WEST NILE VIRUS INFECTIONS IN ROMANIA – PAST, PRESENT AND PERSPECTIVE

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Summary

West Nile virus (WNV) is currently the most widely distributed arbovirus in the world, occurring on all continents and causing sporadic cases and outbreaks of human and equine disease in Europe (western Mediterranean and southern Russia between 1962 and 1964, Belarus and Ukraine in the 1970s and 1980s, Romania in 1996 and 1997, Czechland in 1997, and Italy in 1998). Environmental factors, including human activities that enhance population densities of vectors (heavy rains followed by floods, irrigation, higher than usual temperature, or formation of ecologic niches that enable mass breeding of mosquitoes) could increase the incidence of West Nile fever. The outbreak of West Nile fever in and near Bucharest in 1996–1997, led to more than 500 clinical cases in human with a case-fatality rate of nearly 10% (15).The article presents a short review of the WNV evolution in Romania and discuss the results of our researches. We have made a serosurvey in horses and birds which are the indicators for the West Nile virus presence. The results underline the evolution of West Nile Virus in the south east region of Romania in the last two years and the lack of any antibody detection in horses sampled from the north east region. We have registered a seroprevalence ratio in horses from the south east area varying between 33, 53%, and 31,88% (for the IgG detection) with positive results for IgM also. In birds the seroprevalence was 7, 46% with a percent of in 21, 95% Corvidae, which are incriminated as virus reservoir.

Key words: West Nile Virus, horse, birds, serosurvey

West Nile virus (WNV), is a member of the Flavivirus genus of the family Flaviviridae, which contains approximately 70 members, most of which are transmitted either by mosquitoes or ticks (2). WNV is classified within the Japanese Encephalitis serological complex on the basis of cross-neutralization (2) and molecular genetic studies (6). The WNV virion, like that of other flaviviruses, is enveloped, spherical, approximately 40–60 nm in diameter, with an electron-dense core (10).

The virus was the first isolated from a febrile woman in 1937 in Uganda (14) and subsequently was associated with sporadic cases of diseases well as major outbreaks in Africa, Eurasia, Australia and the Middle East. Epidemics documented prior to 1996 generally involved hundreds to thousands of cases in mostly rural populations with few cases of severe neurological disease (4).

However, beginning in the 1990s, outbreaks began to occur more frequently, especially in the Mediterranean Basin, and were associated with

increased numbers of cases with severe disease including viral encephalitis and neurological symptoms (8).

Between 1996 and 1999, three major WNV epidemics occurred in southern Romania, the Volga Delta (in southern Russia) and the north-eastern United States, all of which involved hundreds of cases of severe neurological disease and fatal infections. These were the first epidemics reported in large urban populations. In 2000 in Israel, a country-wide outbreak occurred with a case fatality rate of 8.4% (17) and then neurological disease was observed in 2001 in Russia (18) and in 2003 in Tunisia (1).

Transmission cycle

Throughout its world wide distribution, WNV is maintained in nature in an enzootic cycle between ornithophilic mosquitoes, predominantly *Culex* species and birds. Naturally, the clinical simptomatology is rare excepting the horses. The incubation period in the case of equine encephalitis preceding the transmission by mosquitoes is estimated at 3-15 days, a floating viremia with a low virus titre precedes the installation of clinical signs. The encephalitis is clinically manifested at a low percent of the infected horses and the symptomatology is characterized by ataxia, muscular fasciculation and symptomatology correlated with the damage of cranial nerves functions (11, 16).Fever is not constant. The infection in horses is a good indicator of the virus presence and infection evolution.

West Nile virus in Romania

The 1996-1997 outbreak of West Nile fever in and near Bucharest, with more than 500 clinical cases and a case-fatality rate approaching 10% (7,13,15), was the largest outbreak of arboviral illness in Europe since the fever epidemic caused by Sindbis virus (a member of the *Togaviridae* family, in the *alphavirus* subfamily) in northern Europe in the 1980s (5). This latest outbreak reaffirmed that mosquito-borne viral diseases may occur on a mass scale, even in temperate climate.

In early October 1996, entomologic and avian investigations of the epidemic were conducted in Bucharest and nearby rural areas. Thirty of 73 domestic fowl sampled (41%), had neutralizing antibody to WN virus, including 5 of 13 ducks (38%), 1 of 1 goose, 19 of 52 chickens (37%), 1 of 1 peahen and 4 of 6 turkeys (67%). Serum collected from one of 12 Passeriformes, an *Erithacus rubecula*, was positive for neutralizing antibody to WN virus. A total of 5.577 mosquitoes representing seven taxa were collected. *Culex pipiens pipiens* accounted for 96% of the mosquitoes collected. A single virus isolate, RO 97-50, was obtained from a pool of 30 *Culex p. pipiens* females aspirated from the wall sand ceiling of a block house located near the center of Bucharest, resulting in a minimum infection rate of 0.19 per 1,000. The nucleotide sequence of RO 97-50

was identical to the sequence of a WNV isolate obtained from *Culex Neavei* mosquitoes from Senegal and *Culex Univittatus* mosquitoes from Kenya. The phylogenetic analyses were compatible with the introduction of virus into Romania by birds migrating from sub-Saharian Africa, to northern Africa and into southern Europe (13).

Following the 1996 epidemic, Romania implemented a surveillance program for continued WN viral circulation and human disease. During 1997-2000, passive surveillance augmented by alerts to district health departments for human cases of WN encephalitis and meningitis was conducted in Bucharest and elsewhere in southeastern Romania. Serologic tests of serum, cerebrospinal fluid (CSF) or both were employed. A total of 39 cases including 5 fatalities (13%) were diagnosed: 14 in 1997, 5 in 1998, 7 in 1999, and 13 in 2000. Cases occurred from May to September, 82% occurred in August or September. In 37 of the 39 cases, the reported predominant clinical manifestations were central nervous system (CNS) disease: meningitis (24 cases), meningoencephalitis (12 cases) and encephalitis (1 case). The remaining two cases were WN fever with exanthema but without neurologic involvement. Those two cases were diagnosed in hospitalized patients who were initially suspected of having measles (3).

During 1997-2000, domestic fowl (including chickens, ducks, geese, and turkeys) were bled between July and September in Bucharest and three other districts in southeastern Romania and tested for antibody to WNV by neutralization, IgG ELISA or both. The overall seroprevalence was 34 of 447 (8%), including at least 7 seropositives among 34 young-of-the-year. During July-September 1999, 152 wild birds of 22 species and 6 orders were sampled in the Teleorman and Tulcea districts. Of these, 95% were passerines and 70% were tree sparrows (*Passer montanus*). A total of 12 birds (8%) had neutralizing antibody to WNV, including 7 seropositives among 12 young-of-the-year; all seropositives were non migratory species (3).

In 2006, in Danube Delta area and Dobrodjean tableland researches regarding animal reservoir and host for WNV were made. Birds were investigated and out of 1.047 samples, 173 were positive for WNV, the seroprevalence being 16.52%. The highest seroprevalence (9) was found in Hooded Crow (24.24%), Rook (23.07%), Great Reed Warbler (22.1%) and House Sparrow (20%).

In 2007, investigations regarding WNV presence in horses were made in Tulcea and Ilfov districts and in Bucharest area (12). The overall seroprevalence in Bucharest and Ilfov district was 14,4% (182/1267) and 32,1%, (214/666) for Tulcea district.

Materials and methods

Our research followed a serosurvey of West Nile virus in horses and birds and the isolation of the virus from mosquito and bird samples.

The horse sera was evaluated for the presence of anti-WNV antibodies by an ELISA ,, Kit for detection of West Nile anti prM-E antibodies in horse sera" made by ID.VET Innovative Diagnostics, for the detection of IgG and a house made ELISA assay for the detection of IgM. The collection and stock of serum samples were made following a strict protocol.

In a first phase we sampled horses from the south east of Romania in five districts with an important epidemiological role in WNV cycle: Braila, Constanta, Galati, Ialomita and Tulcea including the Danube Delta area. From the 508 horses sampled in the south east area, 167 serum specimens were tested. We have sampled also 215 horses from the north east region, in Lucina and Radauti stud farms, to see if the infection spreads in a colder region where the vectors are less probable to live.

In order to test the reproductibility of our assay we have chosen 76 sera samples from the Tulcea and Braila district, to be reexamined in France for Ig G, using the same kit. We also reexamined all the 215 samples from the north east. For the IgM, analyses were made by our researchers in UMR 1161 Virology laboratory of Alfort, France using a house made ELISA assay.

In a second phase, the serological investigations were made in Tulcea and Braila districts were the seroprevalence was higher in 2006 on 68 samples and also in Lucina stud farm in Suceava County where we intend to test a vaccine against West Nile virus in horses. The samples were tested both for Ig G and IgM twice.

We also have collected bird samples from Danube Delta : Mila 23, Iazurile, Mila 26, Sulina, Dunavățul de Jos, Sălcioara, Maliuc, Grindul Lupilor. We have sampled both domestic and wild birds.

Finally in a third phase sera samples collected from birds were tested using a seroneutralisation assay. We also started a virusologic examination of mosquitoes and brain tissue from birds. The brain was collected in carbon ice and after this stored at -80° C. The mosquitoes collected were divided in pools of 50 and kept at -80° C. We started a viral isolation assay on Vero cells.

Results and discussions

In the first phase, the biggest number of positive samples was found in Tulcea district: 34 samples from 73 analyzed the next district as seroprevalence is situated Braila 10 positive samples from 20 analyzed and after this Galati 8, Ialomita 3 and Constanta only one positive sample. Although the global seroprevalence was 33.53%, in Tulcea (46.57% prevalence) and Braila (50% prevalence) districts the results are concludent and perfectly related to the presence of the mosquito and to the climate.

The 76 samples analyzed in duplicate from Tulcea and Braila district gave results that matches in a 94.45% the one obtained in UMR 1161 Virology laboratory, with a 47.36 % prevalence in Romania and 44.73% prevalence

obtained in France. The difference is due probably of the transport conditions that damaged some IgG in sera but the results are comparable and attest the accuracy of the tests made in Romania.

Table 1

Comparative analysis of the results obtained in France and Romania

Laboratory	Romania	France
Total samples tested	76	76
Total positive samples	36	34
Total negative samples	40	42
Prevalence	47.36%	44.73%

The 215 samples tested from the stud farms placed in the Carpathians Mountains were negative, both in Romania and in France laboratories.

The IgM presence was pointed out in one sample from Sulina, this confirm the presence of West Nile 2006/2007 in this area.

In 2007 we collected samples from horses again but in a smaller number: 34 samples from Lucina stud farm in Carpathians and 68 samples from Tulcea and Braila County. We tested the samples from the study areas using the Id Vet Kitt for IgG detection and a home made ELISA for the detection of IgG in horses provided from the Virology Laboratory UMR 1161. The results for the IgG detection were negative for all 34 samples from the stud farm in Carpathians but in the inferior area of Danube we have found again seropositive horses with 32.35% prevalence (22/68).

The most important aspect of the research in this phase is that we have obtained two positive responses for IgM in horses from Sulina region and 2 positive samples for IgM in Braila that indicates a recent infection in the 2007 summer.

Table 2

Results of the serological investigations in Tulcea and Braila Counties in 2007

County	Localities	No. of samples analyzed	Positive results IgG	Percent of positive samples for IgG	Positive results IgM	Percent of positive samples for Ig M
Braila	Chişcani	3	0	58.82%	0	5.88%
	Gropeni	6	4		0	
	Tichileşti	2	1		1	
	Tufeşti	6	5		1	
Tulcea	Sulina	11	4	36.36%	2	
	Mahmudia	40	8	20.00%	0	
Total		68	22	32.35%	4	

In the third phase of the project, we have tested the sera samples from birds using a seroneutralization assay. The birds were sampled in Tulcea district, from the Danube Delta localities, in the summer and autumn of 2007.

If we refer to the spatial distribution of the seropositive cases, we have obtained a 2% seropositivity in Iazurile (2/100), 10,52% in Sulina (2/19), 3,33% in Dunavățul de Jos (1/30), 21,95% in Sălcioara (5 Rook and 4 Hooded Crow from 41) and 2% in Maliuc (2/10). From the positive birds three were young of the year (*Corvus frugilegus* in Sălcioara, *Larus cachinnans* in Sulina, *Passer domesticus* in Dunavățul de Jos).

Table 3

Results of the seroneutralization assay performed on bird sera sample from Danube Delta area

Species of birds	No. of samples analyzed	Positive results	Negative results	Percent of positive samples
Gallus gallus	11	2	9	18,18%
Palmipedae	9	0	9	0%
Passer domesticus (House Sparrow)	123	5	118	4,06%
Passer montanus (Tree Sparrow)	21	0	21	0%
Larus cachinnans (Yellow-legged Gull)	19	2	17	10,53%
Acrocephalus scirpaceus (Reed Warblers)	5	0	5	0%
*Emberiza schoeniclus (*The Reed Bunting)	8	0	8	0%
Lanius collurio (The Red-backed Shrike)	1	0	1	0%
Sturnus vulgaris (European starling)	1	0	1	0%
Corvus frugilegus (Rook)	31	5	26	16.13%
Corvus c. cornix (Hooded Crow)	10	4	6	40.00%
*Streptopelia decaocto (*Collared dove)	2	0	2	0%
Total	**241**	**18**	**223**	**7.47%**

From the mosquitoes pools and crows internal organs (brain, heart, digestive tract), sampled in Danube Delta area, we have isolated on Vero cells a cytopathogenic virus which will be identified and characterized using molecular biology methods in our further researches.

Table 4

Preliminary results of the virus isolation assay on Vero cell

No. crt.	Sample type	Total no. of samples	No. of positive samples	No. of negative samples
1	crows	24	4	20
2	mosquitoes	15	1	14

Conclusions

WNV is well established in the south east of Romania and its activity indicated by the IgM detection in horses and seropositivity in birds in young of the year birds in 2007 will most likely continue at levels determined by virus, host and environmental factors in the next years.

The absence of an antibody response in horses from the north east area of the country indicates a region free of West Nile virus infection, due to the colder climate and the lack of migrating birds and mosquitoes.

The tests made in Romania have proven to be reproducible in comparison with the ones made in the French laboratory and give us the certitude that we are ready in the future for the implementation of a surveillance program in horses which are a good indicator of the existence and persistence of West Nile virus infection. This provides us the possibility to prevent another epidemic episode with simple tests which doesn't need a high level of biosecurity.

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