Epidemiology and Diagnosis of West Nile Virus Infection

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Abstract

West Nile (WN) virus produces a mosquito-borne infection manifesting in fever and, in serious cases, encephalitis. The virus that causes WN infections, WN virus, belongs to the family *Flaviviridae*, genus *Flavivirus*. The WN virus emerged in New York City in August, 1999, as the first case on the American continent, and seven human deaths were reported. Along with humans, deaths were observed among horses and various birds, such as crows. The WN virus epidemic expanded to the southern United States in 2000 and most of the rest of the country by 2004, extending as far as Argentina in 2007. Since there is a possibility of the WN virus spreading to Japan, where the Japanese encephalitis virus is also prevalent, the TaqMan assay was adapted to develop a sensitive, specific diagnostic test to differentiate WN virus from Japanese encephalitis virus.

Key words: epidemiology, diagnosis, West Nile virus infection

1. Epidemiology

1.1 Global epidemiology

The WN virus (WNV) was first isolated in 1937 from the blood of a febrile patient in the West Nile district of Uganda. WNV has since been found endemic over a wide range of areas in Africa, the Middle East, western Asia and Australia. Outbreaks of varying size occurred in Israel in 1941 and 1951-1954, and in Africa in 1974. After that, no large outbreaks were observed for 20 years, but from 1994 to 2000, WNV outbreaks occurred among humans and horses in Algeria in 1994, Morocco in 1996, Romania in 1996, Tunisia in 1997, the Czech Republic in 1997, the Congo in 1998, Italy in 1998, Israel in 1997-2000, Russia in 1999, France in 2000 and the United States in 1999-2002 (Marfin & Gubler, 2001).

1.2 Epidemiology on the American Continent

During the five years following the first diagnosis of WN encephalitis in August, 1999, in New York City, the WNV epidemic spread over 41 states. The total number of diagnosed patients was 26,997 and the total number of deaths was 1,008 (Table 1). (CDC (Centers for Disease Control and Prevention), 2008). Deaths in the United States were seen most often among persons older than 50 years and the average mortality rate was ca. 3.73%. Between 1999 and 2006, WNV was detected in 62 species of mosquitoes, with *Culex* species accounting for more than 98% of the total reported. Over the same period, 317 species of WNV-positive dead birds were reported, with American crows and blue jays accounting for more than 62% of the these cases. WNV outbreaks have also been reported among islands in the Caribbean Sea and in Argentina. The means of importation of the virus into the United States is unknown.

Table 1 Reported WNV disease cases in humans, United States, 1999-2007. (modified from CDC 2008)

Year	Total cases	Deaths
1999	62	7
2000	21	2
2001	66	9
2002	4,156	284
2003	9,862	264
2004	2,539	100
2005	3,000	119
2006	4,269	177
2007	3,022	76
Total	26,997	1,008

2. Causal Virus

WNV belongs to the family *Flaviviridae*, genus

Flavivirus and is included in the Japanese encephalitis (JE) virus serocomplex group. This group of viruses includes the St. Louis encephalitis virus of the United States and the Murray Valley encephalitis virus of Australia. The viral genome consists of single-stranded positive RNA and encodes three structural proteins (C, prM and E) and seven non-structural proteins.

WN virus isolates have been subjected to phylogenetic analysis, and can be subdivided into two major lineages. Lineage 1 includes most of the virus isolated since 1996, the American strains identified in 1999 and 2000, and the strains identified in Romania in 1996, Israel in 1999, and Volgograd, Russia in 1999. Lineage 2 includes most of the strains prevalent in Africa (Lanciotti *et al*., 1999).

3. Transmission Cycle and Infection Route

The major transmission cycle of WNV occurs between mosquitoes and birds. The model of virus transmission proposed for the United States is shown in Fig. 1. Birds are the virus-amplifying hosts, and mosquitoes of the *Culex* species are the major vectors in many outbreaks. Humans and horses are infected by bites from virus-carrying mosquitoes, and while they show clinical symptoms such as fever and encephalitis, they are called 'dead-end hosts' as they do not serve as an infection source for mosquitoes, because the viremia in humans and horses is not high enough to infect the mosquitoes. Major vector species of mosquitoes in the United States are *Cx. pipiens, Cx. quinquefasciatus, Cx. restuans, Cx. salinarius* and *Cx. tarsalis*. Crows are often used as sentinel animals to monitor virus activity in the United States.

Routes of human infection other than mosquito bites include fetal infection through the placenta, infantile infection via breast feeding, infection due to blood transfusion or organ transplantation and laboratory-acquired infection during handling of the live virus or infected materials.

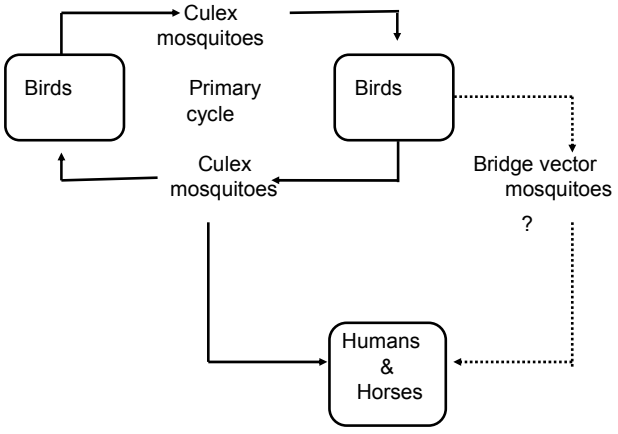

Fig. 1 Transmission cycle of WN virus. (modified from Campbell *et al*. 2002)

4. Clinical Symptoms

In humans, the incubation period of WNV is three to fifteen days following infection. Most cases are asymptomatic, but in some cases, fever and fatal encephalitis may develop. Mild cases show a febrile period of three to six days, headache, backache, myalgia, fatigue, rash and lymphadenopathy. Severe cases develop acute myelitis and encephalitis. The mortality rate of the outbreaks recorded in the United States has been approximately 3.73%.

In horses, the incubation period is five to ten days. Most infected horses are asymptomatic, but some show clinical symptoms. In the United States, reported symptoms include ataxia, weak legs, lying down, astasia, myospasm, fever, facial neuralgia, lip palsy, facial spasm, gnashing and blindness. The mortality rate, including euthanised cases, is 38%.

In birds, infection is asymptomatic, except in the United States and Israel. In the cases recorded in the United States, high mortality was observed among American crows and blue jays. Symptoms included depression, anorexia, weakness and loss of body weight. Severe cases manifest neurological signs such as ataxia, tremor, revolution and paresis. The clinical signs last for seven days.

5. Diagnosis

Diagnosis of WNV involves virus detection and serological examination. Virus isolation or virus genome detection is performed using sera or cerebrospinal fluids. IgM-captured ELISA, neutralization test, hemagglutination inhibition test and complement fixation test are used to detect WNV-specific antibodies. Acute phase sera, plasma, cerebrospinal fluids or post-mortem brain materials can all be used for virus isolation. For serological examination, paired sera from patients in the acute phase and convalescent phase should be collected and tested. Differential diagnosis should be considered for other flavivirus infections including JE, St. Louis encephalitis, tick-borne encephalitis, Murray Valley encephalitis and dengue. Other viral infections, such as alphavirus infection and herpesvirus infection, should be also considered.

5.1 Development of a sensitive diagnostic test for differentiation of WNV and Japanese encephalitis (JE) virus

Like WNV, JE virus is a mosquito-borne flavivirus from the JE virus (JEV) serocomplex, and causes encephalitis in humans and horses; it is widespread throughout most of Asia (Igarashi, 1992; Rosen, 1986). In areas where JEV is endemic, such as Japan, distinguishing between WNV and JEV is critical for correct identification of a WNV invasion. However, JE serocomplex flaviviruses cross-react antigenically and are thus not readily differentiated by serological methods (Martin, *et al*., 2000). Molecular diagnostic methods

are therefore preferred, and the reverse transcriptase polymerase chain reaction (RT-PCR) has been used to develop sensitive and specific assays for the identification of WNV (Igarashi, *et al*., 1994; Porter, *et al*., 1993). Recently, more sensitive assays, such as fluorogenic real-time (TaqMan) PCR, SYBR Green-based real-time PCR and loop-mediated isothermal amplification (LAMP), have been developed for diagnostic detection of the WNV genome (Lanciotti, *et al*., 2000; Papin, *et al.*, 2004; Parida *et al*., 2004). In areas where JEV is endemic, it is necessary to perform specific assays for both WNV and JEV to make a definitive diagnosis. However, the diagnostic methods described above were designed to detect only WNV or, more specifically, the strain of WNV isolated in the United States. Here, we describe the development of a sensitive molecular diagnostic method that can detect and distinguish between WNV and JEV, using TaqMan RT-PCR analysis with a probe common to WNV and JEV strains (Sirato *et al*., 2005).

To determine the sensitivity of the TaqMan assay, the assay was performed with serially-diluted cDNA from the NY99-6922 strain of WNV (Fig. 2). It was established that a cut-off (Ct) value of <40 and ΔRn signal of >0.5 indicated that WNV was present. The specificity of the primer sets was tested using various flaviviruses (Table 2). The TaqMan assay was performed using the indicated volume of cDNA synthesized from total RNA. The results indicated that the primer set for WNV could detect only WNV strains, including both lineage 1 and 2 viruses. The primer set for JEV could detect only JEV strains, including genotypes 1 (Ishikawa) and 3 (JaGAr01, Nakayama, and Beijing). The MVE virus, which is one of the JE serocomplex viruses, was not detected by either primer set. Flaviviruses of other serocomplexes, such as TBE virus, Dengue virus, Langat virus, and Powassan virus, were also not detected by either primer set.

To measure the sensitivity of the TaqMan assay for other WNV and JEV strains, the assay was performed

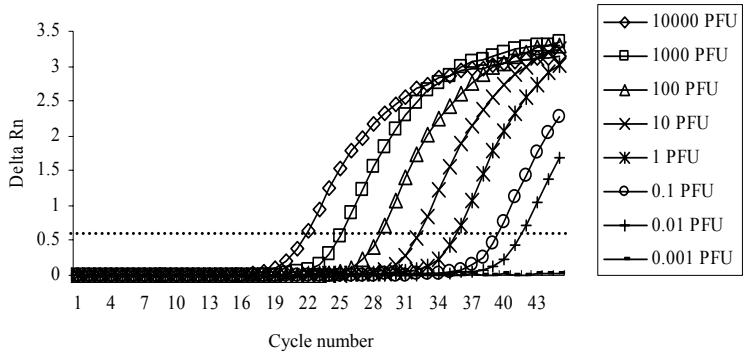

Fig. 2 Determination of the cut-off values for the TaqMan assay. The assay was performed with serially diluted cDNA from the NY99-6922 strain of WNV. The dotted line is drawn at ΔRn = 0.5. (Data from Shirato *et al*. 2005.)

Table 2 Specificity of the multi-probe TaqMan assay. (modified from Shirato *et al*. 2005)

		Primer set used			
		WNV primers		JEV primers	
Virus	Amount of template	Ct*	Result**	Ct	Result
WNV					
Lineage 1					
NY99-6922	1 μg of total RNA	16.8 ± 0.18	Pos	> 40.0	Neg
NY99-A301	1 μg of total RNA	17.7 ± 0.15	Pos	> 40.0	Neg
BC787	1 μg of total RNA	17.6 ± 0.03	Pos	> 40.0	Neg
6-LP	1 PFU	33.6 ± 0.00	Pos	> 40.0	Neg
6-SP	1 PFU	31.2 ± 0.07	Pos	> 40.0	Neg
Eg101	1 μg of total RNA	17.0 ± 0.04	Pos	> 40.0	Neg
Kunjin (OP393)	100 PFU	34.7 ± 0.20	Pos	> 40.0	Neg
Lineage 2					
FCG	1 μg of total RNA	17.2 ± 0.03	Pos	> 40.0	Neg
JEV					
Genotype 1					
Ishikawa	1,000 PFU	> 40.0	Neg	23.5 ± 0.03	Pos
Genotype 3					
Nakayama	1 μg of total RNA	> 40.0	Neg	20.1 ± 0.19	Pos
Beijing	1 μg of total RNA	> 40.0	Neg	15.8 ± 0.2	Pos
JaGAr01	1 μg of total RNA	> 40.0	Neg	16.3 ± 0.11	Pos

Pos=positive; Neg=negative
* Data are represented as the mean ± standard deviation.
** Ct values < 40 with ΔRn signals of > 0.5 were considered positive.

using serially diluted cDNA samples from titrated virus stocks (Table 3). At least 10^{-1} plaque-forming units (PFU) of virus was required for fluorescence detection by the TaqMan assay with WNV or JEV primer sets. However, the sensitivity for detection of Kunjin virus was lower than that of other viruses; at least 10 PFU of Kunjin virus were required for detection. Although the TaqMan assay was first performed using a 50 µl reaction volume, there was no change in sensitivity when the assay was performed using 25 µl or 12.5 µl reaction volumes. The TaqMan assay could detect 10^{-1} PFU or more of virus even in these smaller reaction volumes (data not shown). Therefore, all TaqMan assays were performed using a 25 µl reaction volume thereafter.

We also examined whether the primer sets used in this study could detect viruses in animal tissues. As Japan is a WNV-free country, we were unable to obtain archived clinical samples from animals naturally infected with WNV. Therefore, the TaqMan assay was performed using tissues from experimentally-infected mice. BALB/c mice were infected with 10^6 PFU of the NY99-6922 strain of WNV via the intraperitoneal route, and tissue samples were collected on the indicated days post-infection (p.i.). Total RNA was extracted, and cDNA was synthesized and tested using the TaqMan assay. The assay detected viral RNA in blood, spleen and brain samples (Table 4). Viruses were detected at both the early (three days p.i.) and late (eight days p.i.) stages of infection. Plaque assays were also performed to detect viruses in infected animal tissues.

This paper describes a highly sensitive genetic diagnostic method that can detect WNV and JEV using TaqMan RT-PCR analysis with a single probe that is common to both WNV and JEV strains. WNV strains of both lineage 1 and 2 were successfully detected when using the primer set for WNV, and genotype 1 and 3 JEV were detected when using the primer set for JEV. Viral RNA was detected in experimentally-infected animal tissues. Therefore, it is considered likely that the method described here is sufficiently sensitive and specific for detecting WNV and JEV strains in both human and animal samples.

Table 3 Sensitivity of the multi-probe the TaqMan assay. (modified from Shirato *et al*. 2005)

	Virus dose (PFU)					
Virus	10^3	10^2	10^1	10^0	10^{-1}	10^{-2}
WNV						
Lineage 1						
NY99-6922	Pos*	Pos	Pos	Pos	Pos	Neg
6-LP	Pos	Pos	Pos	Pos	Pos	Neg
6-SP	Pos	Pos	Pos	Pos	Pos	Neg
Eg101	Pos	Pos	Pos	Pos	Pos	Neg
Kunjin (OP393)	Pos	Pos	Pos	Neg	Neg	Neg
Lineage 2						
FCG	Pos	Pos	Pos	Pos	Pos	Pos
JEV						
Genotype 1						
Ishikawa	Pos	Pos	Pos	Pos	Pos	Neg
Genotype 3						
JaGAr01	Pos	Pos	Pos	Pos	Pos	Neg

Pos=positive; Neg=negative
Ct values < 40 with ΔRn signals of > 0.5 were considered positive.

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Table 4 Detection of WNV and JEV in experimentally infected mouse tissues using the multi-probe TaqMan assay. (modified from Shirato, 2005)

						Primer set			
						WNV		JEV	
Infecting virus	Number	Days p. i.	Clinical signs	Tissue	Virus titer*	Ct	Result**	Ct	Result
NY99-6922	1	3	No signs	Blood	1.3	37.5	Pos	> 40.0	Neg
				Spleen	3.6	30.2	Pos	> 40.0	Neg
	2	3	No signs	Blood	1.0	39.9	Pos	> 40.0	Neg
				Spleen	3.8	30.6	Pos	> 40.0	Neg
	3	3	No signs	Blood	1.0	37.1	Pos	> 40.0	Neg
				Spleen	3.6	31.1	Pos	> 40.0	Neg
	4	8	Dead	Spleen	2.3	32.0	Pos	> 40.0	Neg
				Brain	> 7.0	14.4	Pos	> 40.0	Neg
	5	8	Moderate encephalitis	Blood	1.3	38.3	Pos	> 40.0	Neg
				Spleen	2.8	30.2	Pos	> 40.0	Neg
				Brain	5.6	24.5	Pos	> 40.0	Neg

Pos=positive; Neg, negative; n.d.=not detected
* The viral titer in blood samples is expressed as log PFU/ml and that of tissue samples is expressed as log PFU/g. The detection limits were 10 PFU/ml (blood) and 100 PFU/g (spleen and brain).
** Ct values < 40 with ΔRn signals of > 0.5 were considered positive.

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