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Evidence of West Nile virus circulation in horses and dogs in Libya

Kholoud Khalid Ben-Mostafa[1,2], Giovanni Savini[3,*], Annapia Di Gennaro[3], Liana Teodori[3], Alessandra Leone[3], Federica Monaco[3], Mohammed Masoud A. Alaoqib[4], Abdunnabi A. Rayes[5], Abdunaser Dayhum[6] and Ibrahim Eldaghayes[1,*]

[1] Department of Microbiology and Parasitology, Faculty of Veterinary Medicine, University of Tripoli, Tripoli, Libya; benmostafakholoud@yahoo.com (K.K.B); ibrahim.eldaghayes@vetmed.edu.ly (I.E.)
[2] National Center for Animal Health, Tripoli, Libya
[3] Department of Virology and Tissue Culture, Istituto Zooprofilattico Sperimentale dell'Abruzzo e del Molise "G.Caporale", Teramo, Italy; g.savini@izs.it (G.S.); a.digennaro@izs.it (A.D.G.); l.teodori@izs.it (L.T.); a.leone@izs.it (A.L.); f.monaco@izs.it (F.M.)
[4] Department of Internal and Infectious Diseases, Faculty of Veterinary Medicine, Omar Al-Mukhtar University, Albaida, Libya; lhlh77_m@yahoo.com
[5] Department of Internal Medicine, Faculty of Medicine, University of Tripoli, Tripoli, Libya; drabdurayes@gmail.com
[6] Department of Preventive Medicine, Faculty of Veterinary Medicine, University of Tripoli, Tripoli, Libya; adayhum@yahoo.com
* Correspondence: g.savini@izs.it (G.S.); ibrahim.eldaghayes@vetmed.edu.ly (I.E.)

Abstract: West Nile virus (WNV) is a global important mosquito-borne Flavivirus causing West Nile disease (WND). In Libya, evidence of WNV circulation has been reported in humans but never in animals. The aim of this study was to determine the seroprevalence of the WNV infection in horses and dogs in Libya. A total of 574 and 63 serum samples from horses and dogs, respectively, were collected from healthy unvaccinated animals between 2016 - 2019. A commercially available competitive ELISA (c-ELISA) kit was initially used to test the collected samples for the presence of WNV Ig-G antibodies. Positive and doubtful sera were also tested by using the more specific virus neutralization assays to confirm whether the ELISA positive results were due to WNV or Usutu virus (USUV) antibodies. The seroprevalence of WNV IgG ELISA antibodies was 13.2% (76/574) and 30.2% (19/63) in horses and dogs, respectively. Virus neutralization test (VNT) showed that 77.5% (62/80) and 89.5% (17/19) of positive and doubtful horse serum samples and dogs serum samples, respectively, were positive with WNV neutralising titers ranging from 1:10 to 1:640. The results of the present study provided novel evidence about the WNV circulation in Libya.

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1. Introduction

West Nile is single-stranded RNA virus belonging to the Flavivirus genus within the Flaviviridae family. The virus was first isolated in 1937 from a febrile patient in the West Nile province of Uganda [1]. Following its first isolation, WNV has spread in Africa, Middle East, Europe and America [2-4]. It is responsible for neurological symptoms in humans and animals and is currently considered as a serious public health problem worldwide causing outbreaks and fatal casualties in humans [5].

The virus is maintained in nature in an enzootic cycle involving competent mosquitoes and a wide variety of reservoir host bird species [6,7]. Being a vector borne disease, many environmental factors contribute to the occurrence and emergence of WNV including weather patterns, virus adaptation to local vectors and bird-migration. WNV has been isolated from numerous bird species. In some of them, the infection has been

shown to cause specific pathological changes in various tissues particularly central nervous system (CNS) [8].

Various animals including domestic, companion and wildlife species may be infected with WNV [5]. When infected, most mammalians, including humans and horses, usually act as incidental and dead-end hosts [9]. In other words, due to the low level of viraemia, they are not able to transmit WNV to the competent vectors. Exception in that regards is the possibility of WNV transmission among humans through blood transfusion, organ transplantation or, in the laboratory, by handling live virus [5].

In humans and horses, most WNV infections are asymptomatic. Clinical manifestations occur at very low incidence and may involve the neurology system. West Nile neuroinvasive disease (WNND), West Nile meningitis (WNM), West Nile encephalitis (WNE) and West Nile acute flaccid paralysis (a poliomyelitis-like syndrome) known as West Nile poliomyelitis (WNP) [5] have been described in humans whose severity depends on age and the immune status of the patient [10]. In a study in South Africa, 52% WNV positive cases had fever, 92% displayed neurological signs, and 39% experienced mortality [11].

In other mammal and not mammal species including dogs, cattle, sheep, goats, camels, deer, squirrels and reptiles, WNV infection can elicit antibodies [12]. Sharing the domestic environment with humans, dogs which can be accidentally infected with WNV, can be important sentinel by indirectly indicating the viral circulation in urban and suburban area even before the onset of human cases in the population [13,14].

WNV outbreaks have been observed in many North African countries like Algeria, Morocco and Tunisia [14]. Among equids, symptomatic infections and fatalities have been reported in Morocco [15]. However, no information is available on WNV circulation in Libya. There was only one study published on WND seroprevalence in humans in Libya in 2017 showed 11 positive samples out of 400 samples tested (2.75%) by ELISA [16].

Enzyme linked immunosorbent assays (ELISAs) was the most commonly used diagnostic method for the detection of anti-WNV antibodies in humans and animals [17]. However, the use of the ELISA without the more specific WNV neutralization test might result in false positive results due to the potential of cross-reactivity with closely related pathogens, such as the Usutu virus, the St. Louis encephalitis virus, or the Japanese Encephalitis virus.

The aim of this study was to get information on the circulation of WNV in Libya through a seroprevalence study carried out on horses and dogs. In addition to this, we aim to assess the risk factors associated with the WNV seropositivity in animals. To the best of our knowledge, this is the first study on WNV seroprevalence in animals in Libya.

2. Materials and Methods

2.1 Study design

A cross sectional study was conducted between 2016-2019 to investigate the serological prevalence and exposure to WNV in apparently healthy horses and dogs in some of the western and eastern regions of Libya. This area was selected based on the ecological environment suitable for the life cycle of the virus. No detailed sampling program was planned due to the unavailable epidemiological data on animals from Libya. The study was based on the available published information from the regional and neighbouring countries.

2.2 Targeted animals and sampling strategy

Samples were collected from dogs and horses of various breeds, with no clinical signs related to the WNV-associated disease. The owners declared the animals spent all their life in the area where they were sampled. So, we were able to assign a specific area to each sample. Animals were bled once. Local information on the epidemiological status of the sampling areas was obtained from animal health centre in Tripoli.

Collection of data was done using a questionnaire in which the most common variables that are typically associated with WNV infection were considered. Questions on location, age in years and months, sex, breed, and use of each animal were asked. Information on clinical signs and vaccination strategy within the last 3 months was also collected. Other data on the breeding system used and the migratory birds present in the area were also obtained.

2.3 Samples data

In total, 574 samples from horses and 63 samples from dogs were collected. The horse age ranged between 2 and 240 months, while the dogs aged between 3 and 72 months. Male horses represented 49.3% of the total study group. Breeds of horses included in the study were: Arabian (n=145), Local Thoroughbred (n=202), Imported Thoroughbred (n=93) and Local Libyan (n=35) (Table 1). Horses originated from seven Libyan cities including: Al-Marj (n=99), Gasr Ben Ghashir (n=140), Al-Swani (n=68), Zuwarah (n=56), Tripoli (n=157), Al-Zawia (n=26) and Surman (n=28). All dog samples were collected from Tripoli. Male dogs represented 49.2% of the total sampled animals.

Table 1. Some demographic data on the study sample animals.

Total Samples	Age (months)	Sex	Animal Breed	Area
Horses (n=574)	2 - 240	49.3% Male (n=283) 50.7% Females (n= 291)	Arabian (n=145/ 25.3%)	Western Libya
			Local Thoroughbred (n=202/ 35.2%)	
			Imported Thoroughbred (n=93/ 16.2%)	
			Local Libyan (n=35/ 6.1%)	
			Mixed (n=99/ 17.2%)	Eastern Libya
Dogs (n=63)	3 - 72	49.2% males (n=31) 50.8% females (n=32)	Many breeds	Tripoli

2.4 Collection and processing of blood samples

A single blood sample of 5 mL blood was collected in a plain dry tube through venipuncture of the jugular vein using a sterile needle and syringe directly after clinical examination. Samples were transported at 4° C to the laboratory for serum separation within 24 hours. In the laboratory, they were centrifuged at 3000 rpm for 10 minutes, sera were transferred into two Eppendorf tubes and then stored at -20°C until further use.

2.5 Serological tests

The serological assays were performed following the recommendation of the WOAH Terrestrial Manual 2018. Both, competitive ELISA (c-ELISA) and virus neutralization test (VNT) were performed at the WOAH Reference Laboratory for West Nile Disease, Istituto Zooprofilattico Sperimentale "G. Caporale", Teramo, Italy.

2.6 Competitive enzyme-linked immunosorbent assay (c-ELISA)

Dog and horse serum samples were screened by c-ELISA (ID Screen® West Nile Competition Multi-species, IDvet, Grabels, France) for the presence IgG antibodies against Flaviviruses. The test was designed to detect multi-species antibodies directed against an epitope of E protein common to WNV and other members of the Japanese Encephalitis serocomplex. The ELISA procedure was performed adopting the manufacturer protocol. ELISA results were interpreted by calculating the O.D. and the

Sample/Negative control ratio (S/N% value) as reported in the manufacturer guidelines. Serum samples with a S/N ratio less than or equal to 40% were considered positive, between 40% and 50% inconclusive (doubtful), and greater than 50% negative. In addition to the positive and negative controls of the manufacturer, internal control sera were also used as a tracer according to quality assurance system of the laboratory.

2.7 Virus neutralization test (VNT)

ELISA positive or doubtful samples were also screened for WNV and USUV neutralising antibodies using the VNT as described by Di Gennaro et al. [18]. This technique based on the capability of the test serum to neutralise the cytopathic effect of the virus is more specific and reduces false positive results. Apart from detecting specific neutralising antibodies, the technique is also capable of determining the neutralising titer.

This technique was performed in cell culture micro plates, using four wells per serum dilution. After inactivation for one hour at 56°C, 50 μL two-fold serum dilutions (from 1:5 to 1.640) were mixed with an equal volume of the virus containing 100 tissue culture infectious doses 50% (TCID50). Plates were then incubated at 37°C with 5% CO2 for 1 h. Positive and negative control sera were included in each plate. Vero cells grown in Dulbecco's Modified Eagle's Medium supplemented with 5% foetal calf serum were added to obtain confluence in 48 h.

For testing its activity, four replicates of the virus at concentrations of 1, 10, 100, 1000 TCID50 doses were included in each performed VNT. Reading was carried out on the fifth day by observing the presence and extension of the CPE in each well. Sera with a neutralizing titre equal or greater than 1:10 were considered positive.

2.8 Statistical analysis

All the collected data were incorporated, organized using Microsoft Excel® spreadsheet and then analysed using descriptive statistics. Chi-square analysis was used to evaluate any significant association between the variables considered in this study (significance at $p \leq 0.05$).

3. Results

3.1 Seroprevalence of WNV in horses (10.8%; 95% CI:8.5-13.6%)

Out of 574 horse sera samples tested by ELISA, 76 (13.2%) were found positive for WNV antibodies and four samples tested doubtful (Table 2). To confirm whether the reactivity to ELISA was due to the presence of WNV antibodies, ELISA reactive samples (n=80: 76 positive samples and 4 doubtful samples) were tested by VNT. Out of 80 samples tested, specific WNV neutralising antibodies were detected in 62 serum samples (77.5%) representing 10.8% (n=62/574) of the total tested horse samples with titres ranging from 1:10 to 1:640 (Tables 2 and 3). No USUV antibodies were detected in the tested samples.

Table 2. West Nile virus seroprevalence in Libyan horses and dogs.

Total Samples	Positive samples		VNT Titre range
	c-ELISA	VNT	
Horses (n=574)	13.2% (n=76/574)	Of positive ELISA: n=62/80 (77.5%) Of Total samples: n=62/574 (10.8%)	1:10 - 1:640
Dogs (n=63)	30.2% (n=19/63)	Of positive ELISA: n=17/19 (89.5%) Of Total samples: n=17/63 (26.9%)	1:10 - 1:320

(c-ELISA): Competitive Enzyme-Linked Immunosorbent Assay; (VNT): Virus Neutralization Test.

Table 3. c-ELISA results and WNV neutralizing titers for horses.

Samples NO.	C-ELISA (IgG)	VNT (Titre)	Samples NO.	C-ELISA (IgG)	VNT (Titre)
1	POS	1:20	41	POS	1:10
2	POS	1:10	42	POS	NEG
3	POS	1:20	43	POS	1:20
4	POS	1:10	44	POS	1:40
5	POS	1:20	45	POS	1:10
6	POS	1:10	46	POS	1:20
7	POS	1:40	47	POS	1:160
8	POS	1:10	48	POS	1:10
9	POS	1:10	49	POS	1:320
10	POS	1:40	50	POS	1:80
11	POS	1:20	51	POS	1:20
12	POS	1:20	52	POS	1:40
13	POS	1:10	53	POS	POS
14	POS	1:40	54	POS	NEG
15	POS	1:80	55	POS	1:320
16	POS	NEG	56	POS	1:40
17	POS	1:40	57	POS	1:160
18	POS	1:160	58	POS	1:40
19	POS	1:40	59	POS	NEG
20	DOUBT	NEG	60	POS	NEG
21	POS	1:10	61	POS	1:640
22	POS	1:20	62	POS	1:40
23	POS	NEG	63	POS	NEG
24	POS	1:10	64	DOUBT	1:10
25	POS	1:20	65	POS	1:40
26	POS	1:40	66	POS	1:20
27	POS	1:20	67	POS	1:40
28	POS	1:40	68	POS	NEG
29	POS	1:10	69	POS	1:80
30	POS	1:20	70	POS	1:40
31	POS	1:20	71	POS	NEG
32	POS	1:20	72	POS	1:40
33	POS	1:10	73	POS	1:10
34	POS	POS	74	POS	POS
35	POS	NEG	75	POS	1:10
36	DOUBT	NEG	76	POS	1:40
37	POS	1:40	77	DOUBT	1:10
38	POS	NEG	78	POS	1:20
39	POS	1:20	79	POS	1:40
40	POS	1:80	80	POS	NEG

(POS): Positive; (NEG): Negative; (DOUBT): Doubtful.

3.2 Seroprevalence of WNV in dogs (27%; 95% CI: 17.6-39.1%)

Out of 63 dogs' serum samples tested by ELISA for the presence of WNV antibodies, 19 samples (30.2%) were found positive (Table 2). Then, all the 19 ELISA positive sera samples were tested by VNT. Specific WNV neutralizing antibodies were detected in 17 serum samples (89.5%) representing 27% (n=17/63) of the total tested dog samples with titers ranging from 1:10 to 1:320 (Tables 2 and 4).

Table 4. c-ELISA results and WNV neutralizing titers for dogs.

Samples NO.	c-ELISA (IgG)	VNT (WND Titre)
1	POS	1:320
2	POS	1:80
3	POS	1:40
4	POS	1:20
5	POS	1:10
6	POS	1:320
7	POS	1:320
8	POS	1:320
9	POS	1:40
10	POS	1:160
11	POS	NEG
12	POS	1:80
13	POS	1:80
14	POS	1:20
15	POS	1:320
16	POS	NEG
17	POS	1:20
18	POS	1:160
19	POS	1:40

(POS): Positive; (NEG): Negative.

3.3 Risk factor analysis (IgG-ELISA horses)

Concerning WNV seroprevalence related to the horse breed, Arabian horses showed the highest percentage of IgG seropositivity (20%; 29/145) followed by the Thoroughbred horses (14.5%; 43/295) and local Libyan horses (11.4%; 4/35) (Table 5).

Table 5. West Nile virus seroprevalence in Libyan horses according to breeds in the Western part of Libya.

			ELISA IgG		Total
			Negative	Positive	
Breed	Arabian	Count	116	29	145
		% within Breed	80%	20 %	100.0%
		% of Total	24.4%	6.1%	30.5%
	Local Thoroughbred	Count	172	30	202
		% within Breed	85.1%	14.9%	100.0%
		% of Total	36.2%	6.3%	42.5%
	Imported Thoroughbred	Count	80	13	93
		% within Breed	86.0%	14.0%	100.0%
		% of Total	16.8%	2.7%	19.6%
	Libyan	Count	31	4	35
		% within Breed	88.6%	11.4%	100.0%
		% of Total	6.5%	0.8%	7.4%
Total*		Count	399	76	475
		% within Breed	84%	16%	100.0%
		% of Total	84%	16%	100.0%

* The statistical analysis excluded samples from Al-Marj (n=99) due to seronegative results.

However, these differences were not significant (p= 0.341). When horses were grouped by geographic areas, Al-Zawia (n=11/26; 42.30 %) was the area with the highest percentage of positive animals, followed by Al-Swani (20.5%; n=14/68), Gasr ben ghashir (15%; n=21/140), Tripoli (14%; n=22/157), Surman (10.7%; n=3/28) Zawarah (8.9%; n=5/56) and Al-Marj (0%; n=0/99) (Table 6). Unlike breeds, a significant difference was found between the WNV seroprevalence in the western area and the eastern area of Libya (p= 0.000).

Table 6. West Nile virus seroprevalence in Libyan horses according to the geographic area.

Animal	Animal origin	ELISA (IgG) testing	
		Negative	Positive
Horses	Al-Marj[a] (n=99)	n=99 (100%)	n=0 (0%)
	Gasr Ben Ghashir[b] (n=140)	n=119 (85%)	n=21 (15%)
	Al-Swani[b] (n=68)	n=54 (79.4%)	n=14 (20.6%)
	Zawarah[b] (n=56)	n=51 (91.1%)	n=5 (8.9%)
	Tripoli[b] (n=157)	n=135 (86%)	n=22 (14%)
	Al-Zawia[b] (n=26)	n=15 (57.7%)	n=11 (42.3%)
	Surman[b] (n=28)	n=25 (89.3%)	n=3 (10.7%)
	Total (574)	n=498 (86.8%)	n=76 (13.2%)
Dogs	Tripoli[b] (n=63)	n=44 (69.8%)	n=19 (30.2%)

([a]): located in Eastern region of Libya; ([b]): Located in Western region of Libya.

For the purpose of this study, horses were organized into five groups based on the age range as follows: younger than 6 months (n=16); from 7 to 18 months (n=122); from 19 to 48 months (n=355); from 49 to 72 months (n=50) and older than 72 months (n=31). It was observed that the WNV seropositivity increases as age increases (p= 0.000) (Table 7).

Table 7. West Nile virus seroprevalence in Libyan horses according to age.

			ELISA IgG		Total
			Negative	Positive	
Age Group	< 6 Months	Count	16	0	16
		% within Age Group	100.0%	0.0%	100.0%
	7 - 18 Months	Count	117	5	122
		% within Age Group	95.9%	4.1%	100.0%
	19 - 48 Months	Count	304	51	355
		% within Age Group	85.6%	14.4%	100.0%
	49 - 72 Months	Count	40	10	50
		% within Age Group	80.0%	20.0%	100.0%
	> 72 Months	Count	21	10	31
		% within Age Group	67.7%	32.3%	100.0%
Total		Count	498	76	574
		% within Age Group	86.8%	13.2%	100.0%

4. Discussion

WNV has re-emerged globally as an important pathogen affecting humans and horses with distinct epidemiology and irregular epidemiological scenario [19-21]. Although recent global surveillance data showed that the WNV incidence of neurological disease increased and expanded geographically causing recurrent horse and human epidemics in many regions, the data available from Africa are still scanty. There may be many reasons behind this lack of data, even if we reckon that the lack of funding is most probable cause. However, even though the real burden of WNV infections in Africa is not well known, the few information currently available is sufficient to provide evidence that WNV originated and is circulating in the continent [22].

Our results confirmed that the virus has circulated or is circulating in Libya. WNV antibodies were in fact detected in both horses and dogs. Only few African countries have investigated the presence of WNV antibodies in dogs, most of them were sub-Saharan. A survey on dogs in South Africa, revealed that 46% of them were positive for haemagglutination-inhibition antibodies against WNV [23]. In Morocco, a study on military working dogs and horses reported similar seroprevalences (62%) in dogs and horses (60%) indicating that both species can be efficiently used as sentinel animals [15]. Interestingly, in this study, the prevalence found in dogs was significantly ($P<0.05\%$) higher than that detected in horses. For their life style, dogs can be regarded as good sentinels for monitoring the WNV urban life cycle whereas horses are appropriate to monitor the WNV rural life cycle. In our survey, the WNV prevalence value recorded in the urban area was significantly higher than that found in the rural area.

Different prevalence values were also found between western and eastern regions of Libya as the western regions were more affected compared with no positive cases in the eastern regions. One of the explanations for that is that the majority of samples collected from horses in the eastern region were from horses of young age. These findings proved that the WNV circulation in Libya is not uniformly distributed. Apart from providing figures on the WNV circulation in the urban area, monitoring of dog populations living near human populations may give valuable information on the level of human exposure to WNV. Based on our findings, it seems that the Tripoli population has been highly exposed to WNV infection. This high value is rather worrying. A previous seroprevalence study on the presence of WNV IgG ELISA antibodies in humans in Tripoli performed in 2017 and involving 400 people, found a prevalence of 2.75% (11/400) much lower to the value detected in this study [16]. Therefore, even if blood samples from people in contact with the sampled animals were not taken because this was beyond the scope of the current study, our results indicated that in the Tripoli urban area, WNV circulation is significantly increasing in the last years. This should raise concern about a possible increase of human cases.

Although these relatively high prevalence values, WND clinical cases were not evidenced neither in the sampled population nor in humans. As observed by other authors in many African countries, particularly in the North African countries facing the Meditaerranean basin, WND seems to be endemic causing only mild, self-limited febrile condition [19, 24-28]. The absence of severe WND cases in Libya could also be a consequence of the circulation of relative mild strains of WNV. The only way to confirm this hypothesis is to uncover the genome characteristic of the WNV strain circulating in Libya, this can be achieved only through a thorough epidemiological investigation focused on humans and a comprehensive monitoring of vectors and reservoir hosts [16]. We then strongly encourage to do more research on WND in humans in Libya, focusing on the areas where there was evidence of the presence of WNV among the animals. Evidence from Europe suggests that accurate identification of mosquito species in an area is important to reveal and predict the emergence of WNV for urban or rural environment. This is true in any surveillance strategy including those for other zoonotic arboviruses [29].

Our survey demonstrated that dogs and horses have been exposed to WNV and, possibly, other closely related Flaviviruses. The VNT is the gold standard method for WNV serology being able to identify WNV-specific or cross-reacting antibodies. In our case, the negative results of the VNT against USUV excludes the possible false positive results due to the co-circulation of this WNV cross-related virus. The epidemiology of WNV and USUV has undergone dramatic changes over the recent decades showing increase in the number of sporadic cases and the occurrence of outbreaks in different European countries [2]. In Africa, the virus was serologically detected in horses and dogs as well as different animal species such as bats, squirrels, wild boar, deer and lizards [30]. Zoonotic concern of USUV has been reported with increasing frequency in causing neuroinvasive disease in humans in different countries [31]. In the current study, all samples were seronegative for USUV antibodies proving that this virus has not circulated or is not circulating in Libya despite suitable environmental conditions. We have also seen how variables like geographic areas and different settlements have influenced the WNV prevalence. In the current study, another factor, which increased the risk to be exposed to WNV infection, is age. This finding has widely been reported in horses. A study from Egypt has revealed that horses of age ≥ 15 years, stallions breed, and those of mixed breed are potential risk factors associated with high seroprevalence rate of WNV [32]. Another survey showed that age together with other variables like presence of ponds, use of insecticides and presence of both rice fields and ruminants in the same properties increase the WNV exposure of horses and wild birds [33]. Other factors were frequently identified to be associated with WNV seroprevalence in horses including low number of horses within the holding, transportation and presence of mosquitoes [34] and the presence of dead birds and other ill animals on the property, the use of fans and a stable construction of solid wood or cement [35].

5. Conclusions

The present study provides novel evidence about the occurrence of WNV in horses and dogs in Libya. It also demonstrates the circulation of WNV in animal/vector populations, and in certain environments of the country. It adds new knowledge to the ongoing documented endemic status of the virus in North Africa and its possible emergence as an important human health problem.

Horses and dogs are good sentinel species for monitoring WNV circulation. Since horses generally live in the countryside, they can give useful information on the WNV circulation in the rural area, on the other hand, dogs are useful in monitoring the WNV circulation in the urban area and in places shared with the owners, as they commonly spend most of their live in close contact with humans. In both cases, this is a good example on how human and animal health are connected and emphasize the importance of the "One Health" approach. Multidisciplinary interventional teams including virologists, ornithologists, entomologists, climatologists, veterinarian, physicians, and policymakers should then be involved.

There is an urgent need for continuous monitoring programs on humans, horses, mosquitoes and birds including migrating avian species to provide essential epidemiological data for early detection of WNV circulation. Equally, it is also crucial to increase public and professional awareness about WNV and associated clinical problems in animals and humans.

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