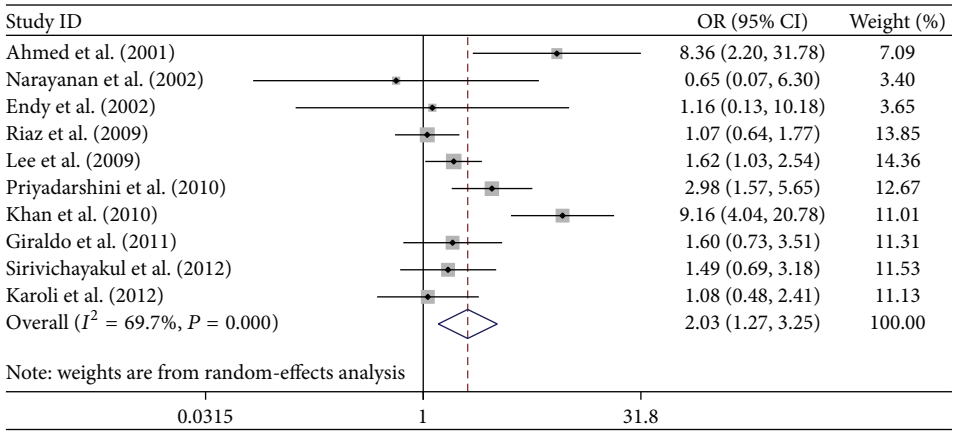
3.3. Heterogeneity Analysis. Metaregression analysis was conducted to examine which factors could have brought heterogeneity across the studies, and 11 clinical symptoms/signs were analyzed, including vomiting/nausea, abdominal pain, skin rashes, bleeding (epistaxis and gum bleeding), lethargy, retroorbital pain, diarrhea, hepatomegaly, and tourniquet test. It turned out that two factors, study design and population, contributed to the heterogeneity in the studies of gum bleeding ($P < 0.10$) and epistaxis ($P < 0.10$). Based on the subgroup analyses, the epistaxis ratio was not significantly different between DF and SDD groups in children ($P = 0.562$), while the difference was significant in adults ($P < 0.001$, OR: 14.139, 95% CI: 6.622, 30.187). Similar results applied to the subgroup analysis of gum bleeding based on the retrospective and prospective studies (data not shown).



(a) Vomiting/nausea

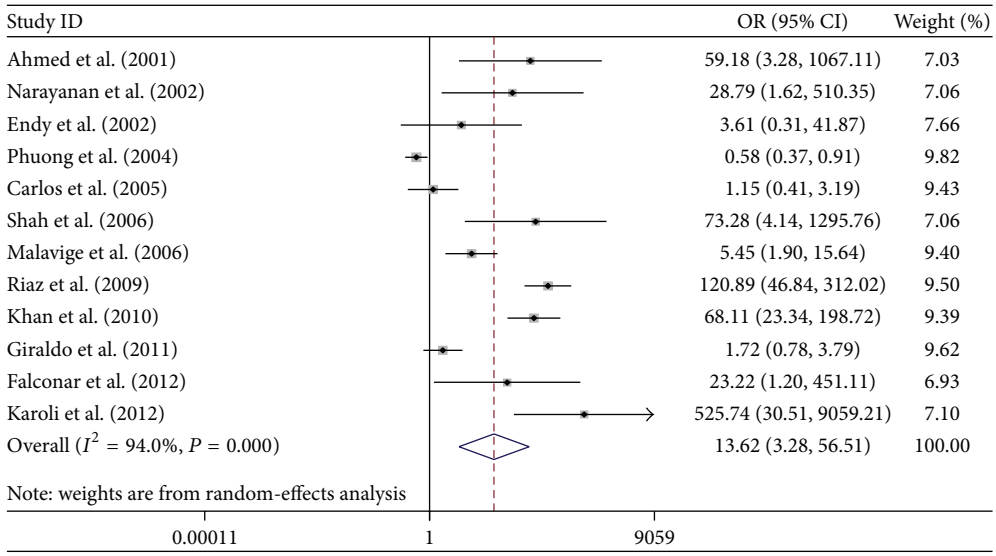


(b) Abdominal pain

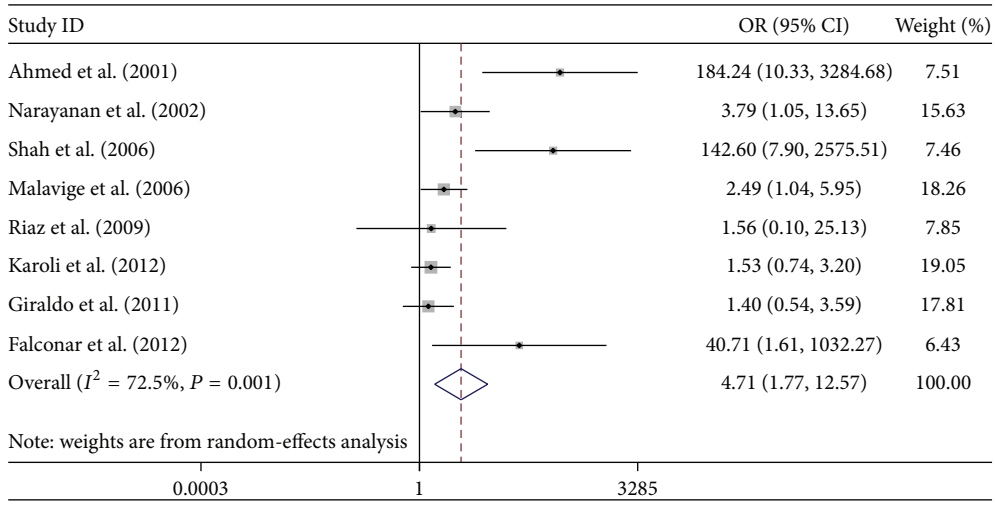


(c) Skin rashes

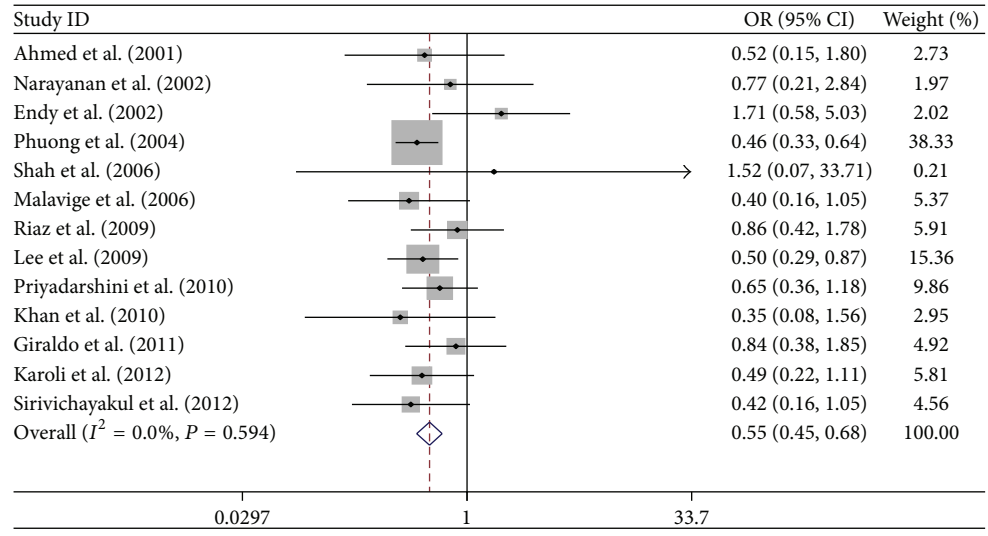
Figure 2: Continued.



(d) Bleeding

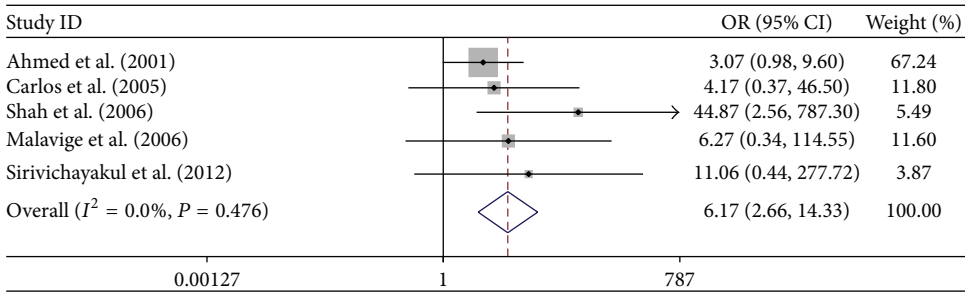


(e) Hepatomegaly

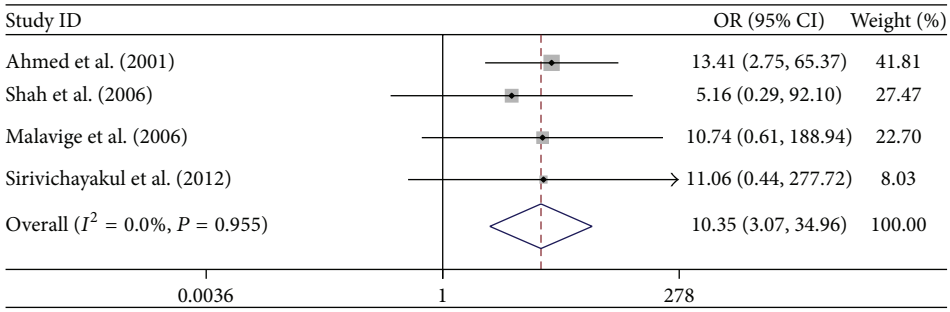


(f) Headache

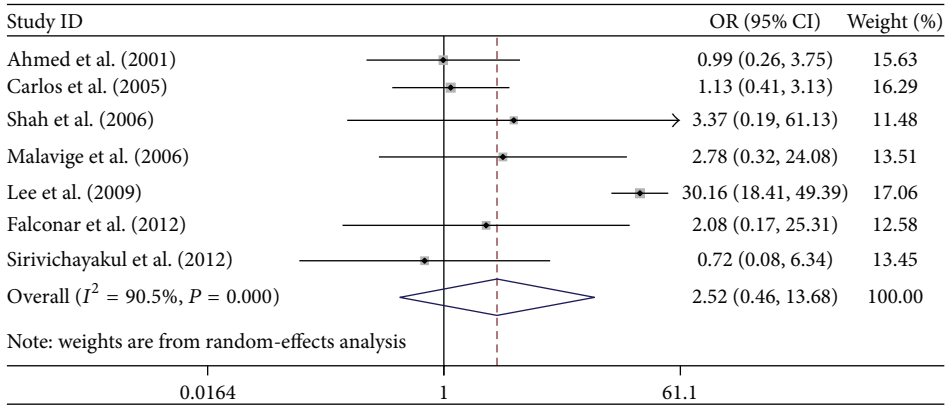
Figure 2: Forrest plots of the relationship between DF and the risk of SDD. (a)–(e) Pooled ORs of SDD are greater than one in 5 symptoms and signs with vomiting/nausea, abdominal pain, skin rashes, bleeding, and hepatomegaly; (f) pooled OR of SDD is smaller than one in headache.



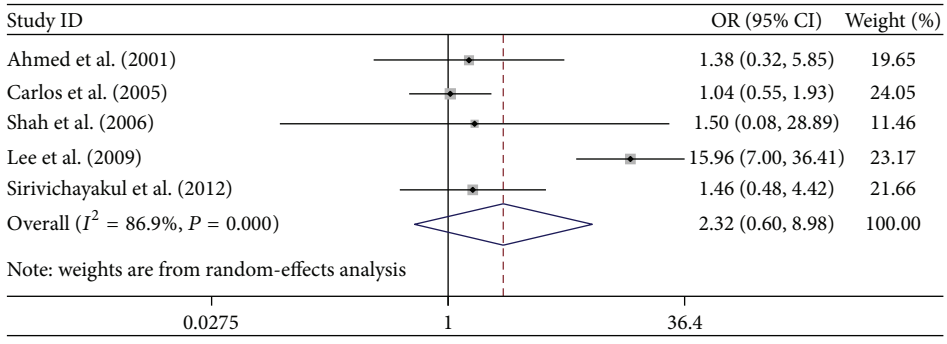
(a) Hematemesis



(b) Melena



(c) Gum bleeding



(d) Epistaxis

Figure 3: Forrest plots of four kinds of bleeding. (a)-(b) Pooled ORs of SDD are greater than one in hematemesis and melena; (c)-(d) pooled ORs of SDD are not significantly different from one in epistaxis and gum bleeding.

3.4. Publication Bias. Funnel plots showed no publication bias in the studies covering vomiting/nausea, abdominal pain, skin rashes, bleeding, or retroorbital pain (Figure S1 and Table 2). The P values of Egger's and Begg's tests also suggested that publication bias had little impact on the results. There were three signs, bleeding, gum bleeding, and hepatomegaly showing publication bias (Egger's test: P = 0.041, 0.058, 0.014).

4. Discussion

The present study is the meta-analysis to comprehensively evaluate the correlation of clinical symptoms/signs with the development of SDD in patients with DF. The results showed that a total of five symptoms/signs significantly predict dengue patients progressing into SDD: vomiting/nausea, abdominal pain, skin rashes, bleeding, and hepatomegaly. The other five factors were not associated with the disease progression, including tourniquet versus nontourniquet, female versus male patients, lethargy, retroorbital pain, and diarrhea. We found that patients with bleeding after DENV infection had approximately a 14-fold increased risk for progression into SDD (including DHF and DSS). When compared with the frequencies of leucopenia and thrombocytopenia, haemorrhagic manifestations, such as gum bleeding, epistaxis, and gastrointestinal bleeding, are less frequent, but not rare [28]. Our analysis included four kinds of bleeding: hematemesis, melena, gum bleeding, and epistaxis. Previous studies showed that the frequencies of hematemesis, melena, gum bleeding, and epistaxis were higher in SDD patients than in DF patients, but none of them were related to the risk of development of SDD in patients with these symptoms [1, 17, 18, 25]. A recent study also showed that the gastrointestinal bleeding was associated with DSS, although it is not a strong association (OR = 1.84) [29]. According to our meta-analysis, the two kinds of gastrointestinal bleeding that strongly predicted SDD were hematemesis (OR: 6.174; 95% CI: 2.66, 14.334; $P < 0.001$) and melena (OR: 10.351; 95% CI: 3.065, 34.956; $P < 0.001$), while the other two kinds of bleeding were not significant risk factors. The other four clinical symptoms and signs proved significant for predicting the progression into SDD are vomiting/nausea (OR: 1.692; 95% CI: 1.256, 2.280), abdominal pain (OR: 2.278; 95% CI: 1631, 3.182), skin rashes (OR: 2.031; 95% CI: 1.269, 3.250), and hepatomegaly (OR: 4.751; 95% CI: 1.769, 12.570). Although the vomiting/nausea, abdominal pain, and skin rashes showed a weak association with SDD compared with DF patients, these warnings must be taken seriously as recent studies demonstrated that these symptoms were associated with the mortality caused by dengue [30, 31]. We found that patients with hepatomegaly after DENV infection had approximately a 5-fold increased risk of progression into SDD; however, the CI was with a wide range. The possible reason is that the rate of hepatomegaly was significantly higher in adults than the elderly [32].

The most accepted hypothesis for progression of DF is that subneutralizing levels of DENV-specific antibodies exacerbate the disease by means of an antibody-dependent enhancement of infection (ADE) [33], which induces a complicated immunopathogenesis in the host. The extent of vascular permeability is enhanced as a result of ADE [34] and patients with SDD as well as alterations of endothelial cells have been shown to experience thrombocytopenia and coagulation disorders [35]. These significant symptoms/signs, especially the bleeding (hematemesis/melena) and hepatomegaly, are manifested in patients with SDD as a result of the aforementioned alterations. In the *in vivo* model for ADE-induced SDD, gastrointestinal bleeding and viral RNA increased in the liver were observed [36]. Additionally, based on skin biopsies, IgM, beta 1 C-globulin, dengue antigen, and fibrinogen deposits were found to be present within or about blood vessel walls of dermal papillae or in the blood vessels [37], implying that skin rashes that appeared in DHF were caused by an immunopathologic process. So in patients with SDD, the host immune system plays a central role in triggering symptoms like bleeding, hepatomegaly, and skin rashes, which could be used to triage patients in need of intensive care.

The unassociated factors/manifestations were gender, lethargy, retroorbital pain, diarrhea, and positivity of a tourniquet test. However, the World Health Organization (WHO) has published guidelines stating that positivity of a tourniquet test may be included in the clinical case definition of dengue haemorrhagic fever [38], and an altered level of consciousness such as lethargy should be paid extra attention [2]. Although the results from this meta-analysis showed unexpected absence of associations, relaxing vigilance over the patients with these symptoms/signs is not recommended, because the results were generated from a random-effect model that tends to be overconservative.

Furthermore, headache was a protective factor against SDD after DENV infection (OR: 0.555; 95% CI: 0.455, 0.676), implying that dengue patients with headache had a lower probability to develop into SDD. The protective effect has been proved by a retrospective cohort study [25]. However, in another study, BALB/c mice were infected with different strains of DENV which were isolated from DSS or DF patients, respectively, and in the mice infected with the strain from DSS patients, DENV-1 isolates appeared to be primarily neurotropic, whereas in the cases of other strains the virus turned to mainly infect lung and liver [39]. Suggesting that high frequency of headache could occur in patients with SDD.

There are limitations in the present study. Firstly, the results will not apply to multicenter prospective studies, since the present meta-analysis only included retrospective and single-center prospective studies. These designs could not eliminate recall and selection biases. Hence, the true associations between these symptoms/signs and the development of SDD might have been distorted. Secondly, the definitions of DF and SDD within these studies may have varied, which brought uncertainty into determining cases. Lastly, some of the results were based on a random-effect model that might weaken the validity of the analysis. Nonetheless, this study explored a new approach to identify the correlations of the symptoms/signs after DENV infection with the risk of progression into SDD, which can greatly facilitate the prevention of SDD.

5. Conclusions

This meta-analysis identified clinical symptoms and signs that significantly predicted DF patients progressing into severe dengue. DF patients with vomiting/nausea, abdominal pain, skin rashes, bleeding (hematemesis/melena), and hepatomegaly were more likely to develop SDD, while patients with headache had a lower risk of progression into SDD. Other factors such as gender, lethargy, retroorbital pain, diarrhea, and positive tourniquet test are not associated with SDD. Further studies, especially ones with larger sample sizes and prospective, are warranted to confirm the findings.

Abbreviations

DENV: Dengue virus
DF: Dengue fever
SDD: Severe dengue disease
DHF: Dengue haemorrhagic fever
DSS: Dengue shock syndrome
NOS: The Newcastle-Ottawa Scale
ADE: Antibody-dependent enhancement of infection
WHO: The World Health Organization.

Conflict of Interests

On behalf of all authors, the corresponding author states that there is no conflict of interests.

Authors' Contribution

All authors were involved in the study design, including developing the search strategies and project protocol. H. Zhang, H. J. Peng, X. H. Zhang, F. Y. Zhou, Z. H. Liu, and X. G. Chen were responsible for supervising the project and performing the literature search and data extraction. H. J. Peng, F. Y. Zhou, and Z. H. Liu assessed the quality of studies. H. Zhang performed data analysis and drafted the paper. X. H. Zhang was responsible for the language editorial. Y. P. Zhou and X. G. Chen revised the paper.

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