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Farzaneh Ahmadnejad

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Farzaneh Ahmadnejad. Circulation du virus West-Nile dans les populations équine d'Iran : impact épidémiologique de l'environnement et du climat. Sciences agricoles. Université de Grenoble, 2012. Français. NNT : 2012GRENS001 . tel-00683646

HAL Id: tel-00683646

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THÈSE

Pour obtenir le grade de

DOCTEUR DE L'UNIVERSITÉ DE GRENOBLE

Spécialité : "Modèles, méthodes et algorithmes en biologie, santé et environnement"

Arrêté ministériel : 7 août 2006

Présentée par

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Thèse préparée au sein du Laboratoire "Techniques de l'Ingénierie Médicale et de la Complexité - Informatique, Mathématiques et Applications de Grenoble" (TIMC-IMAG) - École Doctorale "Ingénierie pour la santé, la Cognition et l'Environnement" (EDISCE)

West Nile Virus Circulation in Equine Population from Iran: Epidemiological Impact of Environment and Climate

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ACKNOWLEDGMENTS

This doctoral thesis would not have been possible without the support of many people. First of all, I would like to acknowledge my committee members:

Philippe Sabatier, who provided me, supported and taught me much valuable lessons throughout my study. The opportunity to work as a student with him was very rewarding and unforgettable.

Vahid Otarod, without whose support I would not have obtain the samples and start epidemiological work.

Sylvie Lecollinet and Stephan Zientara who provided me with technical support and laboratory facilities in France and accepted to review and examine my PhD thesis.

Christophe Peyrefitte, for accepting to evaluate my work and his helpful comments to improve the work.

Emmanuel Drouet for agreeing to chair my thesis defence and Jacques Demongeot for accepting to review and examine my PhD thesis.

My deepest gratitude also goes to Fereshteh Firouzi for her help and assisting me during my study and travels.

A warmly thanks to Mohammad Hosein Fallah who provided me with technical support and laboratory facilities in Iran.

A very special thanks goes out to Philippe Pourquier (ID Vet Company) for providing, gracefully, the ID SCREEN® competitive ELISA kits for the detection of West Nile Virus anti-pr-E antibodies in the sera and also to Agnes Leblond, Francis Geiger and Marc Grandadam for their helps in the preliminary study.

My heartfelt appreciation goes for Benoit Durand for statistical advices and review of the articles.

I express sincere appreciation to Amanollah Fathnia and Ali Ahmadabadi for their teaching me using Geographical Information System and other related softwares for the data analysis.

I would like to thank Steeve Lowenski, Josiane Maingault, Mehdi Kamyabi, Saeid Rahimizadeh, Alireza Gholami, Alireza Zavareh, Iman Vazirian, Alireza Janani, Saeid Jodairy-Eslami, Hassan Amiri, Ali Moradi-Joshaghan, Hossein Najafi, Celine Bahuon, Matthieu Chaumien, Dominique Bicout and Martine Meyer, for help with laboratory analyses, their technical assistance and helpful consultation.

I place special thanks to Mr. Amini and Ms. Elahi Rad regarding bird data.

My deep gratitude goes to Sirous Zeinali, Ahmad Fayaz, Gholamreza Ebadi and Mohammad Reza Siavashi for their official support.

Acknowledgments

I would like to sincerely thank Hooshng Ghaemi, for providing partially meteorological data.

I would like to acknowledge the Service de Coopération et d'Action Culturelle (SCAC) of the Embassy of France in Tehran for partly financial support and also to The Center for International Scientific Studies and Collaboration (CISSC) Ministry of Science, Research and Technology of Iran.

Thanks also go out to all the veterinarians who were involved in field support for this study.

Special thanks to Iman Vazirian for editing and improving my thesis and all the help he gave to me.

I am thankful to my friends, Elham Mazaheri-Tehrani and Nargess Miandehi for all the moral support they provided and for always being there.

Lastly, I would like to thank my family for their support, encouraging me and be patient. In addition, they have always encouraged me to reach higher and this work is a result of their encouragement.

I dedicate this thesis to the spirits of my father and older brother who always encouraged me and I lost them at the beginning and the end of this project, also to the other members of my dearest family.

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GENERAL INTRODUCTION

Despite considerable success in controlling communicable diseases, emerging and reemerging diseases have caused and still cause detrimental effects in different continents. Zoonotic infectious diseases include a large portion of this group. They involve the transmission of the etiologic agents to humans from an ongoing reservoir life cycle in animals or arthropods.

The zoonoses are a group of diseases that can be passed from animals to humans. This may be through direct contact with infected animals, contamination of water and food, infected aerosols, or by the bite of an infected arthropod vector which is an important route of transferring diseases to human (Hubalek 2003).

West Nile (WN) infection is an arboviral zoonotic disease, transmitted by mosquitoes, which is not restricted by international borders and involves all continents; causing illness and deaths in humans and horses.

This virus belongs to *Flaviviridae* family, genus *Flavivirus* and is maintained in a transmission cycle involving bird and mosquito species. Although humans and horses are considered as incidental hosts, and infection is often inapparent or mild, recently it has been shown that the infection can lead to severe disease or even deaths (Gubler *et al.* 2007)

Since the increase in the frequency and severity of outbreaks in humans and animals, particularly horses, in the Middle East, European and African countries, and also its introduction in 1999 in USA and rapid spread throughout the western hemisphere, more research has turned toward the biological and ecological aspects of WN virus (WNV), especially when it was shown that the isolated strain in USA was very close to the isolated strain in the Middle East (Lanciotti *et al.* 1999, Deubel *et al.* 2001, Ruiz *et al.* 2004, Zeller & Schuffenecker 2004).

The Middle East, because of its geographical situation, is extremely prone to the introduction of diseases and the spread of endemic infections and zoonotic diseases. This part of the world is a crossroad between different continents. WN disease or circulation of the virus has been reported from different countries in the region; such as Egypt, Israel, Lebanon, Iraq and the United Arab Emirates (UAE) (Hubalek & Halouzka 1999b, Chowers *et al.* 2005, Alfaresi & Elkoush 2008).

Iran is located at the eastern most edge of the Middle East region and the first report of circulation of the virus goes back around forty years ago when sera from residents of Khorasan and Khuzestan provinces were found positive for WNV neutralizing antibodies

(Naficy & Saidi 1970). There are other studies, all before 1980, indicating the presence of antibodies against WNV in healthy children from the Caspian area (Saidi S. 1974) and in six of 7 provinces in the country (Saidi *et al.* 1976). According to these evidences, Iran has been included in the category of infected countries with WNV; but after that time, there are no more facts relating to the situation of the virus and its presumptive detrimental effects in the country.

The present work aims to document the epidemiology of WNV in Iran, by: (i) assessing the spatial circulation of WNV; (ii) analyzing the impact of the environmental and climatic factors on WNV circulation. Chapter 1 explores the environmental conditions, particularly the distribution of the habitats of mosquitoes and hosts, and the climatic conditions, particularly temperature and rainfalls, which are favorable to the contacts between hosts and vectors. Indeed, these factors affect the abundance and activity of mosquitoes and hosts, but also the force of infection of infective species (Thomson & Connor 2000, Mellor 2004, WHO 2004, Reisen *et al.* 2006). Chapter 2 assesses the circulation of WNV in Iran. For this purpose, we considered equines, facilely captured and sampled, as sentinel hosts for the virus circulation survey in the country. We study the WNV seroprevalence and spatial distribution of seropositivity in equines from different Iranian regions. Moreover, Chapter 3 analyses the effects of environmental and climatic factors on the spatial circulation of WNV. Iran has diverse ecological conditions, resulting in biodiversity and consequently differential epidemiological transmission patterns. For this purpose, we have to identify ecological and climatic parameters related to WNV circulation in Iran. This analysis allows us to explain the relation between epidemiology of WNV and ecology of infected areas by an “oasis effect”.

Chapter 1.

***ENVIRONMENTAL AND CLIMATIC
CONTROL OF WEST NILE VIRUS
TRANSMISSION***

1. Introduction

First described in Uganda in 1937 (Smithburn 1940), West Nile virus (WNV) is responsible for infections in humans and horses, the virus has a transmission cycle involving mosquitoes as vectors and birds as amplifying hosts (Work *et al.* 1955, Taylor *et al.* 1956). For a long time, WN disease was endemic in the Middle East and Africa with sporadic outbreaks reported in Asia and Europe; so that it was not expected that the disease would reach the Western Hemisphere and cause death of many humans, horses and birds (Murgue *et al.* 2002). In Europe, several outbreaks have recently occurred in horses and humans in different countries including Romania (1996, 2010), Italy (1998, 2011), Russia (1999, 2007) and France (2000, 2003, 2004) (Hubalek & Halouzka 1999a, Murgue *et al.* 2002, Platonov *et al.* 2008, Bagnarelli *et al.* 2011). Since its introduction in North America (CDC 1999), WNV is recognized as the most widespread of the flaviviruses, with worldwide distribution.

Infection with WNV shows different effects on the hosts. Most infected hosts have no symptoms. A few percentages of infected individuals develop mild flu like symptoms. Neuroinvasive disease rarely occurs, however, is the acute form of WNV infection in humans and horses. It is estimated that about 20% of people who become infected will develop WN fever with mild symptoms, including fever, headache, myalgia, and occasionally a skin rash on the trunk of the body. About 1 of 150 WNV infections (<1%) result in meningitis or encephalitis. Case fatality rates among patients hospitalized during recent outbreaks have ranged from 4% to 14%. (Petersen & Marfin 2002). The infected birds may or may not become ill. Birds generally do not have symptoms of West Nile until the virus has caused encephalitis, or inflammation of the brain. Some bird species, like crows, showed a high susceptibility with death during WNV outbreaks in the US (Eidson *et al.* 2001).

Even if the epidemiology remains unclear, birds are known to take part in various aspects of WNV circulation (McLean *et al.* 2001, Malkinson & Banet 2002). Several studies have proved the essential role of birds as reservoir and spreader of WNV (Reed *et al.* 2003, Jourdain *et al.* 2007). Iran has "important wetlands", which support large breeding populations of many species of birds; in addition, each year, millions of birds coming from different countries migrate to these areas to pass the winter or use them as staging areas on their way to and from wintering areas. Iran appears to be a privileged area for the WNV transmission cycle. Likewise, the objective in this study is to identify the biotic and abiotic factors

involved in the circulation, transmission maintenance and spreading, of WNV in the environment.

2. Geographic dissemination of WNV

West Nile virus belongs to the *Flaviviridae* family, *flavivirus* genus. The viruses belonging to this family possess a single strand, positive-sense RNA genome. Within *Flavivirus* genus, based on cross-neutralization and molecular genetics, WNV is classified within the Japanese Encephalitis serological complex (Kuno *et al.* 1998). WNV is currently distributed in many parts of the world. The virus was first recognized in 1937 after it was isolated from the blood of a febrile woman in the West Nile District of Uganda (Smithburn 1940). Since then, it has been reported from the Middle East, Africa, Europe, Asia, Australia and from American countries (Hayes 2001).

2.1. Worldwide circulation of WNV

In South Africa, the first evidence of WNV was documented in 1958. The largest human outbreak of the disease was reported in 1974, which involved tens of thousands of human cases in the Karoo and Northern Cape Provinces (Murgue *et al.* 2002).

The first report of WNV in Europe was the detection of specific virus antibodies in Albania in 1958 (Bardos *et al.* 1959). In France, the first outbreaks of WNV occurred in the south, in 1962, with several nervous system abnormalities reported in the Camargue region in humans and horses. The region is characterized by wetlands and marshes, with a great number of migratory and resident bird species and large populations of mosquitoes (Hubalek & Halouzka 1999a, Murgue *et al.* 2001b).

After 38 years, another outbreak of WN disease was reported in horse population in southern France in 2000. During this outbreak, 76 of 131 equines with neurologic disorders were laboratory-confirmed; most of the cases were located in a region with large marshes (Murgue *et al.* 2001b). In Italy, during the late summer in 1998, an outbreak of neurologic disease occurred among horses residing in a large wetland area. The case-fatality rate of this outbreak (43%) was similar to the outbreak in 2000 in France (34%) (Autorino *et al.* 2002). In 2008, the WNV infection re-emerged in Italy; the isolated strains of these outbreaks (1998/2008) fell in the same sub-cluster with the isolates from Romania and Russia (Savini *et al.* 2008). However, phylogenetic tree analysis of the complete genome sequence of WNV

strains isolated in 2009 in Italy showed that the strains were closely related to the two WNV strains isolated from magpies in Italy in 2008. The 2008-2009 Italian WNV isolates had a higher degree of divergence from the eastern European strains isolated in Romania in 1996 and in Russia in 1999 and from the American/Israeli cluster (Barzon *et al.* 2009).

In 1996, 94 equines were affected in Morocco; 42 of which died. WN disease was reported in all age categories and it caused a higher mortality rate (45%) than the other almost concurrent outbreaks. There was a silent period until 2003, when an outbreak of WNV occurred again among stabled horses. Based on phylogenetic analyses, the virus isolates of Morocco in 2003 were similar to the 1996 isolate and to other strains responsible for equine outbreaks in the western Mediterranean basin. Recently an outbreak of WNV has been reported in horses in Centre region in Morocco (Schuffenecker *et al.* 2005, GAH 2010).

Since the outbreak of WNV in France, there have been several reports of West Nile virus activity in other European countries such as Spain (in the 1960s and the 1980s, 2010, 2011) and Austria (1960s and 70s). The activity or outbreaks of the virus have been detected in Portugal, Italy, Greece, Bulgaria, Romania, Hungary, Slovakia, Belarus, Ukraine, and Czech Republic (Hubalek & Halouzka 1999a, Samoilova *et al.* 2003, Esteve *et al.* 2005, Hubalek *et al.* 2006, Krisztalovics *et al.* 2008, Barros *et al.* 2011, Danis *et al.* 2011, Garcia-Bocanegra *et al.* 2011, Lelli *et al.* 2011).

In 1996, the most serious European outbreak of WNV occurred in Romania where the first activity of the virus dated back to the 1960s (Campbell *et al.* 2001). Between July and October 1996, 835 patients were hospitalized with suspected central nervous system infection. Among 509 patients with samples for testing, 393 (77%) were laboratory-confirmed to have WN virus antibodies, including 286 from Bucharest. There were 17 fatalities (4.3%) in patients older than 50 years (Tsai *et al.* 1998).

Currently there is no evidence that WNV causes disease in the UK, nevertheless in 2003, Buckley *et al.* reported the presence of virus-specific neutralizing antibodies to WNV (22.7 %) in the sera collected from 30 different species of wild or farm birds (resident and migrant birds) in the UK (Cambridgeshire, Dorset and south Wales), implying that the virus was being introduced to UK birds, possibly by mosquitoes. It seemed that the virus is being introduced into UK-resident birds, apparently without causing any obvious reduction in the bird population. In addition, other activities included surveillance for WNV infections in humans, and in dead birds, mosquitoes and horses, carried out between 2001 and 2005, have found no positive cases of UK-acquired WNV. Between 2001 and 2006, tissue samples were

collected from over 1800 wild birds by the laboratories of SAC in Scotland and the Veterinary Laboratories Agency (VLA) in England and Wales. The samples were tested by VLA for the presence of WNV by virus isolation and PCR. No evidence of WNV infection was found (Buckley *et al.* 2003, Morgan 2006, Pennycott & Cert 2011).

In 1999, WNV found its way to North America as an important pathogen and has spread rapidly throughout the country, presenting a threat to both human and animal health. During this outbreak, among 62 human cases, 59 (95%) were reported as West Nile meningitis or encephalitis (neuroinvasive disease), 3 (5%) were reported as West Nile fever (milder disease) and seven patients died (CDC 2007).

The case fatality rate for horses in New York was 25% and thousands of birds of as many as 18 species died during this outbreak (Steele *et al.* 2000). In the later years WNV continued to cause disease in humans, horses, and a variety of bird species in New York City and within several years, it became firmly established in U.S and gradually made its way to Latin America, the Caribbean and Canada (Komar *et al.* 2003b, Mattar *et al.* 2005). In Canada, the first reports of WN virus activity were notified in 2001 when the virus was found in dead birds and mosquito pools in Southern Ontario and the first human and horse cases were confirmed in the following year, 2002 (Weese *et al.* 2003).

The source of virus introduction to North America is unknown; potential sources include an infected vector, human, bird, or other vertebrate hosts (Lanciotti *et al.* 1999). The close genetic relationship between WN virus isolates from Israel and New York suggests that the virus was imported into North America from the Middle East (Lanciotti *et al.* 1999).

Overall, we can classify epidemic and/or epizootic occurrence of WNV to two distinct periods: before 1990 and after 1990. The years after 1990 apparently have had suitable conditions for the circulation of the virus as a higher occurrence of disease among human, horse and bird population has been reported in these years (

Figure 1). Circulation of WNV, like other vector-borne-diseases, is so dependent on the climate and the difference between two parts of time can be related to the global climate change. A millennial study (1000-2000) of climate change has revealed that the 1990s was the warmest decade and 1998 the warmest year (Jones 2000).

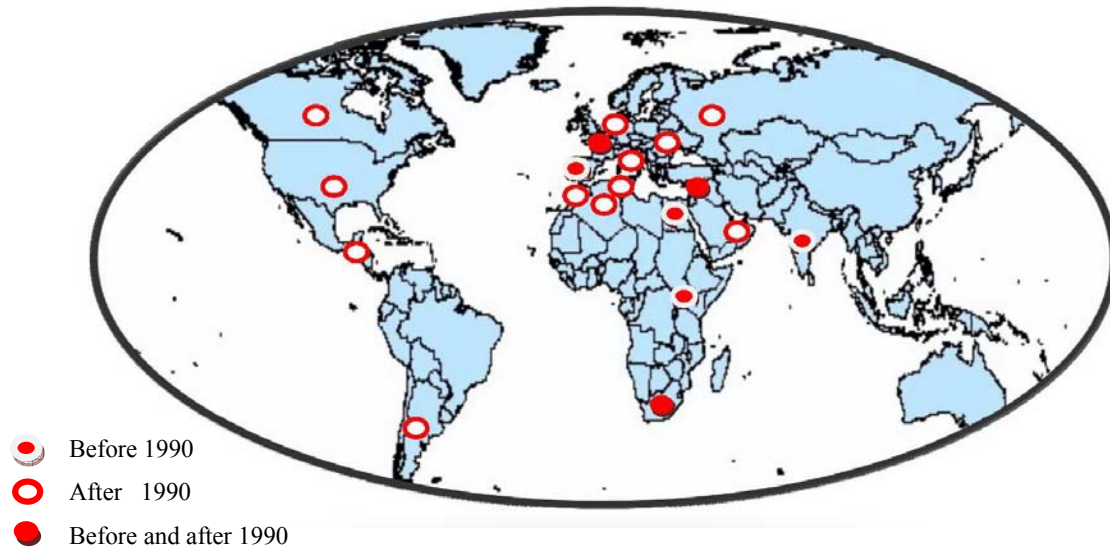


Figure 1. Occurrence of West Nile virus epidemic and/or epizootic in the world

2.2. Geographical distribution of the lineages

Evidence from phylogenetic studies suggests that there are at least eight genetic lineages of WN virus:

- **Lineage 1:** these strains have a broad geographical distribution, isolated from over 20 countries. The viruses within this lineage are divided into two clades:

- **1a:** the viruses isolated from Europe, Africa (north, West, central), India, Asia, Middle East and the United States;
- **1b:** the virus isolated in Australia, also known as Kunjin virus (KUNV).

Most virulent viruses in recent outbreaks belong to clade 1a; however, the lineage contains both virulent and attenuated viruses,

- **Lineage 2:** mainly comprised of virus isolates from endemic areas of west, central and east Africa and Madagascar; although some strains have been found outside Africa. These viruses often cause asymptomatic infections or mild disease, but virulent viruses also exist. One virulent virus has caused encephalitis in raptors in Central Europe (Hungary) (Berthet *et al.* 1997, Lanciotti *et al.* 1999, Savage *et al.* 1999, Lanciotti *et al.* 2002). Recently it has been shown that WNV lineage 2 may cause neurologic disease in horses in southern Africa and in Hungary (Venter *et al.* 2009, Kutasi *et al.* 2011),

- **Lineage 3:** includes the isolates from *Culex pipens* at the Czech Republic/ Austria border (Rabensburg virus),

- **Lineage 4:** the isolate from *Dermacentor marginatus* ticks in the Caucasus Mountain valley, Russia (LEIV-Krnd88-190) makes this lineage (Prilipov *et al.* 2002, Bakonyi *et al.* 2005),

- **Lineage 5:** The Indian isolates had been placed previously within lineage 1 as a separate cluster (clade 1c), but more analyses of genome sequences have classified them as lineage 5 (Bondre *et al.* 2007).

- **Lineage 6:** this lineage has been proposed for the Sarawak Kunjin virus strain (Malaysian isolate), which is markedly different from the other Kunjin viruses.

- **Lineage 7: African** Koutango virus (isolated in Senegal), which is closely related to WNV

- **Lineage 8:** this lineage has been proposed on the basis of WNV sequences detected in *Cx. pipiens* captured in southern Spain (Vazquez *et al.* 2010, Papa *et al.* 2011).



Figure 2. Phylogenetic tree of West Nile Virus (WNV) complete nucleotide sequences (Botha *et al.* 2008).

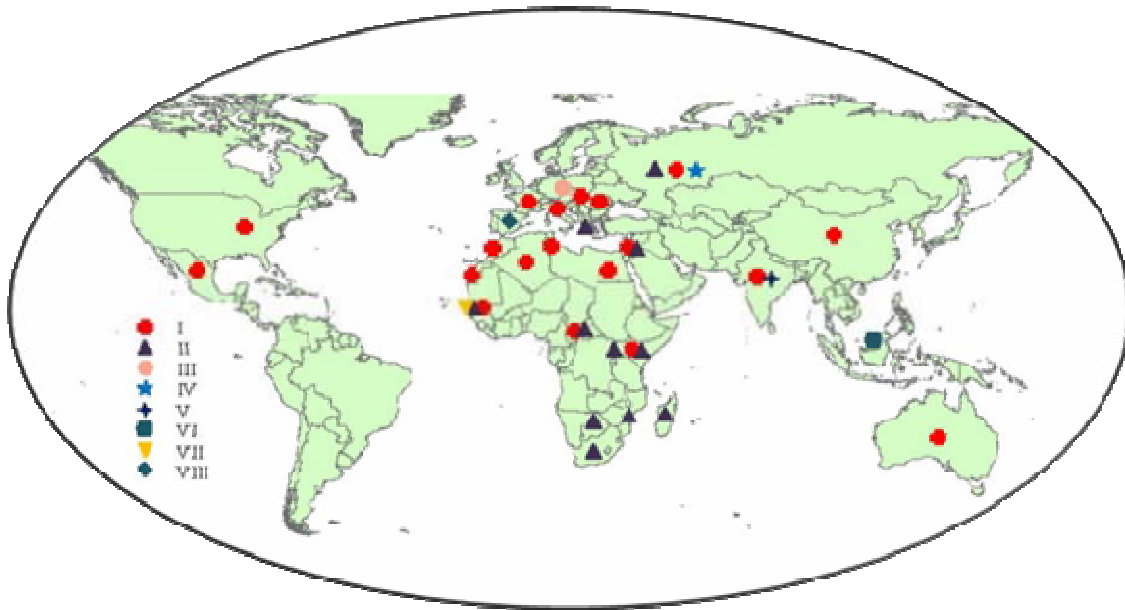


Figure 3. Geographical distribution of different genetic lineages of West Nile virus.

The phylogenetic analysis of WNV strains indicates that co-circulation of different lineages can occur in the same country (Briese *et al.* 2002) (Figure 3). Distribution of several genetic lineages of the virus in different geographic locations indicates the potential importance of birds in viral dispersal (Berthet *et al.* 1997) and also adaptation of the virus with different geographical regions and hosts.

2.3. WNV Circulation in the Middle East

The Middle East is geographically well known as an area where intense migratory activity of birds can be observed during the year. This region is considered as a cross road for the bird species that stop over between Europe and Africa.

Egypt- The first documents about the virus activity and its characteristics are related to the studies in Egypt, where the first epidemiological studies of WN fever have been conducted in the 1950s. Sero-epidemiological surveys in humans, mammals and birds as well as entomological studies carried out at that time characterized the ecology of the virus and the endemicity of WNV in Egypt (Melnick *et al.* 1951, Hurlbut *et al.* 1956, Murgue *et al.* 2002). The results from an endemic area in the Nile Delta suggested that WN infection is essentially a childhood disease, taking place mainly during mid-summer. Although there were no data on

the symptomatology of the infection, it appeared that the infection was mostly self-limited, non-fatal febrile disease rarely associated with manifestations of encephalitis (Taylor *et al.* 1956). Another study was carried out in 1968, among 120 children, attending the fever hospital in Alexandria and complaining of fever. It was noted that although the sera were collected during June to October, all positive sera were obtained in August (Mohammed *et al.* 1970).

A serologic survey conducted in equines (horses, donkeys, and mules) in 1959, showed a high prevalence of WN antibodies (54%) and the first reported WNV infections in a horse occurred in a 12-year-old horse that died and the virus was isolated from the brain tissue. Thereafter experimental infections of equines were conducted in order to study the role of equines as reservoirs of WNV and the duration and magnitude of viremia; surprisingly only two equines, among eleven, developed low grade fever and showed very short-term viremia which suggested that the equines probably were dead-end hosts (Schmidt & Elmansoury 1963).

Natural and experimental studies among different hosts exposed to WNV led to a better understanding on which species are important hosts and produce high level of viremia to infect non-infected mosquitoes. Based on these studies, it was concluded that the main cycle of the virus was probably through mosquitoes and birds; non-migratory birds were considered as hosts responsible for the local dissemination of the virus. The first report of natural WNV-associated morbidity in birds was a sick fledgling pigeon in Egypt in the early 1950s. One study carried out in the mid-1950s in Egypt confirmed the role of passerine birds as important reservoirs in this region. Neutralizing antibodies were found in all species of birds tested from an endemic area in which Hooded Crows and House Sparrows had the highest viral neutralizing antibodies; which suggested these species could be used as natural sentinels for monitoring virus transmission. Experimental infection of birds with WN virus by mosquito bites demonstrated a high mortality rate of crows which had high circulating virus titers and could imply a high mortality in naturally infected crows; but it was also stated that many survived as indicated by the high percentage of antibody positive crows (Work *et al.* 1953, Work *et al.* 1955, Taylor *et al.* 1956, Malkinson & Banet 2002).

The first field studies that implicated mosquitoes as important vectors of WN virus were conducted in Egypt from 1952 to 1954. During this study, 17 isolates of WN virus were obtained from pools of mosquitoes (*Culex* spp.). In addition, a number of pools of other arthropods, including ticks, lice, fleas, mites and flies were tested for WN virus isolation but

were all negative. The persistence of WNV in Egypt was explained by mosquitoes that remained active throughout the colder months (Taylor *et al.* 1956).

Israel- WN fever is endemic in Israel. The virus was first recognized in 1951; although it had probably already been present in that country during previous years. In 1951, an outbreak occurred among the population located at 30 km south of Haifa in Israel where 41% of its 303 inhabitants were patients. None of the cases was fatal; children represented most of the cases that were infected with WNV, and one of them developed mild meningitis (Bernkopf *et al.* 1953).

The occurrence of WNV encephalitis cases among elderly population in Israel in 1957 was the first report that WNV could cause serious central nervous system infections and established a correlation between the age of patients and severity of disease. Encephalitis has been reported from several locations of Hadera area (army camps, urban / rural places and nursing homes), but the death only has been observed in nursing homes by a rate of 8.2% (4/49) in a group of elderly patients (Chowers *et al.* 2001, Murgue *et al.* 2001a, Weinberger *et al.* 2001). There was another outbreak among young soldiers in 1980 with one patient with meningitis and no fatal case.

In 2000, 417 laboratory-confirmed WN fever cases were diagnosed in Israel with 35 deaths, all in patients >50 years of age. The highest incidence was in central Israel; the most populated part. Men and women were equally affected, and their mean age was 54.5±23.8 years (range 6 months to 95 years). This outbreak was different from previously reported outbreaks in Israel because of high rates of encephalitis, countrywide spread, and higher number of hospitalizations (Weinberger *et al.* 2001). At the same time (2000), an outbreak of West Nile virus infection affected the horse population with 76 horses with developing neurological disorder and 15 deaths. (Steinman *et al.* 2002).

During the 2000 WNV epidemic in Israel, specimens from a 72-year-old woman were analyzed and the results indicated closer phylogenetical relationship of this isolate to 1996 Romanian and 1999 Russian than to 1998-99 Israeli or 1999 New York isolates (Briese *et al.* 2002). During the same outbreak, WNV isolated from four patients and their phylogenetic analyses revealed that at least two strains of WN virus circulated in Israel in 2000. This is most likely the result of the movements of migratory birds between Africa and Europe via the Middle East. One sub-clade 1a strain (2 isolates) was similar to WN virus isolates from mosquito, horse, and flamingo during the 1999 NY outbreak. The other sub-clade 1a strain (2

isolates) was similar to the viruses isolated from Romanian (1997) and Russian (Volgograd, 1999) outbreaks, but different from 1996 Romanian isolate (Hindiyeh *et al.* 2001).

Israel is located on the flyway corridor of migrating birds to and from Africa and to Europe, so the virus may have been introduced in this manner. During 1997 and 1998 for the first time, WN virus was reported as the cause of neurologic illness and death among the affected goose flocks. In a study carried out between December 1998 and October 1999, sera were collected from goose farmers and veterinarians working with sick or healthy geese to detect the infection following contact with sick geese. There was a relation between positive serology and contact with flocks of sick geese. Moreover, some farmers who worked with sick geese had developed a flu-like illness, indicating close contact with sick geese as a risk factor (Bin *et al.* 2001).

Another study conducted in Israel revealed again the role of birds for WNV distribution. A flock of 1200 migrating storks arrived outside Eilat in August 1998. Their arrival was attributed to strong, hot westerly winds, which forced the storks to ground in Eilat. Six days after their arrival, three of 11 healthy storks were shown to have WNV-neutralizing antibodies, indicating previous exposure to WNV. The authors concluded that it is improbable that infection had occurred in Eilat because the infected birds were collected within 2 days after arrival. It is likely that hatching time of fledglings and their exposure to WNV had occurred in Europe near the nesting sites or along the route of migration (Malkinson *et al.* 2002).

The phylogenetic analyses indicated a close genetic relationship between IS98 isolate (Israeli WNV strain isolated from a white stork in 1998) and an isolate from goose in Hungary in 2003 (Figure 2). The place where the WNV outbreak occurred in Hungary, is located in the wetland areas that are good habitats for many bird species, supporting the concept of introducing neurovirulent WNV strain from central Europe to Israel (Bakonyi *et al.* 2006).

Turkey- Serologic evidence for the presence of WNV infections dates back to the 1970s based on the detection of hemagglutination inhibiting (HI) antibodies. In one study, WNV prevalence of HI antibodies was 6% in humans and 1.5% in sheep. Another survey indicated a seroprevalence as high as 40% in randomly selected human sera in southeast Turkey (Ozkul *et al.* 2006).

A serological survey, first reported in 2005, indicated the exposure of a wide range of mammals to WNV in Turkey. In this study, 35 of 259 (13.5%) horses and 18 of 88 (20.4%) humans had developed WNV-neutralizing antibodies. Geographical locations of horses showed they were stabled near a lake in a park; a place for migrating birds. The presence of possible human WN disease was suggested among human attending hospitals in southeastern part of Turkey in 2005 (Ozkul *et al.* 2006, Ergunay *et al.* 2007).

Other countries in the Middle East- A study conducted in 2005 among 500 healthy blood donors indicated that WNV infection was not present in the UAE; nevertheless, another study in 2007 demonstrated that WNV had entered the UAE. In the later study among 750 equines, 144 (19.2%) animals had antibodies to WNV. In addition, one horse with antibodies to WNV had clinical signs consistent with WN encephalitis (Wernery 2007, Alfaresi & Elkoush 2008).

In Jordan, a serosurvey was conducted among patients, over 5 years of age, attending a health center in 1998. The seroprevalence of immunoglobulin G antibodies against WNV was 8%. Moreover, authors suggested that the significant risk factor was the proximity to wastewater treatment plants (Batieha *et al.* 2000).

In Oman, 19 clinical cases from a population of 240 horses were reported in 2003 and demonstrated that the disease occurred in only one stable (Al Suleiman 2003). West Nile virus antibodies have also been detected in human sera from Iraq, Lebanon and Syria (Hubalek & Halouzka 1999a).

2.4. WNV Circulation in Iran and Neighboring Countries

The first description of West Nile virus (WNV) in Iran was in the 1970s, when sera were obtained from residents of Khorasan (303 sera) and Khuzestan (60 sera) provinces. The sera were from people of age 8-70 years and were tested for the presence of neutralizing antibodies to WN. A total of 103 (28.4%) cases were antibody positive, of whom 91 (30%) and 12 (20%) were from Khorasan and Khuzestan, respectively (Naficy & Saidi 1970).

In another study reported in 1974, 100 sera from children 1 – 6 years of age, resident of the Caspian area were tested for the prevalence of antibodies against 15 different viruses. The sera were collected during May 1970 to September 1971 and analyzed by hemagglutination-

inhibition (HI) method for detecting WNV antibodies. Ten (10%) children had antibodies against WNV (Saidi S. 1974).

A study conducted in 13 different communities, located in 7 provinces (East-Azarbaijan, Gilan, Khorasan, Tehran, Isfahan, Kermanshah, Khuzestan), was reported in 1976. 186 (26.6%) of 698 human sera had antibodies against WNV. A positive reaction was detected in six of 7 provinces (except East -Azarbaijan province) with highest prevalence in the Dezful-Deigi area located in Khuzestan province (Figure 4) (Saidi *et al.* 1976).

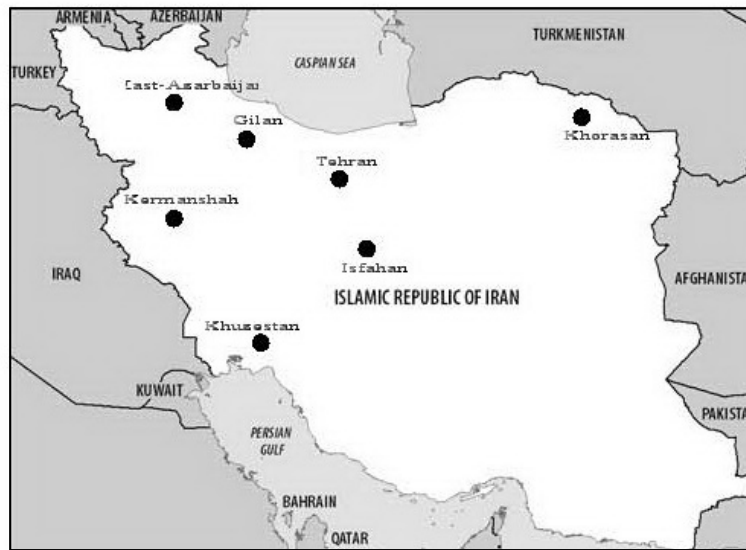


Figure 4. Map of Iran showing the location of 13 communities sampled for West Nile Virus antibodies in 1976 (Saidi *et al.* 1976).

A study of WNV infection in Iranian blood donors, who donated blood at the Tehran Blood Transfusion Center, was reported in 2010, in which 500 sera were examined by ELISA and Real-Time RT- polymerase chain reaction methods. All 500 donors were negative for WNV-specific IgM antibody and WNV-RNA, but 5% of them had IgG antibodies (Sharifi *et al.* 2010).

WNV infection has been reported from Pakistan (eastern neighbor of Iran) and Russia. Russia is the northern neighbor of Iran; and some areas in Iran are considered as wintering places for migratory birds coming from Russia. Siberian Crane is one of these migratory birds that every year migrates from its breeding grounds in northern Siberia to wintering sites in Iran (Kanai *et al.* 2002). Ducks, geese and pelicans are among other birds coming from northern parts to the country.

West Nile fever (WNV) is an endemic disease in Southern Russia and western Siberia-Altai territory. The virus was first isolated from soft ticks (*Ornithodoros capensis*) and hard ticks (*Hyalomma marginatum*, *H. detritum*, *Rhipicephalus turanicus* and *R. muhsamae*), also from birds in the delta of the Volga River of the Astrakhan region in 1963-1968 (Hubalek & Halouzka 1999a, Lvov *et al.* 2000, Fyodorova *et al.* 2006). Russia experienced a large outbreak of WNV in the south of the country (Volgograd, Astrakhan, and Krasnodar regions), in Summer and early of Autumn 1999 with a peak during 21-25 August. Volgograd City is located on the west bank of the Great Volga River, and the total number of suspected WNV cases was estimated to be 480 people in the region with at least 40 deaths. The majority of patients (50%) were over 50 years of age and thirty of the patients (75%) who died were >60 years of age (Lvov *et al.* 2000, Platonov *et al.* 2001). Also during the same period, 89 WNV cases with 5 deaths in Astrakhan Region and at least 38 WNV cases with 3 deaths in Krasnodar Region were reported (Platonov 2001).

During 2001-2004 in the Astrakhan region, sera sampled from 2,884 farming animals were investigated to indicate specific antibodies to West Nile virus. The antibodies were detected in all the examined species of animals (horses, cattle, camels, pigs, and sheep) (Vasil'ev *et al.* 2005).

The epidemiological and entomological studies indicated that the outbreak in the Volgograd area was similar to the epidemic in Romania in 1996 and in New York City in 1999. Phylogenetic analysis also supported this suggestion. Two different genotypes were isolated during the 1999 outbreak, one was similar to isolates of Israel in 1997-99 and New York in 1999, while the other one was similar to the Romania isolate in 1996 (Briese *et al.* 2002, Fyodorova *et al.* 2006). These strains belong to lineage 1, while the virus isolated in 1998, in *Dermacentor marginatus* ticks in the North-West Caucasus Mountain valley of Russia belongs to the lineage 4 (Bondre *et al.* 2007).

3. West Nile virus transmission

WNV is maintained in nature in an enzootic cycle between ornithophilic mosquitoes and birds. Mosquitoes become infected when they feed on infected birds (reservoir hosts) and the virus is transmitted to other birds when the mosquitoes bite again. This is defined as rural or "sylvatic cycle". In a secondary cycle, which is called urban, bridge mosquitoes (feeding

on both birds and non avian hosts) become infected by feeding on an infected bird and then continue spreading WNV to other vertebrates (Hubalek & Halouzka 1999a).

3.1. Epidemiological cycle

Along with the maintenance of virus between mosquitoes and birds in nature, it is also supposed that the virus is transmitted between generations of mosquitoes. Natural transovarial transmission of WNV has been identified in Kenya in *Culex univattatus*. Also in laboratory studies, a female range of *Culex* and *Aedes* mosquitoes have been shown to vertically transmit the virus to offspring (Baqar S. 1993, Miller *et al.* 2000, Turell *et al.* 2001b). This mechanism is important as the virus can persist even during cold weather when there is no adult mosquito.

Vertical transmission is a general terms for transfer of pathogen from one generation to the next; while transovarial transmission means transfer of pathogens from parents to offspring through the ovary and eggs which occurs mainly in arthropods (Schmid-Hempel 1998). Lateral transmission between mosquitoes has also been documented. An infected mosquito and a non-infected mosquito were allowed to feed adjacently on a non-viremic host. After seven days, the non-infected mosquito had viral titers high enough to infect new hosts (Higgs *et al.* 2005). Presence of the virus in hibernating mosquitoes has been also observed (Nasci *et al.* 2001). This issue can lead to the circulation of the virus during the next warm season when mosquitoes become active.

Beside arthropod-borne transmission, vertical and direct transmission of WNV has been observed between vertebrate hosts without the involvement of arthropods. Transmission through transfused blood (Pealer *et al.* 2003), transplanted organs (Iwamoto *et al.* 2003) and transplacental (mother-to-child) transmission of WNV have occurred in humans (CDC 2002b). Transmission through breast-feeding is also likely (CDC 2002c). Laboratory-acquired WNV infections have been also reported among laboratory workers via percutaneous inoculation (CDC 2002a).

Based on recent studies, shedding of WNV RNA has been reported in the urine of some patients, some of whom had a shedding 1.6-6.7 years after the infection (Tonry *et al.* 2005a, Murray *et al.* 2010). It seems that shedding of WNV via urine, as a route of virus transmission, should be considered (Tonry *et al.* 2005a, Tonry *et al.* 2005b).

The possibility of WNV transmission by aerosol among turkey handlers at a turkey trade and among animals raised the possibility of aerosol exposure. Studies showed the possibility of bird-to-bird transmission via saliva and/or fecal aerosols (Banet-Noach 2003, Komar *et al.* 2003a).

Ingestion of infected animals has also been considered as a possible source of transmission. By identification of WNV from a hawk in winter, and from farmed alligators concurrent positive RT-PCR for WNV in their horsemeat, authors concluded that animals probability acquired the infection by eating the infected animals (Garmendia *et al.* 2000, Petersen & Roehrig 2001) (Figure 5).

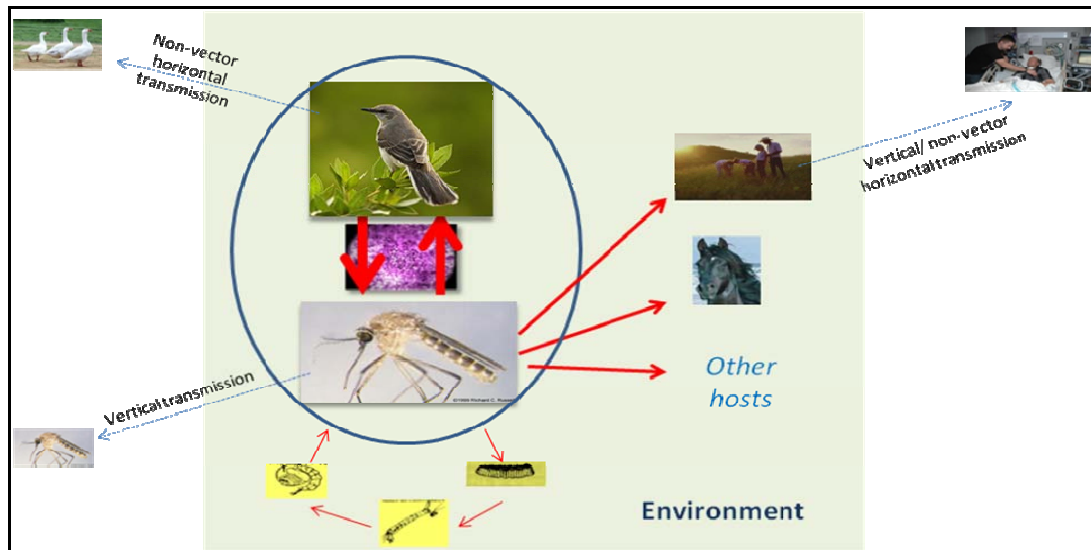


Figure 5. Transmission cycle of West Nile virus

3.2. Vectorial transmission

WN virus has been identified in numerous mosquito species, including members of the genera *Culex*, *Aedes* and *Ochlerotatus*; mosquitoes belonging to the *Culex* species being the primary vector (Turell *et al.* 2001b). The ability of mosquito to become infected after feeding on infected hosts, to replicate the virus and to transmit infection to susceptible hosts is called "vector competence" (Reisen *et al.* 2005).

Female mosquitoes require a blood meal before they can lay eggs. The mosquito can take in as much as twice its body weight in blood, thus creating a relatively good chance of

picking up the pathogens. When a mosquito bites an infected bird, the virus enters the mosquito's bloodstream and finally reaches the salivary glands. After biting, they are capable of transmitting the virus within 10 to 14 days following feeding on an infected bird, so bites after that time are infectious (Jarratt *et al.* 2004).

The host preferences of mosquito species are also important determinants of transmission dynamics. For example, ornithophilic mosquitoes, such as *Culex spp.*, typically feed on viremic birds, whereas *Aedes* prefer to feed on mammals. The species, such as *Ae. vexans*, *Cx. salinarius*, *Ochlerotatus. j. japonicus*, and *Oc. Triseriatus*, known as bridge vectors could become infected when feeding on an infected bird, and then transmit WN virus to another susceptible vertebrate host (Turell *et al.* 2001b).

Moreover, the distribution of principal vectors of WNV differs in different regions. In Egypt, Israel, and South Africa, the main vector is *Cx. univittatus*, while *Cx. modestus* was found to be an important vector for circulation of West Nile virus in the Volga Delta and in populated areas in Russia and in Europe, especially in wetlands of the Camargue in France. *Cx. triaeniorhynchus* and *Cx. vishnui* are the main vectors for WNV based on experimental studies in India. The virus has been isolated from *Cx. Vishnui* complex in Pakistan (Hubalek & Halouzka 1999a, Lvov *et al.* 2000, Paramasivan *et al.* 2003, Balenghien *et al.* 2008). In the winter 2000 after the 1999 outbreak in the US, WNV was isolated from overwintering *Cx. pipiens* mosquitoes collected in New York; providing the mechanisms for viral persistence and re-emergence each spring (Nasci *et al.* 2001, Hayes 2005). The first isolation of an arbovirus from a hibernation mosquito (*Cx. modestus* Fic.) in Europe goes back in 1970 when Tahyna virus (family *Bunyaviridae*) was isolated from the mosquito in Camargue in winter (Chippaux *et al.* 1970).

During the large outbreaks occurring in urban populations of Romania, Volga delta in Russia, and U.S, the significant factor was the involvement of the common house mosquito, *Culex pipiens*, which had not been considered before as an important vector. Proximity to large rivers was a similar parameter between all the sites, presumably providing an attractive place for birds (Hayes 2001). Species in the *Culex pipiens* complex are considered the primary vectors of WNV in the New world because of their abundance in urban areas, occurrence of the disease during their peak and their experimentally confirmed competence. Unlike European *Cx. pipiens*, U.S. *Cx. pipiens* appears to bite readily both avian hosts and humans. It seems that a huge part of the U.S. mosquitoes are hybrids of human-biter and

bird-biter forms and these hybrids caused recently epidemic condition in the U.S. (Fonseca *et al.* 2004).

Some studies have indicated that exposure to infected mosquitoes is a risk factor for acquiring WNV infection. During the 1996 outbreak in Romania, the risk for WNV infection was the time spent outdoors and in flooded basements. In New York outbreak, human cases were clustered in areas with higher vegetation cover, indicating favorable mosquito habitat (Han *et al.* 1999, Brownstein *et al.* 2002).

Other arthropods have been also implicated as vectors of WNV, although their role in the ecology of the virus remains unknown. WNV has occasionally been isolated from hard and soft ticks, and experimental studies indicated that infected ticks (such as *Ornithodoros moubata*, *O. maritimus* and *O. erraticus*) could transmit the virus to the hosts (Hubalek & Halouzka 1999a, Lawrie *et al.* 2004).

3.3. Birds reservoirs

Since viremia in birds can reach high-level titers to infect the mosquito species, birds are considered as the main reservoir host for WNV in nature. However, all the birds do not develop high levels of viremia, and only some of them play an important role in the transmission cycle of WNV; such as passerines (including members of the crow family, Blue Jay and House Sparrow) and charadriiformes (such waders and gulls) (Hubalek & Halouzka 1999a).

Like vectors, several birds in different geographical regions have been implicated as reservoirs, and the virus has been isolated from a variety of wetland and terrestrial bird species. Although their infection usually can be clinically inapparent, that susceptibility to infection varies considerably among species and according to their geographical regions, for example deaths due to WNV has been observed in a pigeon in Egypt, in geese in Israel, in different species, especially corvids in U.S. and in sparrow hawk (*Accipiter nisus*) and several goshawk (*Accipiter gentilis*) fledglings (Lanciotti *et al.* 1999, Bernard *et al.* 2001, Erdelyi *et al.* 2007).

Even though vectors appear to be essential for transmission of WNV among avian species, it seems that birds have also the capability to transmit the virus by alternative modes of transmission. Studies have demonstrated the possibility of bird-to-bird transmission via saliva and/or fecal aerosols, ingestion of contaminated food (Komar *et al.* 2003a).

Among different agents implicated in WNV circulation, infected migratory birds are important in spreading the virus into different, particularly naive, areas along their short or long migrations route (introducing hosts) which is followed by virus adaptation to local vectors and its subsequent amplification through the infection of local bird populations (amplifying hosts).

Migratory birds have long been suspected as the principal hosts for the spread of various pathogens into new regions. The studies show that the probable causes of introduction of WNV in the Middle East, Europe, and U.S. are the birds coming from endemic areas (Rappole *et al.* 2000, Malkinson *et al.* 2002, Jourdain *et al.* 2007).

There is a possibility that the migratory birds, which may be infected in their African wintering areas, transmit the virus northward, in the Middle East and Europe, during spring migration (Cabre *et al.* 2006). On the other hand, as suggested by the similarity between the Israeli isolate in 1998 and European isolate, the virus could have been introduced from central Europe to Israel by migratory birds (Malkinson *et al.* 2002).

3.4. Roles of other vertebrates

Horses, along with humans, are the most susceptible accidental hosts for WNV and like other hosts become infected by biting of infected mosquitoes; and since they are unable to generate a viral titer high enough to infect new mosquitoes, they are considered as “dead-end” hosts. Their infection ranges from asymptomatic to fatal encephalitis. The incubation period, or the time between being bitten by an infected mosquito and the time when clinical signs appear, ranges from three to 15 days (Nash *et al.* 2001).

The estimated rate of clinical disease among infected horses with WNV is 10% with 38-42% mortality rates occurring in different geographical regions. While around 1% of infected humans develop neurological signs with an overall case fatality rate of approximately 10% which may reach 15-29% in people who are over 70 years old (Bunning *et al.* 2002, Castillo-Olivares & Wood 2004, CFSPH 2009).

Few outbreaks of WN disease in horses were documented before the 1990s. The first neurological cases in horses infected with WNV were first described in Egypt and France in the 1960s with mortality rates ranging from 25% to 30% in France (Schmidt & Elmansoury 1963, Murgue *et al.* 2001b). Since then several outbreaks of neurological disease have been reported among horses in Morocco (1996, 2003, 2010), Israel (1998, 2000), Italy (1998,

2008, 2009, 2010, 2011), France (2000, 2003, 2004), Argentina (2006) and U.S.A (1999 through 2009) (Dauphin *et al.* 2004, Morales *et al.* 2006, Macini *et al.* 2008).

Some animals with acute neurological disease die spontaneously, but many severely affected animals are euthanized which imposes economic burden for the countries. In contrast to the human disease, age does not appear to be a risk factor for development of severe neurological disease and death in horses. A study carried out among horses with encephalitis in New York State in 1999 and 2000, revealed that there was no significant difference in the age of horses that died or were euthanized and horses that survived (Trock *et al.* 2001). During a serosurvey of WNV among equines in southern France, age had no effect on seropositivity (Durand *et al.* 2002). However, a study among previously uninfected horses in Texas revealed that older horses were at greater risk of death or euthanasia (Ward *et al.* 2006).

Although the role of the vertebrates other than birds in epidemiology and transmission of WNV is unclear, and they have been considered as accidental hosts, some studies have shown the role of some animals as potential sources of virus for mosquitoes. Several studies have shown the serological evidence of WNV exposure in a broad range of other mammalian hosts such as cats, dogs, feral swine, raccoons, foxes, bats, and squirrels (Komar *et al.* 2001, Dietrich *et al.* 2005, Docherty *et al.* 2006, Gibbs *et al.* 2006a). It seems that the lake frog (*Rana ridibunda*) appears to be a competent reservoir for WNV in Russia (Hubalek & Halouzka 1999a, van der Meulen *et al.* 2005).

4. Environmental and climatic risk factors for WNV circulation

The atmosphere plays a central role for the environmental conditions on earth. Climate refers to the conditions in the atmosphere. The environmental factors impacting public health are related to: (i) atmospheric parameters such as, temperature, precipitation, relative humidity, wind and solar radiation (abiotic); (ii) or terrestrial parameters, such as topography, fresh water ponds, rivers, lakes (abiotic) and hosts (mammals, reptiles, birds), natural predators, parasites, vectors, pathogens, vegetation (biotic) (WHO 1990).

Climate variation has two different effects on ecological processes: (i) direct effects on virus/hosts/vectors, for example, by selecting the variants sensitive, or controlling the physiology of organism such as metabolic processes and reproduction (Gubler *et al.* 2001,

Reiter 2001); and (ii) indirect effect on the habitats, the ecosystems and the relationships among the organisms (Stenseth *et al.* 2002). Mosquito-borne diseases, such as WN, are currently considered the most likely to be affected by both climate and environmental variations (Patz *et al.* 1996).

Spatial patterns of the viral circulation are controlled by the distribution of both vertebrate and invertebrate host/vectors, which is influenced by environmental factors. Therefore, environmental factors play critical roles in the distribution and dynamics of vector-borne diseases. In other words, these diseases are highly susceptible to the ecological and climatic factors because of the interaction of all elements involved in the transmission cycle of the agent with the surrounding environment (Figure 5) (Patz *et al.* 1996, Reiter 2001).

4.1. Direct impact of atmospheric parameters

Climate variation affects the population dynamics of mosquitoes because body temperatures approximate ambient conditions and larvae develop in aquatic habitats created directly or indirectly by precipitation. Temperature governs most mosquitoes biological rates including blood feeding, reproduction, and larval development (Reisen 1995), whereas precipitation determines the quantity and quality of larval habitats and therefore adult population size. Epidemiologically, mosquito population size and reproductive rate are important components of vectorial capacity that determine the frequency of host contact, the rate of pathogen transmission, and therefore the risk of human infection (Smith 1987). Climate variation affects all of these processes at multiple scales. Seasonal cycles create periods of the year with favorable conditions for population reproduction and growth, whereas variation among years determines interannual changes in population size. Interannual patterns of climate have been linked to changes in sea surface temperatures and storm tracks, which, in turn, have been linked to various aspects of human health (Epstein 2002), including patterns in the occurrence of vector-borne disease outbreaks (Gubler *et al.* 2001).

Mosquitoes have a four-stage life cycle (egg, larva, pupa, and adult) which take place in the aquatic and terrestrial environments (Figure 6). Eggs, larvae and pupae need aquatic environment. Adults then complete their life cycle in the terrestrial environment (Jarratt *et al.* 2004).

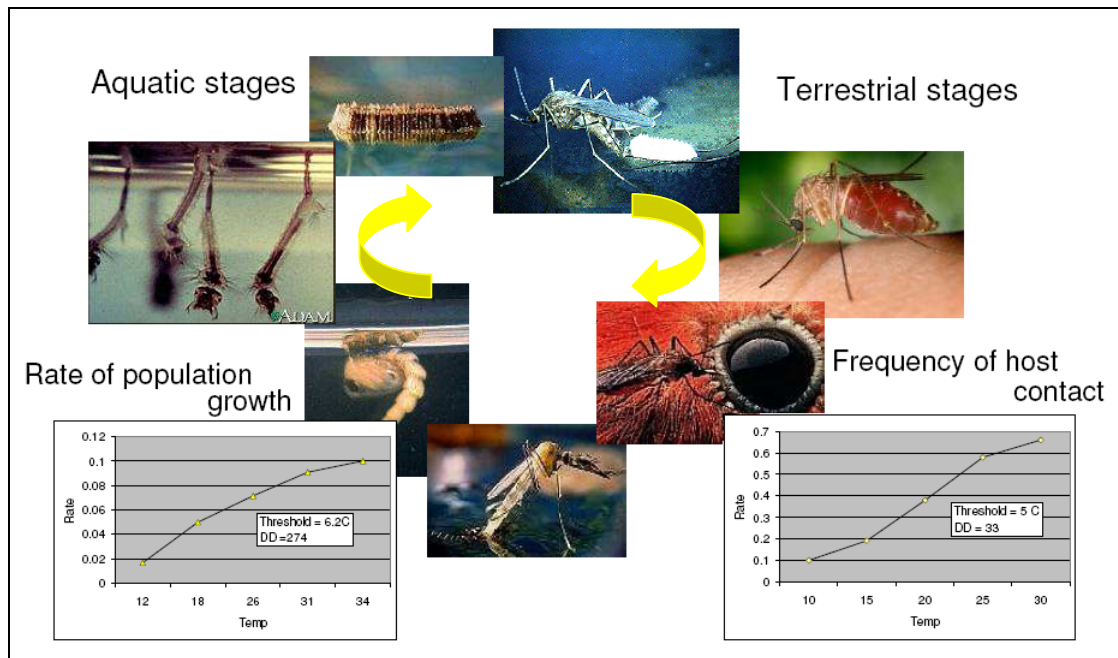


Figure 6. Effects of climatic parameters on mosquito life cycle (Reisen 1995).

Besides intrinsic factors such as physiology and innate behavioral traits, that govern infection of mosquitoes and their ability to transmit the virus, extrinsic factors such as temperature and rainfall can affect virus transmission and its epidemiology. Temperature is important because it governs the rate at which mosquitoes develop into adults, the survival rate of adult mosquitoes, and the incubation time of virus in the mosquito. Temperature is the most important and easily controlled extrinsic factor affecting growth rates of larvae and larvae-pupa ecdysis. There is a specific temperature range for each mosquito species to develop its life cycle. The time between ingestion of an infectious blood meal until a mosquito is capable of transmitting the virus is the extrinsic incubation period (EIP); and the necessary temperature for the pathogens to develop to an infectious stage within the mosquito is called extrinsic incubation temperature (EIT). (Jarratt *et al.* 2004, Richards *et al.* 2007). Experimental studies on WNV indicated that a decrease in temperature from 26°C to 18°C during a 17-day post-infection period caused a decrease in transmission rates from 97% to 48% (Savage *et al.* 1999). The virus was recovered from most studied mosquitoes held exclusively at 26°C, and not from the mosquitoes held at lower temperature (Dohm & Turell 2001). Also, the virus was recovered from most mosquitoes (*Cx. pipiens*) held at 30°C (as

early as 4 days after the infectious blood meal), while disseminated infections were not detected for mosquitoes held at 18°C, until 25 days (Dohm *et al.* 2002).

Humidity is another factor, which has a direct effect on the survival of mosquitoes. Survival rate might be reduced when hot weather is accompanied by low humidity (Reiter 2001). Humidity also affects the risk of exposure to vectors. Usually people wear less clothes in warm and humid climates, and prefer to work and rest in open areas. Therefore, they are more exposed to mosquito bites. On the other hand, due to the abundance of insects they might use more bed-nets or other protective methods, which decrease the risk of effective exposure (Thomson *et al.* 1994). Rain provides the breeding sites for mosquitoes and helps create a humid environment, which prolongs the life of vectors. On the other hand, heavy rains may have a flushing effect, cleansing breeding sites of their mosquitoes (Reiter 2001).

4.2. Indirect impact through terrestrial parameters

The climatic factors have also an effect on environment of vertebrate hosts and mosquitoes. Climate is one of the main determinants of geographic range for many bird species. Distribution, migration activity and arrival times of birds at breeding sites can be influenced by climate changes. Cooler weather can delay bird migration activity and arrival times at breeding sites (Hughes 2000).

Several studies have used environmental and climatic data to assess the risk of WN disease. A study has evaluated environmental factors such as elevation range, physiographic region, and percentage of vegetation cover to determine human WNV risk (Ruiz *et al.* 2004). Another study based on dead bird reports and several environmental variables such as road density, stream density, slope, soil permeability, vegetation, and climatic factors, showed that WNV risk was associated with high road density, low stream density, and gentle slopes (Cooke *et al.* 2006). Gibbs *et al.* demonstrated that temperature, housing density, urban/suburban land use, and physiographic region are important variables associated with the geographic distributions of WNV in Georgia (Gibbs *et al.* 2006b).

These factors include elevation, hydrology, land cover and soil type which most related to the formation and growth of vector breeding habitats and to vertebrate host population sites and their interactions. A study, conducted in Chicago, evaluated the environmental and social determinants of human health risk during the WNV epidemic of 2002. The evaluated risk factors were the presence of vegetation, age, income, race of human population, distance to a

WNV positive bird, age of housing, presence or absence of mosquito abatement, and geologic features. The most notable effect was relevant to mosquito abatement efforts (Ruiz *et al.* 2004).

In Indiana, WNV data in a population of horses were used for clusters in time and space. The authors concluded that there is a significant relationship between normalized difference vegetation index (NDVI) and equine WNV cases. It seems that NDVI may be useful for identifying foci of WNV transmission (Ward *et al.* 2005).

Chlorophyll (green biomass) or density and growth power of green plant can be analyzed by satellite data. Green biomass varies with photosynthetic activities, primary production networks and the vegetative period. Vegetation quality is related to the climatic variations such as increase or decrease of precipitation, temperature and sunshine level.

Plants respond quickly to climate variations. Density and development of vegetation can be changed by climate which can be observed after many years (Stow *et al.* 2004).

NDVI is a simple numerical indicator that can be used to analyze remote sensing measurements and assess vegetation density and whether the target being observed contains live green vegetation or not. It is calculated as a normalized ratio of the red and near infrared wavelengths (Brown *et al.* 2008).

Some researchers believe that finding a WNV positive bird can be a risk factor to indicate that human cases will occur in the site. (Guptill *et al.* 2003). A multi-year analysis study in Connecticut, USA, indicated that considering both environmental variables and animal sentinel data could be useful for predicting human infection. In the same study population density, growing degree-days, temperature, WNV positive mosquitoes, dead birds, and WNV positive birds were significant predictors of human infection risk (Liu *et al.* 2009).

Remotely sensed landscape information was used to determine environmental risk factors with WN disease in horses in Camargue, France. The study indicated that rice fields, dry bushes, and open water were the important risk factors that were associated with the presence of the cases (Leblond *et al.* 2007b). Shaman and *et al.* in a study conducted in Colorado from 2002 to 2007, indicated the relation between hydrologic variability and the incidence of human WN cases. Wetter spring and drier summer conditions in Colorado's eastern plains could predict human WN cases. While this prediction in western parts with drier spring and summer is weaker (Shaman *et al.* 2010).

Abstract:

- WNV is maintained in an enzootic bird-mosquito-bird cycle. However, epidemics/epizootics usually occur after the virus is introduced into the human and animal population by a bridge vector.

- Several strains of the virus with different virulence have been reported from all continents. Phylogenetic analysis showed that the isolated strain in USA was identical to the isolated strain in the Middle East (Israel).

- Migratory birds have a critical role in the virus dissemination in new areas, so that co-circulation of different strains in the same region can be related to starting point location and stopovers habitats where the birds encounter during their flyways.

- Besides the existence of the competence vector and hosts, environmental and climate factors, such as temperature, precipitation, and vegetation, play an important role in the circulation of the virus.

- The environmental factors may divide to: (i) atmospheric parameters such as, temperature and (ii) terrestrial parameters, such as topography. These factors have direct effects (on virus/hosts/vectors) and indirect effects (on the habitats).

- Environmental and climatic factors effect on the mosquito life cycle, host population, virus propagation, as well as on their interaction.

Chapter 2.

***WEST NILE VIRUS
CIRCULATION IN EQUINE
POPULATIONS***

1. Introduction

A recent molecular and serological study was conducted to determine WNV antibody and genome in different species of wild water birds in Iran. Samples were collected from 26 different species (n=519) during 2003-2007, 78 (15%) of which were serologically positive, while no WNV viral RNA-positive samples were found in this study. Most of positive samples were included in common coot (*Fulica atra*) samples (Fereidouni *et al.* 2011). Another study was carried out from January 2008 to June 2009 among patients with fever and loss of consciousness attending the emergency department in Isfahan. Among 632 patients attending the emergency, 249 samples were tested for WNV. Three (1.2%) were positive for both serum and CSF by RT-PCR and six (2.4%) were positive only for IgG antibodies (Chinikar *et al.* 2011).

The objective of this part is the assessment of the circulation of WNV in the equine population in different regions in Iran. This is the first large-scale epidemiological study in Iran, and the first study among equine population in the country (Appendix A). As equines are the most sensitive hosts, next to humans, and their sampling is more feasible than other hosts and vectors, this study focused on this host population involved in the virus circulation.

The aim of this part was to answer the following questions:

- 1) have the equine population contacted with the WNV during their lifespan?
- 2) if so, how much is the prevalence of the seropositivity among the population?
- 3) what are the risk factors for WNV infection among equine population?
- 3) What is the geographic distribution of WNV infected animals?
- 5) when is the probable time of their contact with the virus?

2. Material and Methods

Overall, we can divide equine population in the country into two groups. One group being kept in the clubs including horses; the other group, including horses, donkeys and mules, is kept in the villages. According to the previous reported seropositivity percentages in Iran (10-28%) and by considering a seroprevalence of 15% and precision of 3%, the minimal number of animals to be sampled to discern WNV infection in an infinite population is 545

for each group. 1107 blood samples were collected, but only 1054 samples were tested due to insufficient quantity of serum in 53 samples.

2.1 Study area

The study was carried out in the Islamic Republic of Iran, a country located at the eastern most edge of the Middle East. Iran is representing a total area of 1,640,195 km². It is located between 25°03'-39°47' N and 44°14'-63°20' E and is bordered by the Gulf of Oman, the Persian Gulf, and the Caspian sea. WNV was never reported in horses in the country before.

2.2 Target and study population

The surveys on the equine population were conducted in two hundred and sixty communities located within 27 provinces in the country (Figure 7). All horses were healthy at the time of serum collection.

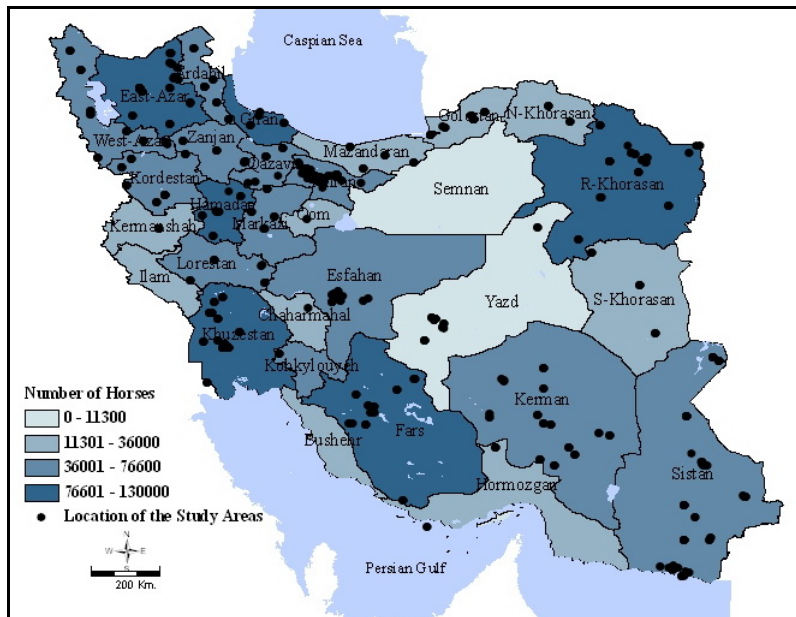


Figure 7. Geographic distribution of equine population by province and geographic location of the collected samples, Iran, 2008-2009.

Throughout a 20-week serosurvey, from september 2008 to February 2009, blood samples were obtained from 1054 equines (844 horses, 9 mules, 201 donkeys). For each animal, a form was completed by a veterinarian. Along with other data (date of sampling, name of the veterinarian and of the animal's keeper), this form mentioned the breed of the animal, its age and sex, location, and the group size, i.e. the number of equines at the same place (Appendix B).

2.3 Treatment of samples

Blood collected from each of the equines' jugular vein were centrifuged at 2000 rpm for 10 minutes. Sera were refrigerated at 4°C until they were transported on ice to the Veterinary Organization. Once samples arrived, they were stored at -20 °C.

2.4 Serological analyses

All equine sera were screened for WNV antibodies with a commercial competition enzyme linked immunosorbent assay (ELISA) test (ID Screen West Nile Competition; ID VET, France) according to the manufacturer's instructions. The reactive sera were confirmed by seroneutralization in 6- well plates. Also the positive sera by Competition ELISA were analyzed for IgM antibodies against WNV.

2.4.1 Competition ELISA. In this method, specific antibody in the sera compete with an enzyme-labelled antibody for binding to the antigen. This method is used for a first preliminary screening of the samples. The kit (ID Screen West Nile Competition; ID VET, France) used in this study is designed to detect horse antibodies directed against WNV envelope protein (pr-E). The microwells were commercially coated with a purified extract of WNV. The samples and the commercial controls are added to the wells. The anti-pr-E antibodies, if present, form an antigen-antibody complex. An anti-pr-E antibody peroxidase conjugate is added to the wells to fix the remaining free pr-E epitopes, forming an antigen-conjugate-peroxidase complex. After washing in order to eliminate the excess conjugate, the Substrate Solution is added. After adding the substrate, it is converted to a colored product by

the enzyme bound to the antibody. The color intensity is measured with a microplate reader and is inversely proportional to the concentration of antibody in the sample. The resulting coloration depends on the quantity of specific antibodies present in the sample to be tested. In the absence of antibodies, a blue solution appears which becomes yellow after addition of the Stop Solution. However, in the presence of antibodies, no coloration appears (Figure 8, Appendix C). This test was done in the laboratory of Iran Veterinary Organization. The kit controls source is not mentioned, but it could be speculated that they are animal sera containing WNV antibody (positive control) and without the antibody (negative control).

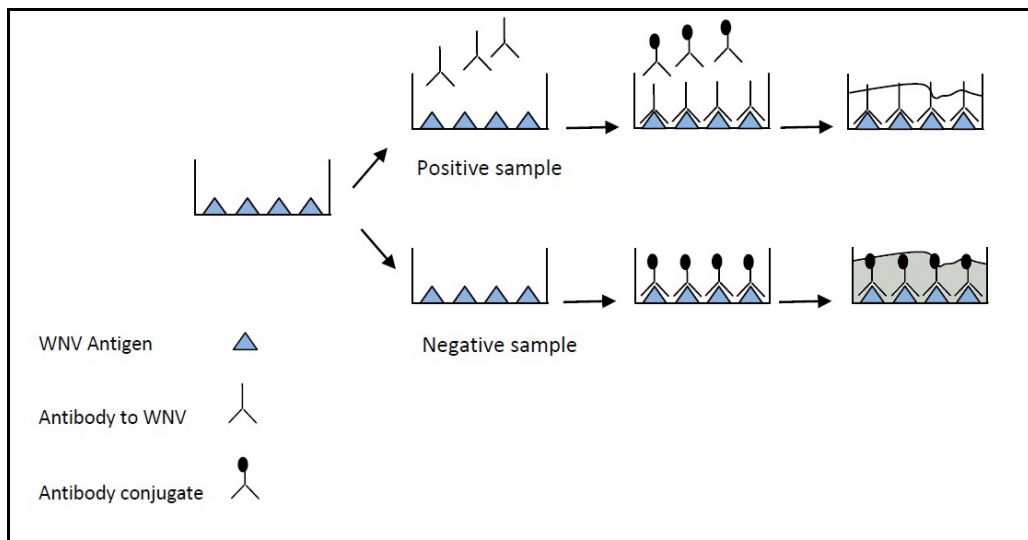


Figure 8. Principle of Competitive ELISA method.

The test was valid if $OD_{NC} > 0.7$ and $OD_{PC} / OD_{NC} < 0.3$. For each sample S/N was calculated which is as follows: $S/N = \frac{OD \text{ sample}}{OD \text{ NC}} \times 100$.

If $S/N \leq 50\%$, the sample was considered positive and if $S/N > 50\%$, the sample was considered negative.

2.4.2 IgM-capture ELISA. This method was used for identification of specific IgM antibody indicating a recent circulation of the virus among the animals. The analysis method is derived from the IgM-capture ELISA method described in the Manual of Diagnostic Tests

and Vaccines for Terrestrial Animals (World Organization for Animal Health, OIE) (OIE, 2008) and was performed as already described in (Murgue *et al.* 2001b). In this method, class M immunoglobulins (IgM), which may be present in the sample, are "trapped" with a specific antiserum (produced on goat).

Briefly, the steps are as follows:

(1) 96-well plates were coated overnight at 4°C with 200ng/well of goat anti-horse IgM antibody (KPL: ref 01-21-03);

(2) The sera found positive by competition ELISA were added into the microtiter plate wells at a 1:100 final dilution in blocking buffer (see Appendix D);

(3) After 1-hour incubation at 37°C and 5 washes with PBST, 2 groups of cells were used: cell culture infected (positive antigen) and not (negative antigen) by West Nile virus. The antigen obtained from infected and uninfected VERO E6 cell cultures (obtained from the French Reference Center for arboviral infections) was added respectively into the test and control wells (positive and negative controls) at a 1:20 dilution and incubated for 3 hours at 37°C;

(4) After 5 washes with PBST, a hyper-immune ascite prepared on mice and containing antibodies directed against WNV (obtained from the French National Reference Center for arboviral infections), diluted 1:2500 in blocking solution, was added into each well and incubated for 1 hour at 37°C;

(5) After five washes, a goat anti-mouse serum conjugated to horseradish peroxidase (Sigma, ref A0168, 0.8mg/mL) was added at a dilution of 1:2000, and incubated for an additional hour at 37°C;

(6) After 5 more washes, 100 µl of chromogenic substrate was added into each well;

(7) The reaction was stopped after 10 min by adding a 10.6% phosphoric acid solution.

Plates were read on an ELISA plate reader at the 450nm wavelength.

The test was considered valid if and only if the following criteria were met:

- OD C (-) / Ag (+) ≤ 0.400
 - OD C (-) / Ag (-) ≤ 0.400
 - OD C (+) 1 / Ag (+) ≥ 0.700
 - OD C (+) 2 / Ag (+) ≥ 0.700
 - OD samples / Ag (-) ≤ 0.400
- } Two positive controls

Then the following ratios were calculated for each sample:

$$R1 = \frac{OD \text{ sample} / Ag(+)}{OD \text{ sample} / Ag(-)}, \quad R2 = \frac{OD \text{ sample} / Ag(+)}{OD C(-) / Ag(+)}$$

If $R1 \geq 2$ and $R2 \geq 2$, sample was considered IgM positive, if $R1 < 2$ and $R2 < 2$, sample was considered IgM negative and if $R1 \geq 2$ and $R2 < 2$, sample was considered doubtful.

2.4.3 Plaque reduction neutralization test (PRNT). PRNT is considered the standard test for confirmation of virus-specific antibodies and is used to quantify the titer of neutralizing antibody for a virus. Because of cross-reactivity of WNV with other members of Japanese encephalitis serocomplex, which can confuse the diagnosis, PRNT is the most specific test and can be used to differentiate WNV infection from other *flavivirus* infections.

The principle of the seroneutralization is to inhibit the cytopathic effect of the virus by specific antibodies. Cytopathic effect is revealed by putting the virus in contact with cells: after several days, the contaminated cells are lysed. On the other hand, in the presence of specific antibodies, the virus cannot infect cells and the cell line remains undamaged. A known volume of virus suspension is inoculated into cell culture and overlaid with an agar or cellulose overlay. Because the spread of the virus is restricted by the overlay, each viral particle grows and forms an area of necrotic plaque. This plaque represents one viral unit and its progeny. The state of the cell line is revealed by using a coloring agent, purple crystal, which color undamaged cells. But, spaces on plate remain colorless; indicating lysis of infected cells by the virus (Adu 2011).

This method is used as a confirmatory test for WNV antibodies by the CDC and several other laboratories. It detects neutralizing antibodies in either serum or cerebral spinal fluid (CSF). The method used in this study was observation of lysis plaques in 6-well plates. If specific West Nile virus antibodies are in the tested serum at the evaluated dilution, they will neutralize viral particles and protect the cells and there are no cytopathic effects.

In this study, PRNT₉₀ was performed to assess the presence of neutralizing antibodies specifically directed against WNV in the positive sera by competitive ELISA method. The method is derived from the OIE PRNT₉₀ method (OIE. 2008) (Appendix E). This method was

conducted in the BSL-3 facilities at Agence Nationale de Sécurité Sanitaire de l'Alimentation, de l'Environnement et du Travail (ANSES).

2.5 Statistical analyses

All the statistical analyses were conducted using R 2.10.1 (R 2009). Some values were missing in questionnaires for sex, breed, age and group size. After having verified that the pattern of missing data was compatible with random missing (Rubin 1987), missing data were imputed using the covariates as predictors (northing, easting, elevation, sex, breed, age, group size and type of stable). Twenty datasets were thus generated using the `aregImpute` function of package `Hmisc` (Horton & Kleinman 2007). All the statistical analyses described thereafter were separately conducted for each of these 20 datasets, results being combined as described elsewhere (Rubin 1987). A logistic model was used to analyze the relation between seropositivity and the covariates. Northing, easting, the corresponding quadratic terms and the northing-easting interaction were included in the model as a proxy for horses' locations. Other covariates were elevation (in meters), breed (grouped in four categories: mixed, thoroughbred, Arab, and other), age (numeric value), sex (male or female), group size (number of animals) and the type of stables (village or club). Performance of the model as a classifier was assessed using a ROC (receiver operating characteristic) analysis and computing the area under the ROC curve (AUC). A cross validation (Stone 1974) was conducted: half of the tested horses were randomly chosen for fitting the model, which was then used to predict the serological status of the remaining animals. Prediction error was evaluated as the usual proportion of incorrect predictions.

3. Results

Of the 1054 sampled animals, 279 were positive by competitive ELISA and 249 (23.7%, 95% confidence interval [CI]: 21% - 26%) were confirmed positive for WNV infection by PRNT. Three sera could not be tested by PRNT because of insufficient sample volume. Seropositive equines were observed in all of the provinces but one, where only 2 animals had been tested (Table 1). Seroprevalence appeared highly heterogeneous and ranged

from 1% to 88%. The most affected provinces were located in the western and southern parts of the country, with seroprevalence rates of >30% (Figure 9). Most of the animals possessing WNV neutralizing antibodies had low PRNT titers as only 73 had a titer >90, corresponding to 29.3% of tested animals (95% CI: 23.6-35%). The majority of these animals were located in provinces with seroprevalence rates >30%. IgM ELISA was performed on 277 competitive ELISA positive sera. Two sera were not tested by IgM ELISA because of insufficient sample volume. Nine sera were positive for IgM antibody. The corresponding four provinces had a PRNT seroprevalence rate >30% (Table 1, Figure 10).

Table 1. Anti-WNV seroprevalence determined by PRNT in equines, Iran, 2008-2009.

Province	PRNT result ^a	Prevalence (95% CI)	Predicted prevalence ^b	IgM	Titer ^c >90
Ardabil	1/32	0.03 (0.00-0.16)	0.07	0	0
Charmahal Bakhtiari	2/2	1.00 (0.16-1.00)	0.21	0	0
East Azarbaijan	7/64	0.11 (0.05-0.21)	0.08	0	1
Fars	16/42	0.38 (0.24-0.54)	0.52	2	5
Gilan	4/16	0.25 (0.07-0.52)	0.11	0	0
Golestan	1/60	0.02 (0.00-0.09)	0.01	0	1
Hamedan	3/23	0.13 (0.03-0.34)	0.14	0	0
Hormozgan	4/8	0.50 (0.16-0.84)	0.70	0	0
Isfahan	7/37	0.19 (0.08-0.35)	0.17	0	0
Kerman	30/62	0.48 (0.35-0.61)	0.43	0	13
Kermanshah	4/5	0.80 (0.28-0.99)	0.41	0	0
Khuzestan	68/77	0.88 (0.79-0.95)	0.79	5	31
Kohgiluyeh-B.	3/5	0.60 (0.15-0.95)	0.66	0	0
Kordestan	6/38	0.16 (0.06-0.31)	0.19	0	1
Lorestan	6/13	0.46 (0.19-0.75)	0.49	1	5
Markazi	2/20	0.10 (0.01-0.32)	0.17	0	2
Mazandaran	1/21	0.05 (0.00-0.24)	0.07	0	0
North Khorasan	1/10	0.10 (0.00-0.45)	0.04	0	0
Qazvin	1/20	0.05 (0.00-0.25)	0.07	0	1
Qom	0/2	0.00 (0.00-0.84)	0.13	0	0

Razavi Khorasan	5/65	0.08 (0.03-0.17)	0.04	0	1
Sistan Baluchestan	55/97	0.57 (0.46-0.67)	0.59	1	5
South Khorasan	3/21	0.14 (0.03-0.36)	0.11	0	0
Tehran	3/205	0.01 (0.00-0.04)	0.03	0	2
West Azarbaijan	6/43	0.14 (0.05-0.28)	0.21	0	1
Yazd	9/46	0.20 (0.09-0.24)	0.20	0	3
Zanjan	1/20	0.05 (0.00-0.25)	0.06	0	1
Total	249/1054	0.24 (0.21-0.26)	0.24	9	73

^a Positive/tested. ^b Average seropositivity probability predicted by the logistic model. ^c PRNT titer.

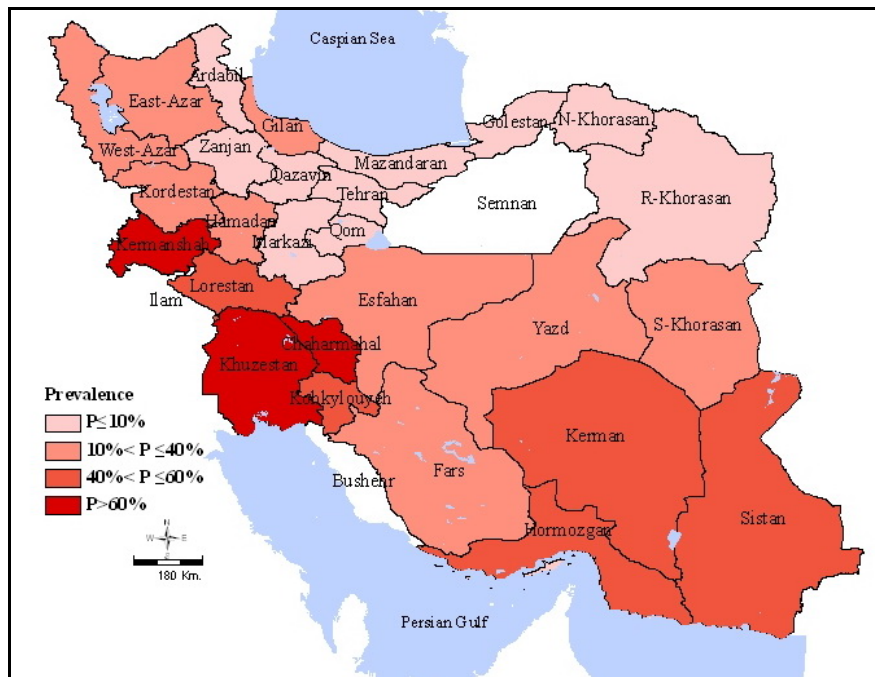


Figure 9. Anti-WNV seroprevalence determined by PRNT in equines, Iran, 2008-2009.

Logistic regression model showed an association between elevation and seropositivity, with a 60% decrease of seropositivity odds when elevation is increased by 1000 m (Table 2). The Arab breed was a seropositivity risk factor, as well as being housed in a village stable (reference: club stable). Seropositivity risk also increased with the age of animals, a difference of 5 years corresponding to a change of seropositivity odds by a factor of 1.3.



Figure 10. Distribution of IgM positive animals in Iran, 2008-2009.

No significant association was observed between sex and seropositivity, nor between group size and seropositivity (Table 2). The ROC analysis resulted in an AUC of 0.887, indicating a relatively good discrimination ability of the model. AUC ranged from 0.884 to 0.889 according to the imputed dataset ($n=20$). Cross-validation showed an individual prediction error of 13.5%: for 86.5% of animals, the predicted PRNT status was thus correct. This prediction error ranged between 12.2% and 14.5% according to the imputed dataset ($n=20$).

Table 2 shows model fit at the province level: predicted seroprevalence rates appeared close to the observed values and were always inside the confidence interval of these observed values.

According to the logistic model, the most exposed animals were mares of the Arab breed, housed in village stables.

No significant association was observed between animal type and seropositivity, while a significant difference was found in the serologic status by geographic distribution. Geographic coordinates had a high effect on seropositivity (Table 3).

Table 2. Logistic model of anti-WNV seropositivity determined by PRNT in equines, Iran, 2008-2009.

Variable	Value	OR (95% CI)	p value
Northing	$\Delta=0.5$ degree	0.37 (0.17-0.80)	0.005 ^a , 0.02 ^b
Easting	$\Delta=0.5$ degree	0.54 (0.32-0.90)	0.008 ^a , 0.001 ^b
Northing-Easting	$\Delta=0.5$ degree	1.22 (1.06-1.39)	0.02
Elevation	$\Delta=1000$ m.	0.60 (0.43-0.84)	0.04
Sex	Female	Reference	
	Male	0.90 (0.61-1.36)	0.32
Breed	Mixed	Reference	
	Arab	3.22 (1.50-6.89)	0.001
	Thoroughbred	1.24 (0.37-4.12)	0.36
	Other	1.50 (0.66-3.38)	0.16
Type of stable	Club	Reference	
	Village	2.18 (1.28-3.73)	0.002
Age	$\Delta=5$ years	1.34 (1.04-1.71)	0.01
Group size	$\Delta=30$ animals	0.80 (0.60-1.06)	0.06

^a Absolute value, ^b Quadratic term

Table 3. Effect of animal kind and geographical coordinates on West Nile virus seropositivity, Iran, 2008-2009.

Variable	Value	OR (95% CI)	p value
Northing	$\Delta=0.5$ degree	0.03 (0.03-0.91)	0.04
Easting	$\Delta=0.5$ degree	0.02 (0.05-0.46)	0.001
Northing-Easting	$\Delta=0.5$ degree	1.03 (1.00-1.03)	0.05
Elevation	$\Delta=1000$ m.	1.00 (0.46-0.89)	0.007
Animal kind	Horse	Reference	0.42
	Donkey	1.40 (0.84-2.34)	0.2
	Mule	1.38 (0.25-7.53)	0.71

4. Discussion

The results of this study, the first large-scale WNV serosurvey in equines conducted in Iran, show that antibody response to WNV is widespread in this country as seropositive animals were found in 26 of 27 provinces. The overall serological prevalence rate of WNV antibodies is high (24%) but varies widely according to the geographic location, southern and western parts of the country being the most affected areas. Nine provinces had a prevalence of $\geq 30\%$, five provinces a prevalence of $\geq 50\%$, and two provinces a prevalence of $\geq 80\%$. We can analyze a double gradient of prevalence between north and south, and between east and west. The huge parts of the provinces with high prevalence are located in a dry area (south) rather than a rainy area (north). A low prevalence was observed in Tehran province (1%). These horses may profit from better management practices, which could reduce their exposure to infectious vectors. These might include health programs and regular cleansing for both animal and environment. Most of these animals are horse racing and there is high probability that they have been infected during their travel to other places. This effect might not be the case in view of the fact that competitions are usually organized in this province.

Logistic modeling confirmed that geographic location was the main seropositivity risk factor. Predicted seroprevalence rates appeared close to the observed values and were always inside the confidence interval of the observed values. Furthermore, seropositivity risk decreased with increasing elevation: this association can be explained by variations in the abundance of the vectors. Seroprevalence also varied with the equines' individual characteristics. Horses had a higher risk for WNV seropositivity when they belonged to Arab breeds. This may be linked with the geographic location of these horses, as they are mainly found in the south- western part of the country. The increase in seroprevalence rate with equine age suggests recurrent circulation of WNV, as described in some serosurveys performed in endemic areas (Cohen *et al.* 1999, Chevalier *et al.* 2006). Considering the fact that seropositive animals were more than one year old, seropositivity may not be related to maternal antibodies.

Besides individual characteristics, seroprevalence varied according to the conditions in which animals were managed. For example, horses housed in village stables appeared to be more exposed to WNV than those in club stables. The number of IgM-positive sera observed

in the present study (n=9) could have been underestimated as only sera positive by competitive ELISA were screened for IgM. The corresponding bias was probably minor as IgG and IgM appear roughly simultaneously after infection (Bunning *et al.* 2002, Castillo-Olivares & Wood 2004). Despite the small number, the positive IgM results revealed a pattern of viral circulation. The corresponding animals were sampled between September 2008 and January 2009 and could thus have been infected from late summer to autumn 2008. Similar observations were made in Europe and the Mediterranean area, with recurring outbreaks in these seasons when the mosquito population density was highest (Tsai *et al.* 1998, Murgue *et al.* 2001b, Trock *et al.* 2001). The latest IgM-positive serum was obtained at the end of January 2009, suggesting that the animal was infected during the last days of autumn or during winter. This animal was located in a desert area, Sistan-Baluchestan province. Considering the mild winter in this province, it would not be surprising to detect active infected mosquitoes at this time of the year.

A few sera positive by ELISA were negative for neutralizing antibodies against WNV. The lower analytical sensitivity of the PRNT test can account for this finding. This was unlikely to be due to assay cross-reactivity as no other related flaviviruses have been reported in Iran. Also, because of no report of other flaviviruses in Iran, they have not been included for seroneutralization test. Although WN infection in horses and humans often causes either no clinical disease or a mild one, but it is not clear if there are any clinical cases in the country or not, sign the potential cases could be concealed because of similarity of this disease with some other diseases.

Circulation and persistence of viruses in the ecosystem depends on environmental, as well as biological factors. WNV is maintained in the nature through a transmission cycle between vector mosquitoes and reservoir birds (Komar 2005, Balenghien *et al.* 2006, Jourdain *et al.* 2007). The present study shows that WNV prevalence is higher in the south rather than the north of Iran. It seems that Khuzestan province has more favorable conditions for propagation and circulation of the virus than the other provinces. Khuzestan has the most important wetlands which host unique flora and fauna. The importance of migratory birds as virus carriers and transfer hosts between different areas has been previously reported (Reed *et al.* 2003, Hubalek 2004). Iran, because of its geographical situation, is located at the crossroads of bird migratory routes, with birds coming from WNV endemic regions (Africa, Europe, and India). Although pathways of migratory birds in Iran are not well documented, it

is specified that Iran supports nearly two fifths of the important wetlands of the Middle East, with many of them corresponding to Important Bird Areas (IBAs). The Iranian wetlands represent wintering areas for millions of migratory waterfowls coming from Siberia, Africa, India and south Asia, which may introduce different strains of WNV encountered elsewhere. The birds from Siberia and northern Europe migrate in winter to the northern parts of the country, around Caspian Sea and some of these species fly to warmer climates in the south of Iran. Phylogenetic studies indicate that different lineages of WNV circulate in different parts of the world; consequently, importation of different strains of the virus by migratory birds is presumably the case.

Vectors also play an important role in the transmission of the virus, and prevalence rate of antibodies can be affected by existence of competent vectors, density of infectious vectors, and their distance to a proper host. Existence of competent vectors and their density, of amplifying hosts and environmental conditions besides the species of migratory birds are the factors influencing distribution of the virus in different districts of the country (Azari-Hamidian 2007). The hot tropical climate of the southern coastal lowlands supports a flora and fauna quite unique compared to those of the rest of Iran. Southwest and southeast areas support fauna which is characteristic of the Afro-tropical and Indo-Malayan subtropical climates, respectively (UNDP. 2003 July).

Since the first reported serological evidence of WNV circulation in the 1950s, the Middle East has been of interest for renewed studies about WNV circulation, especially when it was shown that there was a genetic similarity between the New York strain isolated in 1999 and a strain isolated in the Middle East (Lanciotti *et al.* 2002). Co-circulation of different lineages has been previously reported in the Middle East (Briese *et al.* 2002). However, substantiating the circulation of different lineages in Iran needs further study. Efforts are needed to isolate viruses, to identify vectors and vertebrate hosts, to assess the risk of WNV transmission by migratory birds to free ecosystems and to determine the environmental indicators involved in WNV transmission in Iran.

Abstract:

- The first large scale serosurvey for WNV in the equine population in Iran was conducted, 2008-2009. The samples collected from 260 districts located in 27 provinces.

- WNV seroprevalence was highly heterogeneous in Iran and ranged from 1% to 88%. The overall seroprevalence rate for WNV neutralizing antibodies was 23.7%.

- A double gradient of prevalence has been revealed between north and south, and between east and west. The huge part of the provinces with high prevalence is located in a dry area (south) rather than a rainy area (north).

- The animals housed in village stables appeared to have been more exposed to WNV than those in club stables.

- The presence of IgM-positive animals (n=9) indicated a recent circulation of WNV in several provinces.

- A large circulation of WNV in Iran particularly in south-western provinces where the virus probably circulates every year.

- Co-circulation of different WNV strains in Iran can be speculated and needs further study.

- Although the study present new information on the epidemiology of WNV, but gaps in the circulation knowledge of the virus in Iran still exist. More studies are needed to isolate viruses, to identify competent vectors and vertebrate hosts for WNV in Iran.

Chapter 3.

***IMPACT OF ENVIRONMENTAL
AND CLIMATIC FACTORS ON
WEST NILE CIRCULATION***

1. Introduction

The spatial distribution of most vector-borne pathogens is determined by the distribution and contact between vectors and vertebrate hosts, which, in turn, are influenced by environmental conditions. "The influence of climate and the environment on infectious diseases has been a subject of debate, speculation, and serious study for centuries" (Shope 1991). To understand how climatic and environmental parameters might affect the incidence of WNV, we should consider factors (virus, vector, and host) involved in the infection and the environmental-climatic parameters associated with their life and proliferation.

Therefore, the objectives of this chapter were to determine

- (1) to what extent climatic factors play a role in the WNV prevalence in Iran?
- (2) how does proximity of wetlands influences the risk of WNV infection?
- (3) what is the effect of the Normalized Difference Vegetation Index (NDVI) on the WNV circulation risk?

2. Material and Methods

2.1 Study area

Iran has arid and semiarid climates mostly characterized by low rainfall rate and high potential evapotranspiration. The complex physical conditions of Iran including topography, and vegetation cover have created a diverse climate pattern. Furthermore, the climate is influenced by Caspian Sea in north, coastal areas in south of the country, Mediterranean area and Red sea. Alborz and Zagros mountains also play an important role in determining the non-uniform spatial and temporal distribution of precipitation in the whole country.

Temperature in Iran is an intense function of altitude, latitude and moisture content of the atmosphere, with altitude having a more substantial effect than latitude on temperature. Humidity prevents temperature fluctuations in the north and south of the country. Thermal regime in Iran shows that January with an average temperature of 6°C and July by 29°C are the coldest and the warmest months, respectively (Alijani 1995, Masoudian & Kaviani 2007).

The average annual temperature in Iran is 18 degrees Celsius, and it gets warmer from west to east and from north to south. The effect of latitude is ten times more than that of longitude on temperature differences between places. Increase of temperature from west to east is due to concentration of the mountains at western part of the country while this increase from north to south is because of approaching the equator and increasing of the solar radiation angle.

The rainy period in most of the country is from November to May followed by a dry period between May and October with rare precipitation. The average annual rainfall of the country is about 240 mm with maximum amounts in the north of Iran. Oman coast has warmest climate and is the most humid region in Iran. Four ecological zones with specific plant richness (Hircanian, Khalij-o-Omani, Zagross and Iran-o-Touranian zones) have been established on the basis of environmental factors in Iran (Heshmati 2007) (Figure 11).

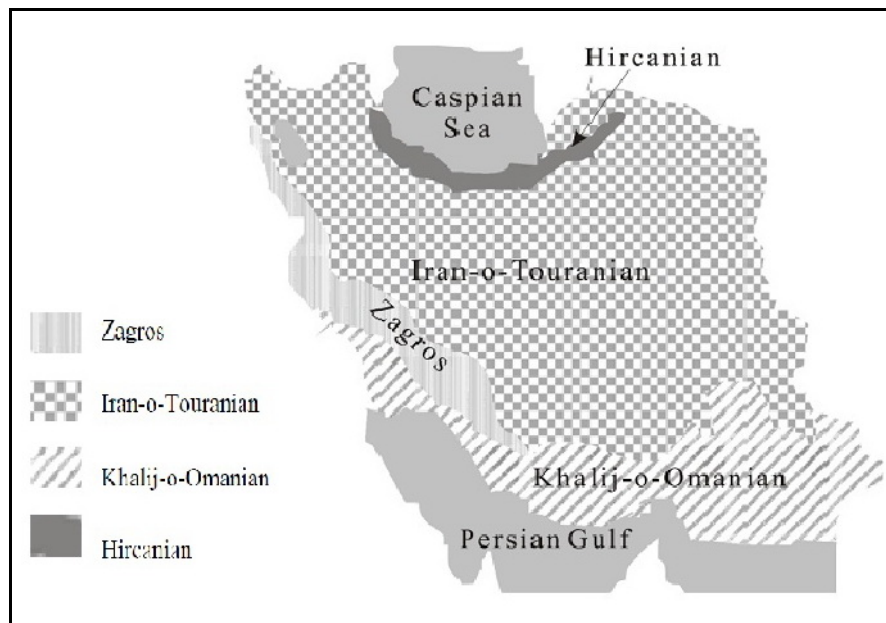


Figure 11. Distribution of four ecological zones of Iran (Heshmati 2007).

Even though the country is dominated by arid and semi-arid regions (southwest coast, central and eastern parts) it possesses a large number and wide variety of wetlands. Wetlands have an important role in the survival of countless species of plants and animals. They support high concentrations of vertebrates (such as birds) and invertebrate species. The

importance of wetlands resulted in formation of the first international contract in the environmental field. The Ramsar Convention was the first treaty about the protection of environment and wetlands, which internationally are important. This treaty began in Ramsar city in Iran, in February 1971, and internationally important wetlands were called Ramsar sites.

Iran supports 63 wetlands, of which 22 were identified as Ramsar sites, approximately 1327920 hectares area. These Ramsar sites constitute 79% of Middle East Ramsar sites and 1.2% of all such sites in the world (Frazier & Stevenson 1999, Unknown 2003). Several millions waterfowl and shore birds utilize these wetlands as feeding and roosting areas.

2.2 Environmental Factors

This section includes the description of the environmental factors associated with WNV infection and seropositivity in equine population.

2.2.1 Climatic factors

2.2.1.1 Data collection

(I) Annual Mean Temperature – As the data of recent years was not available, mean monthly temperature data sets of a 10-year period (1996-2005) was used for analysis between WNV seroprevalence and mean monthly temperature. These data were obtained from I.R of Iran Meteorological Organization (IRIMO). Average temperatures are given in degree Celsius.

(II) Annual Precipitation- The data range and their source were the same as that of annual mean temperature. Precipitation is given in millimeter.

(III) Annual Mean Relative Humidity- The data range and their source were the same as that of mean monthly temperature. Relative humidity is expressed as a percentage.

2.2.1.2 Method of analysis of climatic data

1- Numerical layers of sampling places and meteo-stations were inputted into ArcMap 9.3 software.

2- Coordinate systems of data layers [Datum: D_WGS_1984 and GCS (Geographic Coordinate Systems)] were defined.

3- The stations and their nearest sampling places were defined by Thiessen method. The Thiessen method was used for creating polygons to define geographical influence of each meteorological station. All points of sampling location contained within each polygon are closer to the meteorological station in the same polygon than they are to any other meteorological station (Figure 12).

For studying climatic parameters for the mentioned periods, 103 stations were used for the analyses.

4- Climate data for each stable were organized as monthly (12 months) by Excel software and then yearly data were calculated.

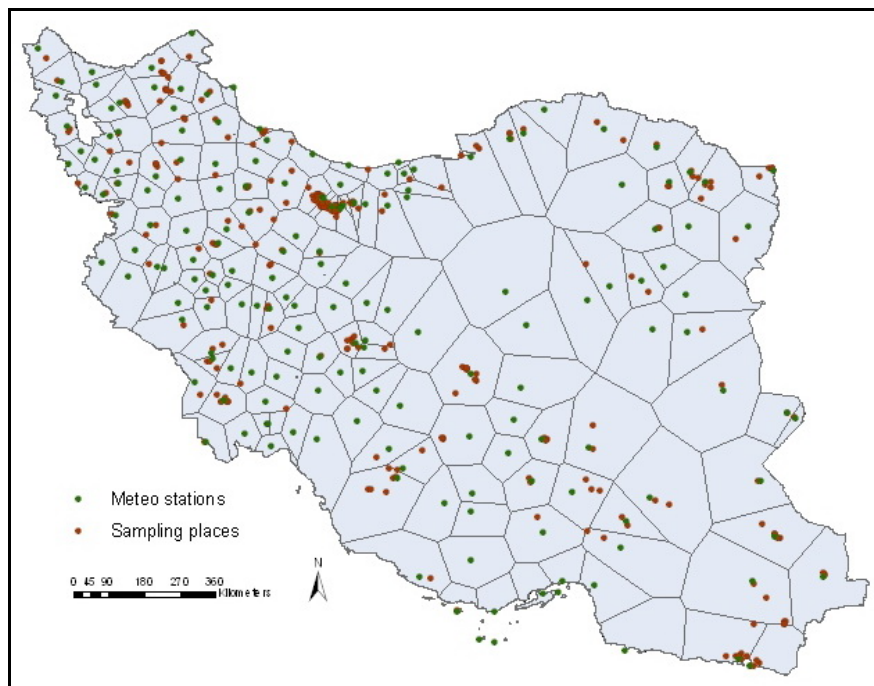


Figure 12. Thiessen polygons analysis.

2.2.2 NDVI

Green plants absorb more solar radiation in the visible electromagnetic spectrum than the near infrared (Figure 13). This unique spectral response of vegetation makes it possible to differentiate vegetation from other surface materials remotely.

The NDVI ratio is calculated by dividing the difference in the near infrared (NIR) and red bands by the sum of the NIR and red bands for each pixel in the image as follows:

$$NDVI = \frac{(NIR - RED)}{(NIR + RED)}$$

NDVI varies between -1.0 and +1.0. Negative values generally represent water body, snow and other non-vegetated surfaces, while positive values represent vegetated surfaces. The NDVI increases as the quality of green biomass increases.

NDVI for green vegetation ranges from 0.1 to 0.8, and is less for plants under stress. NOAA (National Oceanic and Atmospheric Administration) and other organizations have used NDVI as an index for monitoring vegetation health. For instance, NDVI with a value ~0.6, ~0.24 and ~0.09 shows dense, medium and thinning vegetation, respectively. The value ~0.025 and 0.002 shows denuded land and cloud, respectively.

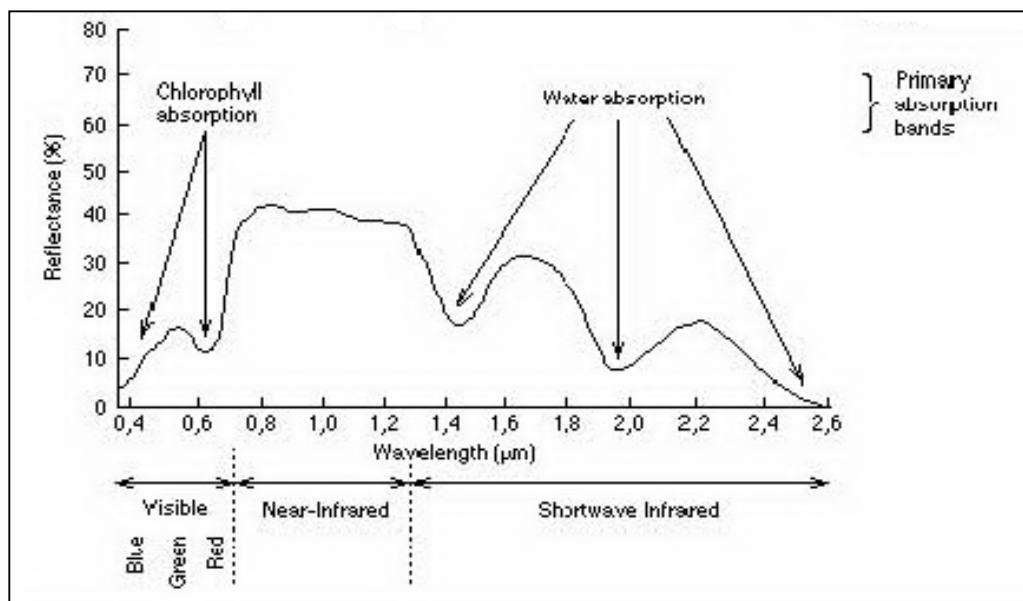


Figure 13. Spectral reflectance of green vegetation (Rahman 2004).

However, clouds can block satellite observations, and numerous studies have explored methods to yield a “cloud free” NDVI, as would be measured at the ground surface. Maximum-value composite images from temporal satellite data have been used to remove errors caused by clouds and other atmospheric perturbations (Holben 1986). This compositing process provides the most cloud-free image possible.

2.2.2.1 Collection and analysis of data

This study used seasonal NDVI images derived from NOAA-18 data. AVHRR (Advanced Very High Resolution Radiometer) is a sensor on board of this satellite which has been sent into space by the National Oceanic and Atmospheric Administration (NOAA).

The NDVI Composites from NOAA/AVHRR data is one of the important data sources for assessing seasonal variations in vegetation condition and changes in earth ecosystem.

The images have been extracted from <http://www.class.ngdc.noaa.gov> based on AVHRR level 1B and resolution of 1.1 km. The images were taken from January 2007 to December 2008 and their selection was according to minimum cloudy.

The collected satellite images were processed as follow (Figure 14):

1- Geometric correction of satellite images. Satellite images cannot be directly combined with other geographic information systems; also the results cannot be directly included into geographic databases and map systems. Geometric correction is necessary to bring the images from ground or slant range geometry into a map reference. In other words, it is the transformation of image data to the scale and projection properties of a map.

For geometric correction, 255 ground control points located in the header files [with an error less than 0.1 pixel (1.1 km)] and ENVI 4.3 software were used

2- Geographic coordinate system Definition. Outputted images were assigned with geographic coordinate systems and Datum: D_WGS_1984 by using ArcMap version 9.3 software.

3- Calculation of NDVI values. By using ERDAS 8.7 software, NDVI values were obtained from channels 1 and 2 NOAA-AVHRR.

4- Scaling of NDVI values to a 8-bit format. As the resulted images had a short range (-1 to +1) NDVI numbers were converted from their original format into the 8 bit format

in order to make it more suitable for analysis with personal computers by a range from 0 to 255.

5- Extraction of monthly maximum NDVI value (January-December). By ERDAS 8.7 software, maximum-value composite images from monthly NDVI have been used to remove errors caused by clouds (Holben 1986). This compositing process provides the most cloud-free image possible.

6- Buffers of 5 and 5-10 kilometers around each sampled point were created with the GIS software and mean NDVI was extracted within each circular buffer. These values were exported into a dbase IV table for further analysis.

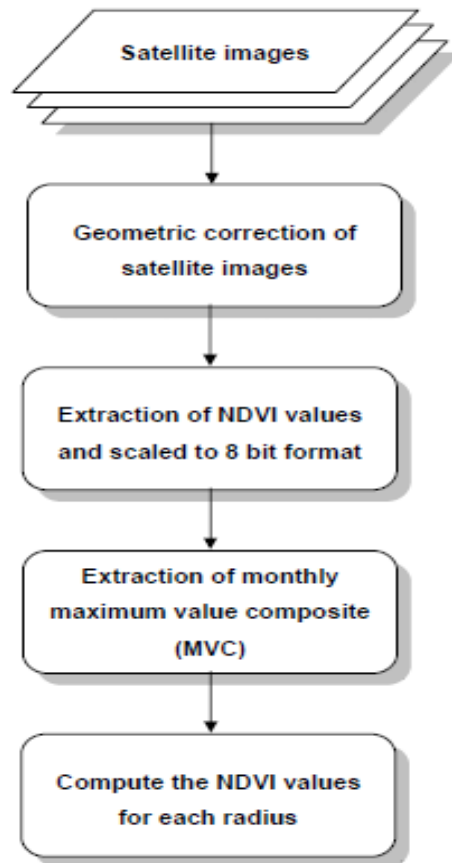


Figure 14. Flowchart of NOAA/AVHRR data processing and calculation of NDVI values for sampling places.

2.2.3) Distance to the nearest wetland area

Another possible environmental risk factor for equines infected with West Nile Virus is proximity to wetlands, which are the most important sites for wild birds, especially migratory birds. The data on wetlands were acquired from Iran Environment protection organization. For this study, we collected the data from 60 important wetlands areas, of which 22 are Ramsar sites (Figure 15). To define proximity to the wetlands, the shape files of wetlands and sampling sites of animals were imported into ArcMap version 9.3. Using the ‘Euclidean Distance’ function, from the spatial analyst extension, nearest wetland and its distance to each stable was calculated.

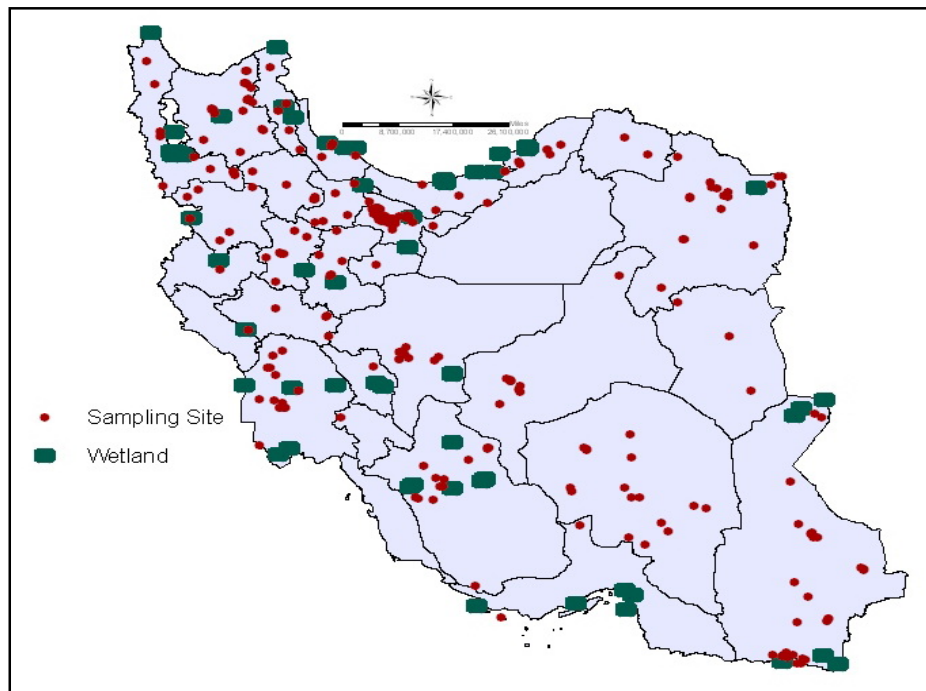


Figure 15. Important studied wetlands and sampling sites.

2.2.4) Type of stable

Since the previous study has shown a high substantial effect of the type of stables (farm or club) on the seropositivity (Ahmadnejad *et al.* 2011), we included this variable in the present analysis.

2.3 Statistical Analysis

Univariate analyses were performed in order to test the relationship between the outcome variable and each explanatory variable. Finally, logistic regression analyses were performed in order to identify which variables were involved in the stables' seropositivity.

Explanatory variables used in logistic regression modeling included annual average temperature, relative humidity and precipitation, distance to the nearest wetland , annual average NDVI, Normalized seasonal differences of NDVI, normalized local differences of NDVI and the type of stable (farm or club) (Table 4). Accuracy (percent of testing sites correctly classified), sensitivity (percent of positive testing sites correctly classified), and specificity (percent of negative testing sites correctly classified) were computed for the model. In addition, the area under the receiver operating characteristic curve (AUC ROC) was calculated as index of the fit of the model.

All the statistical analyses were conducted using R 2.10.1 [18], and maps were produced with a Geographic Information System (ESRI1, ArcGISTM 9.0).

Table 4. Risk factors of West Nile virus infection included for statistical analysis.

Annual mean temperature	Annual mean precipitation	Annual mean humidity	Distance to wetlands
Annual mean NDVI	Seasonal NDVI differences	Local NDVI differences	Type of stable

3. Results

The stables with at least one positive animal were considered as positive stable and 108 of 260 stables (41.5%) were positive for WNV infection (Figure 16). In the training set, on univariate analysis, all the variables were associated with the presence of WNV antibody ($p < 0.05$).

The average minimum and maximum annual temperatures were 5°C and 27.7°C, respectively. Annual mean temperatures for infected and uninfected places were 19.97°C (95% CI 18.97-20.97) and 15.58 (95% CI 15.04-16.11), respectively (Table 5).

Table 5. Statistical results for mean climatic factors in WNV infected and un-infected stables

	Infected stables	Un-infected stables	P value
Annual Mean Temperature (°C)	19.97	15.58	<0.0001
Annual Precipitation (MM.)	258.74	299.18	0.07
Annual Mean Humidity (%)	44.57	47.94	0.46

The average minimum and maximum annual humidity were 25.6% and 83.8%, respectively. Annual mean humidity for infected places was 44.57% (95% CI 42.07- 47.06) and 47.94% (95% CI 45.98- 49.89) for uninfected places. The average minimum and maximum annual precipitation were 50.8 mm and 1759.1 mm, respectively. Annual mean precipitation for infected places was 258.74mm (95% CI 210.58- 306.91), and 299.18mm (95% CI 266.83- 331.53) for uninfected places (Table 5).

The odds ratio and 95% confidence intervals were calculated for seropositivity and all risk factors. Multivariate logistic regression analyses revealed a strong relation between WNV antibody prevalence and annual mean temperature, distance to the nearest wetland, seasonal NDVI differences and Local NDVI differences variables (Table 6).

Table 6. Multivariate logistic model of the presence of anti-WNV seropositive animals in Iranian stables, 2008-2009.

Variable		Odds-ratio (95% CI)	p
Type of stable (village of club)		NS ¹	0.07
Annual mean temperature	$\Delta=5^{\circ}\text{C}$	2.03 (1.10-3.92)	0.03
Annual mean %humidity²	<38%	Reference	
	38-46%	NS	0.12
	46-52%	NS	0.27
	>52%	NS	0.34
Annual mean precipitation²	<154 mm	Reference	
	154-247 mm	NS	0.84
	247-327 mm	NS	0.67
	>327 mm	NS	0.71
Distance to the nearest wetland area	$\Delta=10$ km	0.73 (0.54-0.99)	0.05
Annual mean NDVI		NS	0.77

Seasonal NDVI differences³	$\Delta_{\text{spring-summer}} > 0$	2.37 (1.07-5.41)	0.04
	$\Delta_{\text{summer-autumn}} > 0$	NS	0.96
	$\Delta_{\text{autumn-winter}} > 0$	NS	0.38
	$\Delta_{\text{winter-spring}} > 0$	NS	0.92
Local NDVI differences⁴	spring > 0	4.26 (1.07-17.98)	0.04
	summer > 0	NS	0.11
	autumn > 0	NS	0.35
	winter > 0	NS	0.23

¹NS=Non- significant

²Classes bounds based on distribution quantiles

³Normalized differences of average NDVI for successive seasons. For two successive seasons S1 and S2 and for stable S, value is:

$$(\text{NDVI}_{S,S2} - \text{NDVI}_{S,S1}) - (1/N) \Sigma(\text{NDVI}_{x,S2} - \text{NDVI}_{x,S1})$$

where $\text{NDVI}_{x,y}$ is the average NDVI value observed in a 5km radius area around stable x during season y , and N is the number of stables.

⁴Normalized differences of average NDVI between two areas: a 5 km radius area centred on the stable and the 5-10 km surrounding area. For stable x and season y , value is:

$$(\text{NDVI}_{x,y,0-5\text{km}} - \text{NDVI}_{x,y,5-10\text{km}}) - (1/N) \Sigma(\text{NDVI}_{x,*,0-5\text{km}} - \text{NDVI}_{x,*,5-10\text{km}})$$

where $\text{NDVI}_{x,y,z}$ is the average NDVI value observed around stable x during season y in area z , and N is the number of stables.

The risk of being WNV seropositive was higher for stables located in the areas with higher temperature, as a 5°C increase in mean annual temperature was associated with a statistically significant higher prevalence of WNV infection. Figure 17 shows the aggregation of infected stables within the area with higher temperature.

There was a significant negative correlation between distance to the nearest wetland and seropositivity of stables. The risk of being WNV seropositive for stables increased (27%) with 10km decrease of distance from the nearest wetland area (Figure 18). No significant association was observed between type of stable, annual mean humidity, precipitation, annual NDVI and seropositivity (Figure 19, Figure 20, Figure 21).

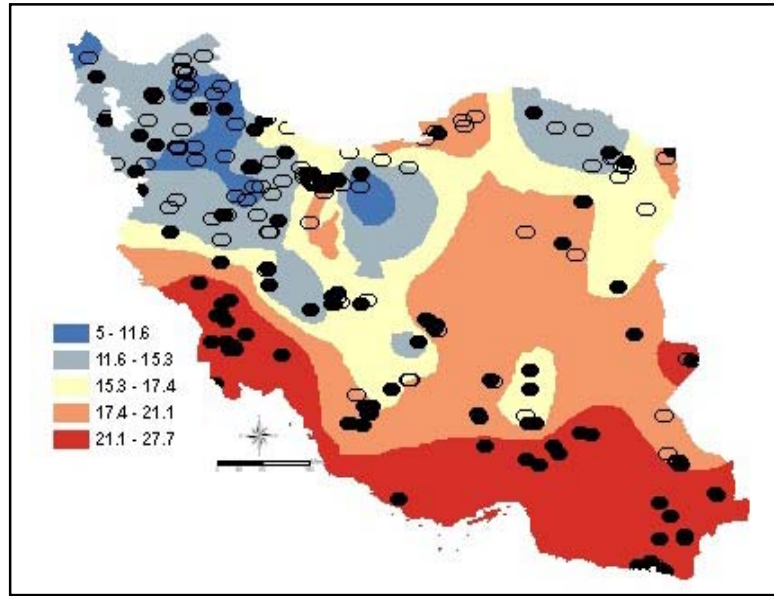


Figure 16. Annual mean temperature and geographical distribution of WNV infected (bold oval) and uninfected (un-bold oval) stables in Iran.

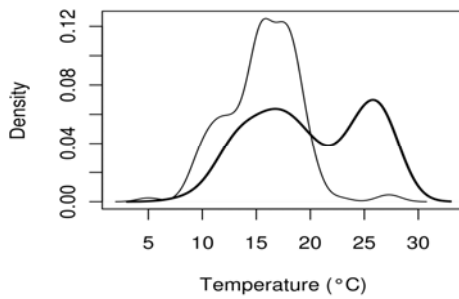


Figure 17. Distribution of positive stables (thick line) and of negative stables (thin line) according to the annual average temperature.

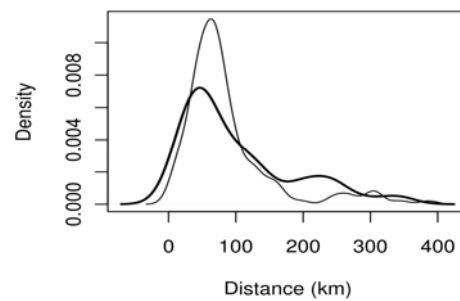


Figure 18. Distribution of positive stables (thick line) and of negative stables (thin line) according to the distance to the nearest wetland.

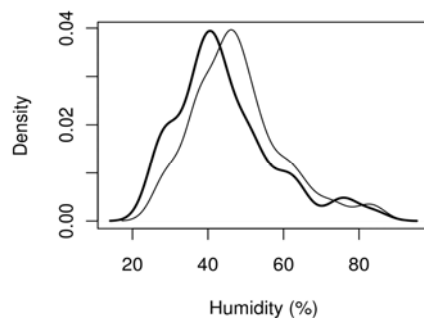


Figure 19. Distribution of positive stables (thick line) and of negative stables (thin line) according to the annual average humidity.

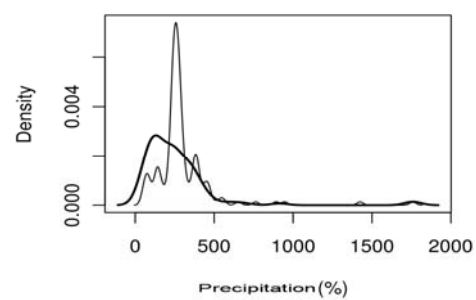


Figure 20. Distribution of positive stables (thick line) and of negative stables (thin line) according to the annual average precipitation.

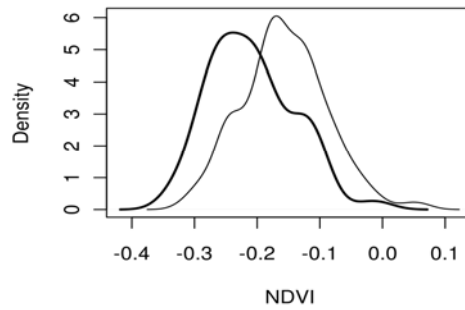


Figure 21. Distribution of positive stables (thick line) and of negative stables(thin line) according to the annual average NDVI.

Figure 22 and show the annual mean NDVI in spring and the distribution of the stables according to the normalized local NDVI differences.

A logistic regression model showed an association between local and seasonal NDVI differences and seropositivity. The difference of NDVI between two areas, a 5 km radius area centered on the stable and the 5-10 km surrounding area, for infected stables was four times higher that of uninfected stables in spring (Figure 23).

A higher significant difference between NDVI of spring and summer within a 5km radius area around the stable increases the seropositivity by two times (Figure 24).

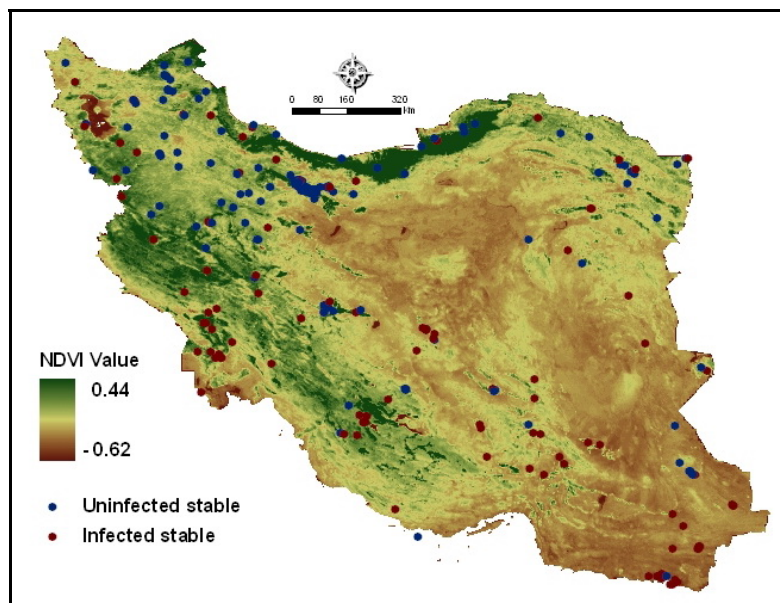


Figure 22. Annual mean NDVI in Spring in Iran, and the geographic distribution of WNV infected (triangle) and uninfected (stars) stables.

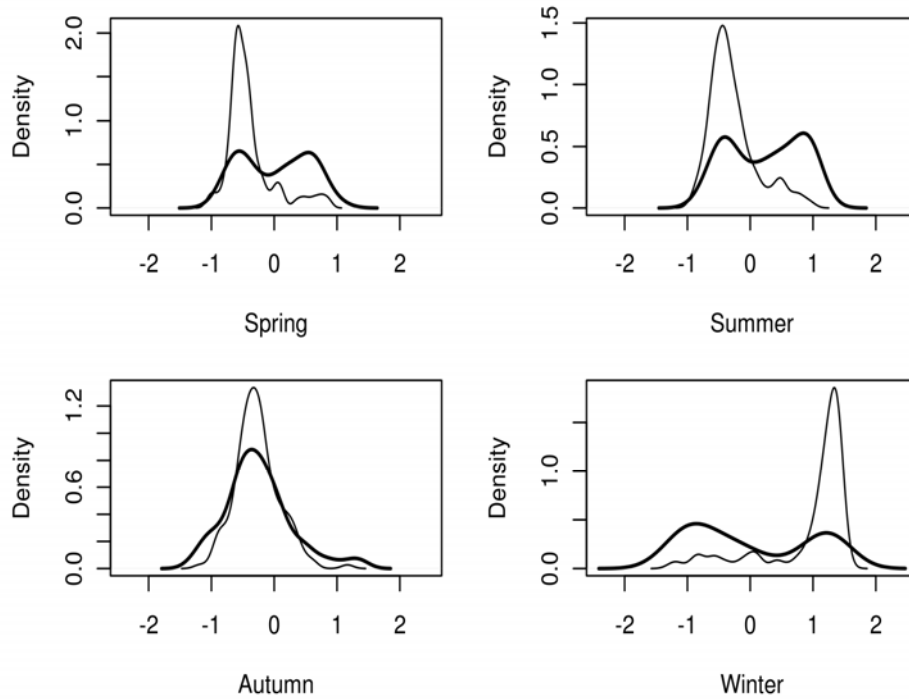


Figure 23. Distribution of positive stables (thick line) and of negative stables (thin line) according to the normalized local NDVI differences.

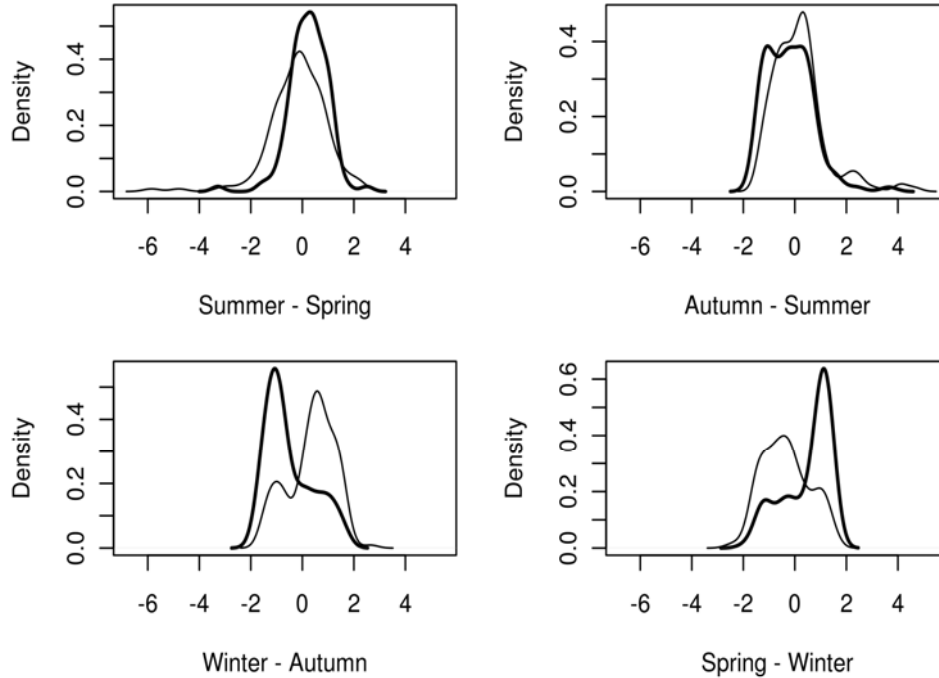


Figure 24. Distribution of positive stables (thick line) and of negative stables (thin line) according to the normalized seasonal NDVI differences.

The ROC analysis resulted in an AUC of 0.838, indicating that the model had relatively good discrimination ability. The model computes probabilities for a cell to have a high WNV circulation status. Computing sensitivity and specificity of this prediction for various probability cut-offs clearly showed that at a decision threshold of 0.4. The model had a sensitivity of 75% and a specificity of 83.6%. Corresponding positive and negative predictive values were 76.4% and 82.5%, respectively (Figure 25).

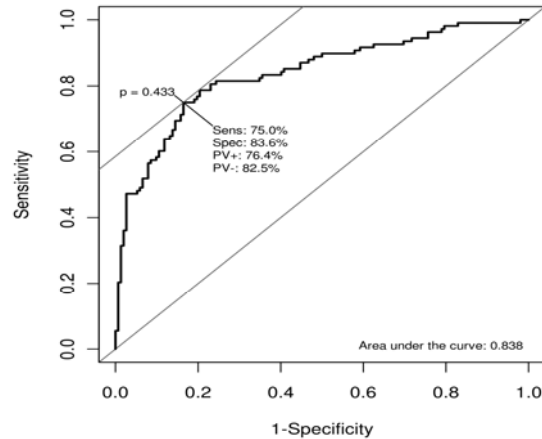


Figure 25. Receiver operating curve of the multivariate logistic model of the presence of anti-WNV seropositive animals in Iranian stables, 2008-2009.

Figure 26 shows the corresponding predicted seropositive map with a higher positivity rate in south and southwestern parts of the country, which has a similar distribution to our finding.

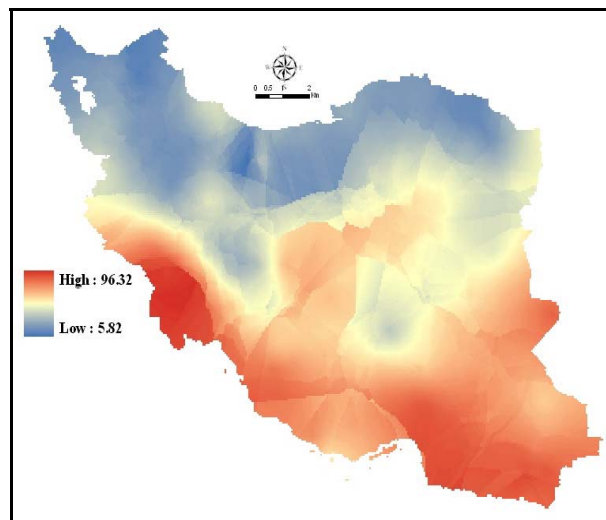


Figure 26. Predicted risk areas of WNV infections in equines, Iran.

4. Discussion

The evidence presented here reveals the geographic factors associated with WNV circulation in Iran, which is reported for the first time in the country. Four of eight studied factors (temperature, distance from wetlands, local and regional differences NDVI) were correlated to the WNV infection of the equines.

Positive anomalies of the temperature in some southwestern provinces appear to have facilitated the mosquito abundance and, consequently, WNV infection in the equines. We observe a north-south gradient of seropositivity following the gradient of annual average temperature. Annual mean temperatures above 22°C were notable in all, except three, of the stables were seropositive. These results are consistent with previous observations made in Egypt (Taylor *et al.* 1956). In southern provinces of the country, where the climate is arid, the infection reaches a higher level. In northern provinces, the situation is quite different: warm, wet summers are common, but droughts occasionally occur in late summer and early fall; and outbreaks of WNV can occur at that time. Temperature in Iran gets warmer from west to east and from north to south. Increase of temperature from west to east is due to concentration of the mountains at the western parts of the country while increase from north to south is because of approaching the equator and increasing of the solar radiation angle. Overall, northern and mountainous parts of the country have higher annual fluctuation and southern areas have relative stability (Alijani 1995).

WNV is endemic in people living in the semi arid regions, where the weather is hot and dry for most of the year. Cases have been regularly reported in sub-desertic areas around the Mediterranean basin: (i) along the Nile Valley (Egypt, 1950; 1994) (ii) along the Syrian-African Rift (Israel, 1951, 1998-2000; Jordan, 2003); (iii) in the Timimoun oasis in the Central Sahara (Algeria, 1994) (Taylor *et al.* 1956, Murgue *et al.* 2001a). The warmer conditions of these areas, along with direct effects that they have on vector and host, cause the standing water with high rich organic materials to attract the mosquitoes and birds, consequently increase the interaction and circulation of WNV between them (Epstein & Defiippo 2001).

Precipitation and humidity could have a major influence on the distribution of mosquito species (Teng & Apperson 2000). Humidity increases vector flight and host-seeking behaviors, and precipitation is necessary for the formation of mosquito breeding habitats

(Shaman *et al.* 2002). However, we could not find a significant relation between WNV infection and annual mean humidity/rainfall. Evidences on relationship between WNV/mosquito abundance, precipitation, and humidity are contradicting, some indicate a weak, some a strong correlations (Landesman *et al.* 2007, DeGroot *et al.* 2008, Soverow *et al.* 2009, Wan Norafikah *et al.* 2009).

Seropositivity was negatively correlated to the distance from wetlands, so being adjacent to wetlands can increase infection of the animals by the virus. This is consistent with the previous studies that showed WNV infection is more strictly linked to wetlands with abundant bird populations, especially migratory birds. Wetlands provide suitable breeding habitats for mosquitoes and birds, and are considered as natural foci of WNV infections in Palearctic. The outbreaks in temperate areas, especially in Europe, have occurred in or near wetlands (Hubalek & Halouzka 1999a, Jourdain *et al.* 2007). In 1998, WNV was isolated from horses suffering from neurologic disease and residing in a large wetland area in Italy. In addition, proximity of wetlands was identified as a risk factor for West Nile virus (WNV) encephalomyelitis in horses living in Indiana (Autorino *et al.* 2002, Moore *et al.* 2003). Moreover, proximity to wetland increase mosquito abundance, as first three stages of their life are aquatic dependent (Tachiiri *et al.* 2006).

The strongest association was detected between seropositivity and NDVI, at seasonal and, especially, local differences. While such relation could not be found with annual mean value of NDVI. The local NDVI's contrast during spring is a major risk factor of the transmission of WNV in Iran. During the early drought season, the stable's seropositivity is increasing with the contrast between the local and regional NDVI environment of the stable. This so-called oasis effect is associated with the attractiveness of the local NDVI environment for WNV's vectors (mosquitoes and birds). This oasis effect disappears in autumn, and the local NDVI's contrast becomes protector (negative impact). This oasis effect is consistent with the seasonal production of vegetation in spring, which is more important compared to the national average (Contrast spring- summer). "Oasis" is a Greek word for watered green fertile land in the desert. These areas have some special characteristics, which make them different from surrounding environment. Influence of vegetation on climatic parameters is widely reported. There is a decrease in temperature in an isolated moisture and vegetated source surrounded by an arid area, which occurs because of a combination of reduced radiation by shading and the evaporation cooling effect. Oasis is a

small rich habitat inserted in the arid regions and attractive habitat for birds, both residents and migrants (Rodriguez-Estrella 2005, Potchter *et al.* 2008).

Droughts and heat wave were found to induce WNV amplification by bringing the hosts and the vectors together. In contrast with the dogma that increasing precipitation predicts mosquito abundance, some authors hypothesize that wild populations should generate outbreaks under drought conditions. Standing water pools become richer in organic materials that *Culex* vectors needs to thrive and the mosquito predators, such as amphibians and dragonflies, are fewer in number (Chase & Knight 2003, Epstein 2004). These findings are consistent with the hypothesis that spring = nestling (in an "oasis", the birds will nest closer to the horses) and autumn = dispersal of juveniles (juveniles are going further if they leave from an "oasis"). These findings are also consistent with the fact that viral circulation is related to the presence of chicks, sedentary and still bare skin in altricial species (Marra *et al.* 2004).

High values of NDVI are related to higher photosynthetic activity and improved ecological condition (Gordo 2007). This suitable condition increases the main food supply, such as arthropods, for most migratory birds. Several studies have used NDVI for assessments of WNV infection foci and its vector habitat. Seasonal differences in NDVI were one of the best predictive factors for determining mosquito distribution abundance in the model studied by Jacob *et al.* (Brownstein *et al.* 2002, Jacob *et al.* 2009). Ward *et al.* investigated association between NDVI and cases of equine WNV encephalomyelitis. They found out that the mean NDVI in biweekly periods with reported cases was significantly higher than the mean NDVI in periods without cases (Ward 2009).

Further studies are needed for better understanding of the ecology of WNV in Iran. The reservoir hosts and vectors are the keys of circulation of the virus and will be the focus of the future work.

Abstract:

- The spatial distribution of most vector-borne pathogens is determined by the distribution and contact between vectors and vertebrate hosts, which, in turn, are influenced by environmental conditions. The objective of this chapter was to determine environmental and climatic factors associated with the risk of WNV equine seropositivity in Iran.

- The studied risk factors were annual mean temperature, precipitation, humidity, type of stable, annual mean Normalized Difference Vegetation Index (NDVI), distance to wetlands, local and seasonal NDVI differences.

- Annual mean temperature, distance to wetlands, local and seasonal NDVI differences were correlated with the WNV infection risk to the equine.

- The effect of local NDVI differences in spring was particularly notable. This was a normalized difference of average NDVI between two areas: a 5 km radius area centred on the stable and the 5-10 km surrounding area

- The model indicated that local NDVI's contrast during spring is a major risk factor of the transmission of West-Nile virus in Iran. This so-called oasis effect consistent with the seasonal production of vegetation in spring, and is associated to the attractiveness of the local NDVI environment for WNV's vectors (mosquitoes and birds).

- The oasis effect of the southwestern provinces of Iran encourages birds to congregate around shrinking water sites and will nest closer to the horses, encouraging viral circulation among hosts and mosquitoes.

GENERAL DISCUSSION

Iran was included in the list of affected countries with WNV by the results of first studies carried out on some healthy human population sera in 1970s. After that, the virus and its effects were neglected in the country. Detrimental effects in Europe, and especially in the US, directed attentions toward the virus, its capabilities and the appropriate ecological conditions. The goals of this research was: (i) developing a better understanding of the virus circulation; and (ii) determining the ecological factors involved in WNV spread in the country and to model how these factors influence its maintenance and transmission.

1. Spatial heterogeneity of the seroprevalence in equines

We found WNV infection among 26 of 27 studied provinces of Iran, which is the second largest country in the Middle East with 30 provinces. These results show an introduction of the virus into different ecological zones. Seropositivity has been found highly heterogeneous, some places with a high circulation of WNV and the others with a low level of circulation. This is as consistent with the fact that Iran is a mountainous country. A large massif, the Zagros mountains runs from the northwest of the country southwards first, to the shores of the Persian Gulf, and then continues eastwards, till the most south-eastern province (Figure 27). Another massif, the Alborz mountain range from sea level to 2800 m altitude, and run from the northwest to the east along the southern edge of the Caspian Sea. Finally, there are several scattered mountain chains along the eastern frontier of the country. We found a low level of circulation, in provinces with mountainous and cold weather. However, the seropositivity is highly heterogeneous.

The distribution of seroprevalence in equine populations is consistent with the spatio-temporal variability of the climate of the Islamic Republic of Iran, characterized by great extremes due to its geographic location and varied topography (Figure 28). Iran is located at high latitudes rendering vast differences between seasons in the country. The summer is extremely hot with temperatures in the interior; over 55 °C has been recorded in some places. The great altitude of much of the country and its continental situation result in far lower temperatures in winter.

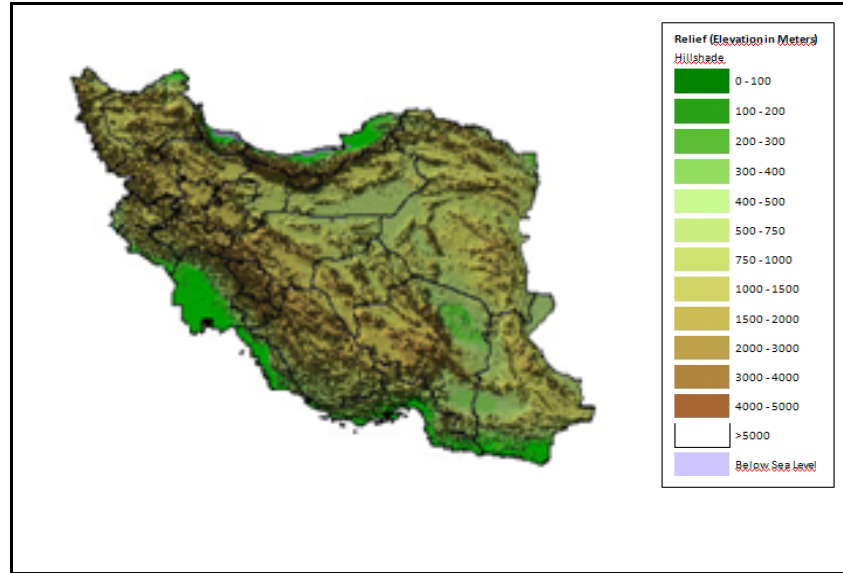


Figure 27. Elevation in Iran(<http://www.fao.org/countryprofiles/Maps/IRN/02/lg/index.html>).

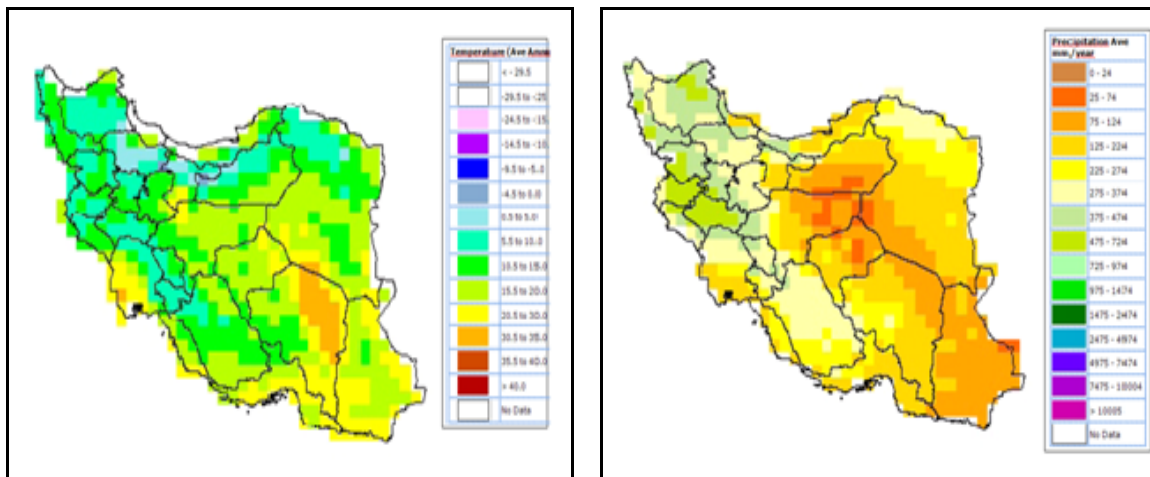


Figure 28. Annual Temperature (left) and Precipitation in Iran (right) (<http://www.fao.org/countryprofiles/maps.asp?iso3=IRN&lang=en>).

Temperatures are colder from west to east and warmer from north to south. Decrease of temperature from west to east is due to concentration of the mountains at western parts of the country while increase from north to south is because of approaching the equator and increasing of the solar radiation angle. Overall, northern and mountainous parts of the country have higher annual fluctuating and southern areas have relative stability (Alijani 1995). Minus Temperatures of $-30\text{ }^{\circ}\text{C}$ can be recorded in the northwest and $-20\text{ }^{\circ}\text{C}$ is common in many places. The average annual rainfall is 228 mm and approximately 90

percent of the country is arid or semi-arid. About 23 percent of the rain falls in spring, 4 percent in summer, 23 percent in autumn and 50 percent in winter.

The high level of seropositivity found in Persian Gulf lowlands, and especially in southwestern provinces (88% in Khuzestan) is consistent with the variability of the Iranian climate, resulting in a complex hydrological and vegetative landscape, with both arid and semiarid areas and marshes in the southwest. Almost half of the country's renewable water resources are located in the Persian Gulf and the Gulf of Oman basin, which only covers one fourth of the country. On the other hand the Markazi basin, covering over half of the country, has less than one third of the total renewable water resources. There are several large rivers, but the only navigable one is the Karun, the others being too steep and irregular. The Karun River flows in the southwest of the country to the Shatt al Arab in Iraq. The few streams that empty into the Central Plateau dissipate into the saline marshes. All streams are seasonable and variable. Spring floods do enormous damage, while there is little water flow in summer when most streams disappear. Water is however stored naturally underground, finding its outlet in subterranean water canals (qanats) and in springs. It can also be tapped by wells (FAO 2008). Climatic parameters influence while indirectly affect the ecosystem and the relationships among the organisms.

2. Variability of the vectorial transmission

Warm temperature of Khuzestan province has both direct and indirect effects on the mosquitoes. Temperature affects directly : (i) the mosquitoes life cycle: development and mortality rates, since vectors are incapable of regulating their body temperature (ii) the gonotrophic cycle: reproduction rates of the mosquitoes, development of eggs within the mosquitoes; (iii) and the trophogonic cycle: incubation time and vector–host contact rate. However, Patz *et al.* suggested that higher temperatures might increase or reduce survival rate, depending on the vector, its behavior, ecology, and many other factors. Thus, the probability of transmission may or may not be increased by higher temperatures (Patz *et al.* 2000); but it has been shown that even a small increase in temperature can have a significant impact on transmission of WNV by mosquitoes. Summer temperature is an important factor for predicting WNV activity (Savage *et al.* 1999, Turell *et al.* 2001a, Paz 2006, Kilpatrick *et al.* 2008). Effect of high temperature on increasing of the mosquito abundance and vector

competency, has also been experimentally proven (Dohm *et al.* 2002). High humidity increase vector flight and host-seeking behaviors. Precipitation has two counter effects, while it is necessary for the formation of mosquito breeding habitats, in heavy conditions it can destroy mosquitoes and their breeding sites (Shaman *et al.* 2002).

Although some papers reviewed the existence of Iranian mosquito species, there is no specific information about WNV vectors. WNV has been identified in numerous mosquito species, including members of the genera *Culex*, *Aedes* and *Ochlerotatus*, but mosquitoes belonging to the *Culex* species are the main vectors for WNV (Turell *et al.* 2001b). The mosquito fauna of Iran includes 64 species and 3 subspecies classified in seven genera and 12 unverified species and the most important vector of WNV, *Culex* spp., includes 19 species. Most of Iranian mosquitoes are palaeartic, but some species are found also in Afrotropical and oriental regions (Zaim & Cranston 1986, Azari-Hamidian 2007). Zaim *et al.* conducted a study among *Culicini* (tribe of *Culex* spp.) mosquitoes collected from 147 counties in Iran and represented geographic distribution of 16 *Culex* spp. (Zaim *et al.* 1985) (Figure 29).

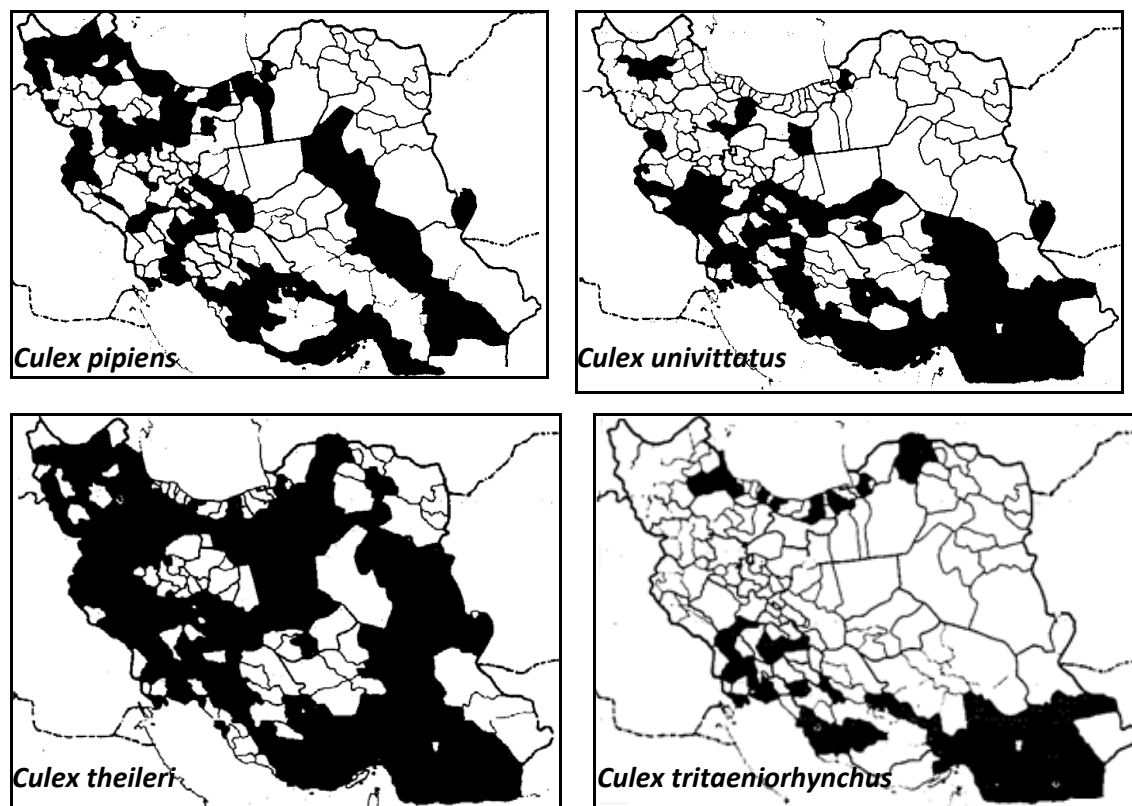


Figure 29. Spatial distribution of four species of *Culex* spp. (Zaim *et al.* 1985).

As expected, *Culex pipiens*, the main vector of WNV in US, has spread in different ecological zones of Iran (Zaim et al. 1985). *Cx. pipiens* larvae have been found in many different natural and artificial habitats including underground ones such as flooded cellars, drains, wells, septic tanks, underground train systems, abandoned mine tunnels, and coal mines (Horsfall 1955, Cranston et al. 1987, Harbach 1988, Service 1993). The wide variety of the breeding places can explain why this species is one of the most abundant and common mosquito species in Iran. Dow (1953), Lotfi (1970, 1976), and Yaghoobi-Ershadi et al. (1986) and Zaim (1987b) found this species mostly in natural habitats.

Cx. univittatus, the main vector in Egypt, Israel, and South Africa has been found in many places in Iran (Zaim et al. 1985). *Cx. tritaeniorhynchus* and *Cx. vishnui* are the main vectors for WNV based on experimental studies in India. The virus has been isolated from *Cx. vishnui* complex in Pakistan (Hubalek & Halouzka 1999a, Lvov et al. 2000, Paramasivan et al. 2003, Balenghien et al. 2008). *Cx. tritaeniorhynchus* has been found in Iran (Zaim et al. 1985). This vector is typically found in rice fields, flood waters, and marshy areas, ponds, river edges, stream edges and some other natural or artificial habitats (Harbach 1988; Lotfi 1970, 1976). Zaim (1987b) found this species mostly (76%) in man-made breeding sites especially rice fields (68.5%), more than any other *Culex species* in Iran, but Azari-Hamidian et al. (2002a) found it mainly (83.9%) in natural habitats.

If we consider Iranian WNV vector within *Culex* spp, it is speculated that other than several species may be involved in WNV circulation in south and southwestern parts of Iran, where there is a high circulation of the virus. However, vector competency studies should be conducted on various species of mosquitoes in order to determine the ability of the mosquito species to transmit WNV and detection of the virus from mosquitoes. Even detection of the virus from a mosquito is not enough to implicate the species as a confirmed competent vector of WNV because they may be unable to transmit the virus. However, population density of mosquitoes is one of the important factors to consider a species as a vector (Sardelis et al. 2001, Turell et al. 2005). Studies are needed to evaluate various mosquito species for their potential to transmit WNV in Iran.

3. Vicinity of wetlands and attractivity for birds

Although the vector is an important actor of epidemiological cycle of WNV, the role of the birds associated to wetlands, cannot be neglected. The study reveals that the higher level of seropositivity of the Kuzestan's stables is linked to a local attractivity that encourages birds to congregate around shrinking water sites, encouraging viral circulation among birds and mosquitoes, while heat accelerates viral maturation. The primary WNV cycle, which is enzootic, involves birds as hosts for the virus and ornithophilic mosquitoes (i.e., mosquitoes that feed on birds mostly) as vectors (Komar 2000). Female mosquitoes get infected when they feed on aviremic bird. The virus reproduces in the mosquito vector and, after a time interval known as the extrinsic incubation period, a new bird can be inoculated. If this bird is receptive to the virus, it becomes viremic and infectious for other vectors for a period that lasts between 1 and 7 days under laboratory conditions (Komar et al. 2003). Locally, birds can consequently be suspected to; (i) amplifying birds are birds that live in areas where ornithophilic mosquitoes are abundant and allow WNV amplification in spring and early summer; and (ii) spreading birds are birds that move from wet to surrounding dry areas in late summer and might spread the virus out.

Besides, this local impact of environment, the study reveals a regional impact associated to the neighboring of wetlands. There is a significant negative correlation between distance to the nearest wetland and seropositivity of stables. The risk of being WNV seropositive for stables increase with the decrease of distance from the nearest wetland area, developing a regional attractivity for many birds of areas in which resources, otherwise scarce in arid southeastern environments (shade, nectar, nest sites, and especially water) are abundant. In epidemic areas, WNV is thought to be introduced by migrating birds and maintained during the periods of mosquito abundance (Komar 2000, Rappole et al. 2000). As epizootics often occur in areas close to wetlands (Hubalek and Halouzka 1999, Rappole and Hubalek 2003), the virus could first be amplified in wet areas and later spread to dry surrounding areas. Besides amplification-dispersion role of domestic birds, long or short distance migratory birds have an importer role in the importation of the virus to new areas (introducing hosts). Birds can consequently be suspected to play third role in the ecology of WNV, namely introduction of viruses during migration from areas where WNV is either endemic or epidemic.

Iranian wetlands are suitable habitats for birds wintering, staging and breeding. A recent study carried out among 26 different species of water birds captured from wetlands, showed 15% seropositivity and the majority of positive birds were common coot (*Fulica atra*) (Fereidouni *et al.* 2011). They did not find any positive samples from Khuzestan province where is the most important region for the virus circulation. Moreover, they had a few samples (n=4) from this province, all of which were birds other than common coot, maybe due to lower abundance of common coot (*Fulica atra*) in this area (Figure 30).

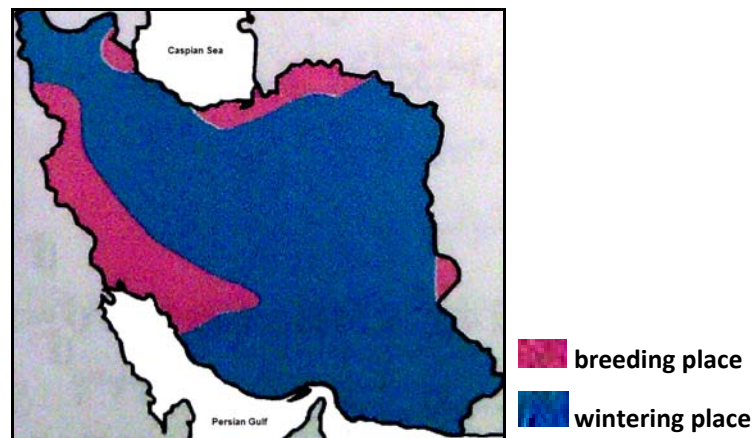


Figure 30. Geographical distribution of *Common Coot* in Iran in breeding and wintering place (Mansoori 2008).

4. Key role of the Mesopotamian marshlands

Located at the southwestern border of the elevated and mountainous part of Iran, Khuzestan comprises an extension of the Mesopotamian plain, which is the most important wetland of the Middle East (Figure 31). In the elevated and mountainous regions of the Khuzestan, moderate summer and cold winter are commonly experienced; while the plain, covered with marshes, is excessively hot and dry (as a desert) in the summer. The coastal winds from the Persian Gulf bring, in summer, high level of humidity and heat. The Mesopotamian marshlands sit in the heart of the vast floodplain wetlands created by the Tigris-Euphrates river system in the lower Mesopotamian basin. Downstream from Baghdad, approximately 520 km from the head of the Gulf, the very weak longitudinal slope of the land causes the two great rivers to meander and split into multiple branches, creating a chain of almost interconnected shallow freshwater lakes, marshes and seasonally inundated

floodplains that typically overflow and merge into larger complexes during periods of high floods. In addition, the perennial Karkheh River as well as a series of seasonal rivers descending from the Zagros mountains in Iran, drain into the eastern marshes, on the eastern bank of the Tigris River (UNEP).

The climate of the Mesopotamian floodplain is arid; mean annual rainfall ranges from 100 mm in the west to 150 mm in the east. The very hot summers, when temperatures can reach over 50°C in July and August, induce high evapotranspiration rates of three meters per annum. The extent of the marshes is highly variable, expanding and contracting with seasonal flooding and annual changes in water flows. Prior to dam construction, discharges into the Tigris and Euphrates peaked in April and May, with flow volumes lowest in August and September. This oscillation, generated by snowmelt flood pulses during the spring and gradual water recession over the summer months, plays a critical role in the dynamics of marshland ecology.

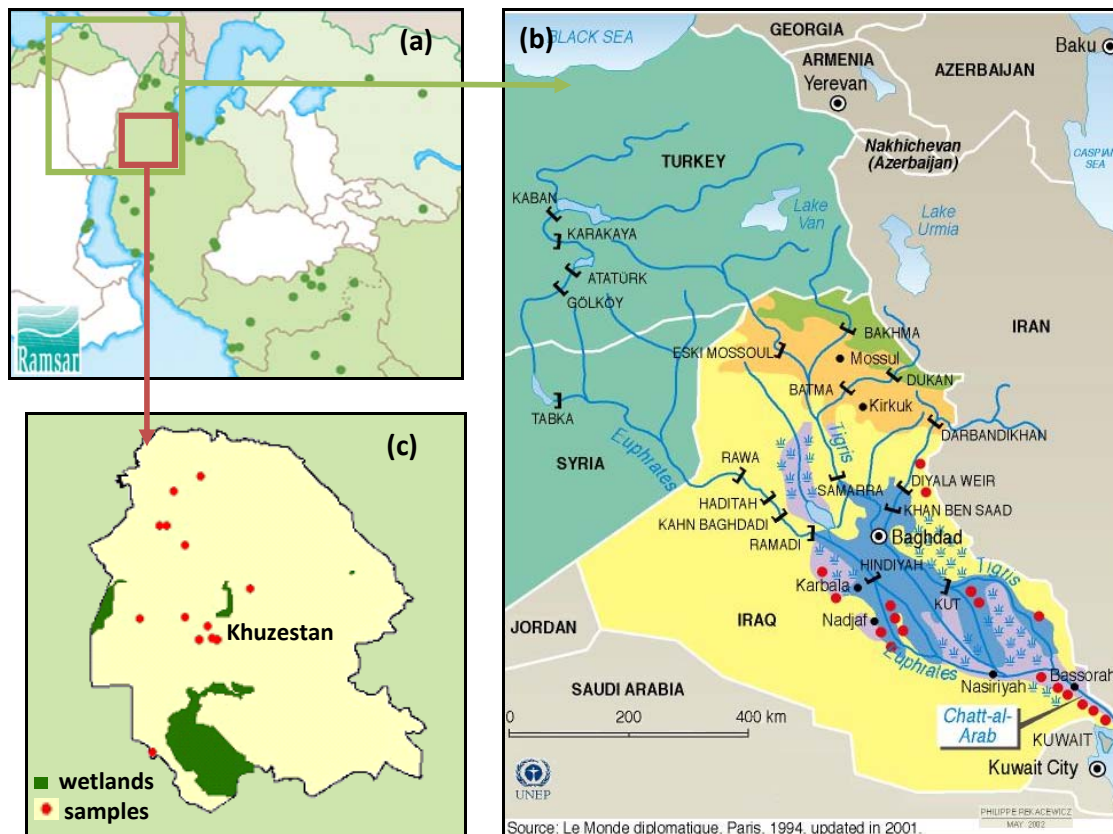


Figure 31. Geographical location of wetlands in: (a) Middle-East; (b) Mesopotamian plain; (c) Khuzestan province, Iran.

The Mesopotamian marshlands constitute the largest wetland ecosystem in the Middle East and Western Eurasia and support a rich biota. They provide a key wintering and staging area for migratory waterfowl on the Africa-Europe/ Asia flyways. Over 50 per cent of 278 species of birds recorded in the region are dependent on these wetland habitats. The marshes historically provided wintering habitat for some of the largest concentrations of waterfowl in the world, estimated at several million. When the marshlands were drained, at least 66 of these bird species were considered to be at risk, of which at least 10 were considered to be regionally or globally threatened. The marshes have also been singled out as one of the eleven non-marine wetland areas in the world with Endemic Bird Area status.

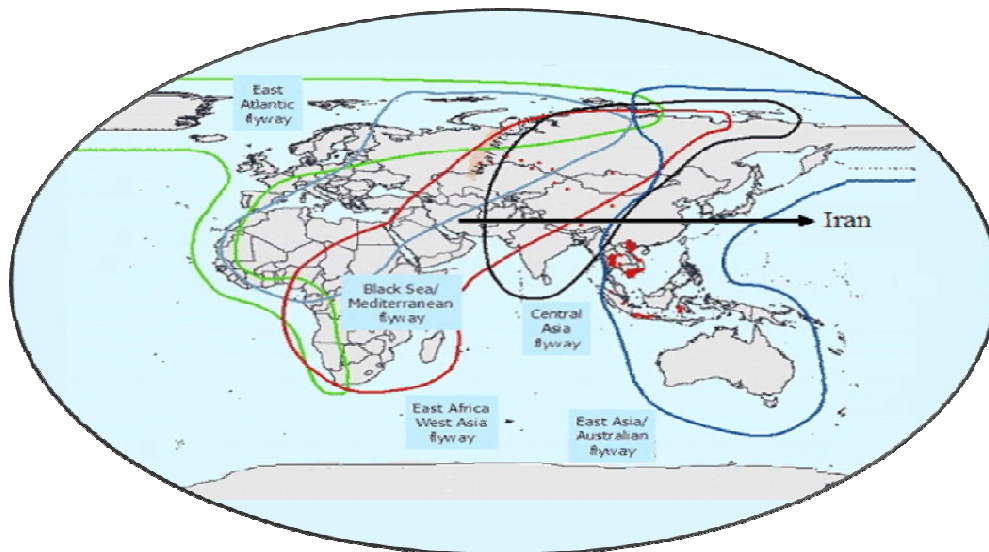


Figure 32. Main flyways of migratory bird around the world
(http://www.fao.org/ag/againfo/foto/EMPRES_Watch_global_flyways.gif).

Mesopotamian marshlands and Iranian wetlands are located in a crossroad of the flyways corridor of migrating birds to and from West Siberian-Caspian-East African and Central Siberian-Indus-South Asian flyways (Figure 32). Migratory birds also play an essential role in the introduction of WNV when they land in wetlands. Iranian wetlands serve as wintering or staging area for migratory birds coming from WNV endemic areas and using the West Siberian-Caspian-East African and Central Siberian-Indus-South Asian flyways (UNDP. 2003 July). In severe winters, a huge part of Russian wintering populations moves south into northern Iran. While in dry years, large number of birds leave the marches dried in

semi-desert areas, such as Khuzestan, central Fars and Sistan, and in winter fly to southeast or southwest (Scott 2010).

5. Preventing new emergences of WNV outbreaks

WNV, still the world's most widely distributed arbovirus, was well known for its sporadic appearances in Europe and the Middle East, with recent notable outbreaks in Romania and Russia. These localized epidemics made perfect sense in light of the migratory habits of Old World birds, which annually fly northward from such tropical havens as the West Nile District of Uganda, where the virus was first isolated in 1937. The current epizootic/epidemic of WNV in North America appears to be the result of a the introduction from Middle-East into the New York City in 1999, spreading across the USA, Canada, Mexico, Central and South America (Smithburn 1940, Dupuis *et al.* 2005, Elizondo-Quiroga *et al.* 2005, Weaver & Reisen 2010). This disease, previously unrecognized in the New World has caused 9843 cases of WN ND (962 deaths) and 13,489 cases of fever in the United States. The WNV outbreak in US resulted in an increasing number of dead birds. Therefore, the Centers for Disease Control and Prevention (CDC) guidelines considered that a system based on the active monitoring of sentinel birds, and/or the observation of the mortality rate of birds, may provide a sensitive method of detecting WNV (Figure 33) (CDC 2001).

However, in Europe and Middle East, contrasting to the United States, birds do not show usually any symptoms when infected with WNV (Guptill *et al.* 2003). Even if seroconversion rates in sentinel birds did eventually been detectable, these seroconversions were generally detected late in the transmission season, after the onset of human cases (Komar 2001). In France, where both passive and active monitoring systems for birds and mosquitoes have been implemented, neither abnormal avian mortality, nor positive pools of mosquitoes were identified (Leblond *et al.* 2007a). So, in this case, the system did not provide an alert before the syndromic or serological surveillance in horses. Furthermore, a disadvantage of such active monitoring system is that the costs may become greater, as well as harder to justify when the target disease becomes rare.

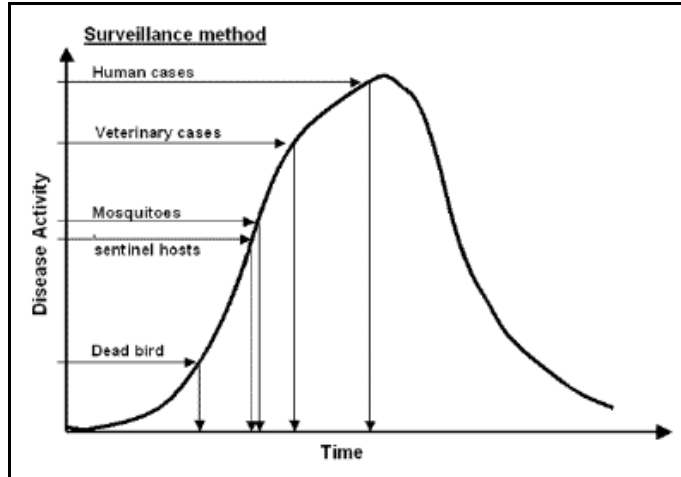


Figure 33. Sensitivity to the surveillance methods for WNV circulation in USA (CDC 2001).

Our study established that epidemiological surveillance of equine population of Khuzestan, located in the vicinity of Mesopotamian wetlands characterized by high annual temperature and affected by oasis effect, could be efficient to monitor the circulation of WNV. Horses seem to be particularly sensitive to WNV. Of horses infected with WNV, approximately 10% present neurological disorders, as compared to 1% of humans (Petersen 2001). Moreover, horses are distributed throughout the Middle East, and several neurological disorders with unknown agents have been reported in different regions (Castillo-Olivares & Wood 2004). Further evaluation of surveillance in equines should be undertaken in areas of the Middle East where extensive populations of horses are maintained and WNV is a potential threat. The implementation of such a monitoring system would allow, in case of danger, to inform both the local populations, and the populations located on the pathways of migratory birds.

Our results indicate that several routes could be pursued for future research. Efforts are needed: to isolate the virus; to identify vectors and vertebrate hosts, and to assess the impact of environment. It is speculated that along with vegetation index, vegetation types may be of importance. The study shows that a WNV infection highly occurred in Khalij-o-Omanian ecological zone, which has a different vegetation cover with specific plant richness (Figure 11). This ecoregion has the lowest plant diversity than the other ecozones and extends throughout southern parts of the country in Khuzestan, Bushehr, Hormozgan and Sistan-Baluchistan provinces, with an area of 2,130,000 ha. The dominated climate is sub-equatorial

and the main plant species of the ecozone are: *Acacia- Prosopia- Ziziphus- Avicennia- Rhizophora- Populus euphatica- Prosopis stephaniana*. The average rainfall of this region is 70-400 mm, with an altitude of 0-1000 meters (Heshmati 2007). Understanding how weather conditions and landscape features influence WNV transmission is critical for effective surveillance and control programs. Additionally, models of virus circulation could be used to predict new areas at risk.

Substantial effort has to be devoted to understanding the molecular epidemiology of WNV, in order to provide a more detailed understanding and modeling of risk assessment. The introduction of WNV into North America in 1999 provided a unique opportunity to prospectively observe the evolution of a novel agent in a naïve environment. The isolated virus in 1999 was most closely related to a strain isolated in Israel a year earlier. Strains collected in New York in 2000 were genetically homogeneous, and strains collected in Texas in 2002 were similar to strains collected previously in the United States (Kramer *et al.* 2008). These studies seemed to confirm the relative genetic stasis of arthropod-borne populations, but contrary evidence began to emerge genetically distinct WNV strains were collected in southeast coastal Texas in 2003 (Ebel *et al.* 2004). Shortly thereafter, it was recognized that a dominant genotype distinct from the introduced 1999 genotype had arisen, which over three years displaced the introduced genotype. This new genotype is now the only WNV genotype recognized in the United States. Its dominance appears to be related to increased transmission efficiency in *Culex spp.* Mosquitoes. Phylogenetic analysis demonstrated that WNV is not constrained geographically, which is harmonious with the virus's rapid geographic spread across the United States (Snapinn *et al.* 2007). Finally, it should be fruitful to consider virus spread, population dynamics, viral determinants of pathogenesis, and mosquito transmission in Southern Iran at the molecular level.

Conclusion

The wetlands of the Garden of Eden, 5,000 year-old heritage of the Babylonians and the Sumerians, attract birds from eastern Africa, southern and central Asia, Siberia, northern and eastern Europe, and Mediterranean basin. Migratory birds are important in spreading virus, as with the introduction of WNV. Birds have been proposed to be the agents of long-distance movement of WNV leading to multiple genotypes circulating concurrently, while

mosquitoes and dispersing birds may move the virus locally. After reviewing ancient accounts and modern theories, John S. Marr and Charles H. Calisher (2003) have concluded that Alexander the Great may have died of West Nile encephalitis in the ancient Mesopotamian city of Babylon, on June 10, of 323 BC after a 2-week febrile illness. The authors considered that it is possible that, in the 3rd century BC, disease caused by West Nile virus arrived in Mesopotamia for the first time in recorded history, killing indigenous birds and an occasional human and causing only incidental febrile illness in many others. Over subsequent centuries the virus may have devolved, becoming less pathogenic for indigenous birds, while retaining its potential as a dangerous human pathogen. What happened in Babylon many years ago might be similar to what has been observed in the ongoing North American epizootic and epidemic. This is speculative, but WNV serves as a model for zoonotic diseases that are emerging, re-emerging, or expanding their ranges globally. It is critical to conduct research on the underlying biological and geographic factor(s) that allows WNV from Mesopotamian wetlands, and specially Khuzestan, to adapt to new hosts and environments. A better understanding will allow improved prediction of risk and approaches to control.

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APPENDICES

APPENDIX A: PUBLISHED ARTICLES

First Article

Epidemiol. Infect. (2011), **139**, 1587–1591
doi:10.1017/S0950268811000173

Spread of West Nile virus in Iran: a cross-sectional serosurvey in equines, 2008–2009

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(Accepted 25 January 2011; first published online 14 March 2011)

SUMMARY

We report the first large-scale serosurvey for West Nile virus (WNV) conducted in the equine population in Iran. Blood samples were obtained in 2008–2009 from 1054 equines collected from 260 districts located in 27 provinces. The overall seroprevalence rate for WNV neutralizing antibodies was 23.7%. Marked geographical variations were observed as province-specific seroprevalence rates ranged from 1% to 88%, the highest values being observed in the southern and western parts of the country. The presence of IgM-positive animals ($n=9$) indicated a recent circulation of WNV in several provinces. Logistic modelling confirmed this result with a significant effect of age on seropositivity. This study revealed extensive circulation of WNV in Iran particularly in southwestern provinces where the virus probably circulates every year.

Key words: Horse, Iran, seroprevalence, West Nile virus.

INTRODUCTION

West Nile virus (WNV; family Flaviviridae, genus *Flavivirus*) is a mosquito-borne virus, belonging to the Japanese encephalitis virus (JEV) serogroup. It is amplified through transmission cycles involving birds as main reservoir hosts and mosquitoes of the genus *Culex* as the principal vectors. WNV, first isolated in 1937 in the West Nile district of Uganda, is now widely distributed throughout different continents [1–3].

On the basis of phylogenetic analysis, WNV has been classified into at least five lineages. Lineage 1 WNV is widespread and most commonly associated with human and horse neurological disease. This lineage has at least two geographically distinct clades. Clade 1a viruses are circulating between Europe, the Middle East, Africa and America via migratory birds [4, 5].

From the early 1950s to the 1990s, several outbreaks, rarely associated with clinical disease in horses and humans, occurred in the Middle East and the Mediterranean Basin. Concurrent serological studies demonstrated that WNV was endemic in these regions [6–8]. Nevertheless, since the 1990s, several outbreaks of neuroinvasive disease occurred in the

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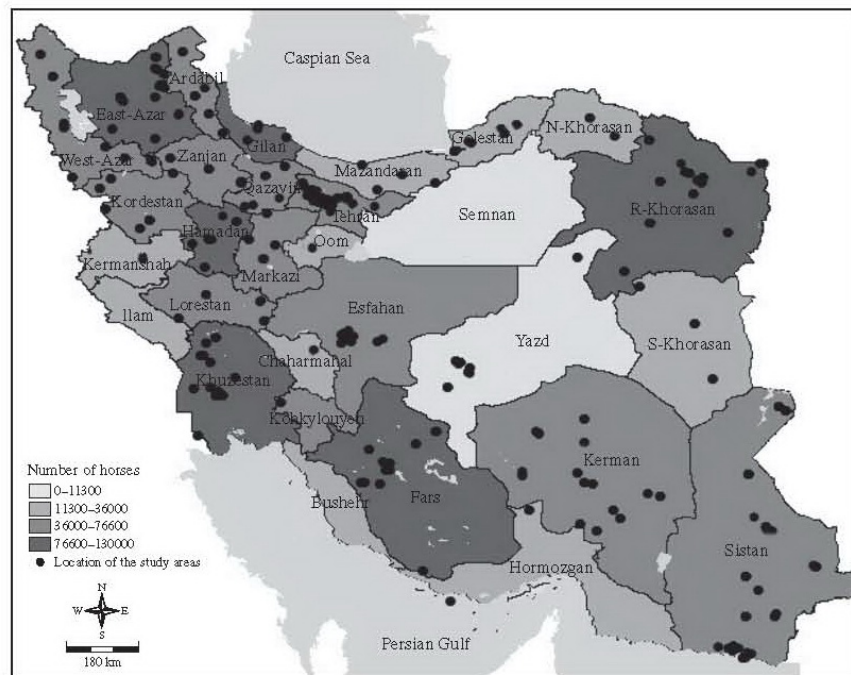


Fig. 1. Geographic distribution of equine population by province and geographic location of collected samples in 27 out of 30 provinces, Iran, 2008–2009.

Middle East and the Mediterranean Basin, extending to Russia and the USA [6, 9–11]. Clade 1a viruses were responsible for these outbreaks; one subclade, associated with considerable bird mortality, caused the recent outbreaks in Israel and the American continent. This subclade subdivision suggested that a virus imported from the Middle East may have caused the recent outbreaks in the USA [4, 12].

The first serological studies conducted before the 1990s, demonstrated the presence of WNV antibodies in the Iranian population. In a study reported in 1969, carried out in Khorasan and Khuzestan provinces, WNV antibody was detected in 28.4% of human sera (103/768) [13]. A study conducted in 2010 demonstrated the presence of WNV IgG in 5% of blood donors in Tehran [14]. While these studies clearly demonstrate human exposure to WNV in Iran, similar findings have not been reported in equines.

The study objective was to assess the level of WNV circulation in the equine population in the Islamic Republic of Iran. A large-scale serosurvey was implemented to evaluate the overall seroprevalence

rate in horses, as well as any geographic variations in provinces.

METHOD

Target and study population

There are around 1 547 100 horses distributed throughout Iran that are involved in sport, farming, educational purposes and local transportation. The overall animal density is about 1 animal/km² and the geographic distribution of the animals is heterogeneous (Fig. 1).

A total of 1054 healthy equines (844 horses, 9 mules, 201 donkeys) were randomly selected from 260 communities located within 27 of 30 provinces in the country (Fig. 1). The survey was performed between September 2008 and February 2009. A form was completed by a veterinarian for each animal. Along with other data (date of sampling, name of the veterinarian and of the animal's keeper), this form recorded the breed of the animal, its age and sex, the

location (easting, northing and elevation), the type of stable (farm or equestrian club) and the group size, i.e. the number of equines living at the same place.

Serological analyses

Enzyme-linked immunosorbent assay (ELISA)

All equine sera were screened for WNV antibodies with a commercial competitive ELISA test (ID Screen West Nile Competition; ID VET, France) according to the manufacturer's instructions.

Reactive serum samples were further tested by IgM-capture ELISA and plaque reduction neutralization test (PRNT). The analysis method for IgM detection was derived from the IgM-capture ELISA method described previously [15] and was performed as described by Murgue *et al.* [16]. Plates were read on an ELISA plate reader at the 450 nm wavelength. The following ratios were calculated for each sample:

$$R1 = \frac{OD \text{ sample}/Ag(+)}{OD \text{ sample}/Ag(-)}, \quad R2 = \frac{OD \text{ sample}/Ag(+)}{OD C(-)/Ag(+)}$$

R1 is the ratio between the absorbance of the test sample in wells containing virus antigen [Ag(+)] and the absorbance of the sample in wells containing control antigen [Ag(-)]. R2 is the ratio between the absorbance of the sample in wells containing virus antigen and the absorbance of negative control serum [C(-)] in wells containing virus antigen. If $R1 \geq 2$ and $R2 \geq 2$, the sample was considered IgM positive, if $R1 < 2$ and $R2 < 2$, the sample was considered IgM negative and if $R1 \geq 2$ and $R2 < 2$, the sample was considered doubtful.

PRNT

PRNT₉₀ was performed to confirm the presence of WNV-specific antibodies and was derived from the OIE PRNT₉₀ method [15], with threefold serially diluted sera and WNV lineage 1 IS98-ST1 strain [17]. Titres were expressed as the reciprocal of the highest serum dilution yielding >90% reduction in the number of viral plaques (PRNT₉₀).

Statistical analyses

Seropositivity risk factor analysis

All the statistical analyses were conducted using R 2.10.1 [18], and maps were produced using a

geographic information system (ESRI, ArcGISTM 9.0). Some values were missing in questionnaires for sex, breed, age and group size. After verifying that the pattern of missing data was compatible with random missing [19], missing data were imputed using the covariates as predictors (northing, easting, elevation, sex, breed, age, group size, type of stable). Twenty datasets were thus generated using the 'aregImpute' function of the Hmisc package [20]. All the statistical analyses described hereafter were separately conducted for each of these 20 datasets, the results being combined as described previously [19].

A logistic model was performed to analyse the relationship between seropositivity and the covariates. Northing, easting, the corresponding quadratic terms and the northing easting interaction were included in the model as a proxy for horses' location. Other covariates were grouped by gender (male or female), breed (Arab, Crossbreed, Thoroughbred, others), age ($\Delta = 5$ years), group size ($\Delta = 30$ animals), elevation ($\Delta = 1000$ m), and the type of stable (farm or club). The performance of the model as a classifier was assessed using receiver-operating characteristic (ROC) analysis and computing the area under the ROC curve (AUC). A cross-validation [21] was conducted: half of the tested horses were randomly chosen for fitting the model, which was then used to predict the serological status of the remaining animals.

The prediction error was evaluated as the usual proportion of incorrect predictions.

RESULTS

Out of 1054 sampled animals 249 [23.6%, 95% confidence interval (CI) 21.1–26.3] were positive by PRNT for WNV infection. Three sera could not be tested by PRNT owing to insufficient sample volume. Seropositive equines were observed in all of the provinces except one, where only two animals had been tested. Seroprevalence appeared to be highly heterogeneous and ranged from 1% to 88%. The most affected provinces were located in the western and southern parts of the country, with seroprevalence rates >30% (Fig. 2). Most of the animals possessing WNV neutralizing antibodies had low PRNT titres as only 73 had a titre >90, corresponding to 29.3% of tested animals (95% CI 23.6–35). The majority of these animals were located in provinces with seroprevalence rates >30%. Two sera were not tested by IgM ELISA because there was insufficient sample volume and one serum was removed because it gave

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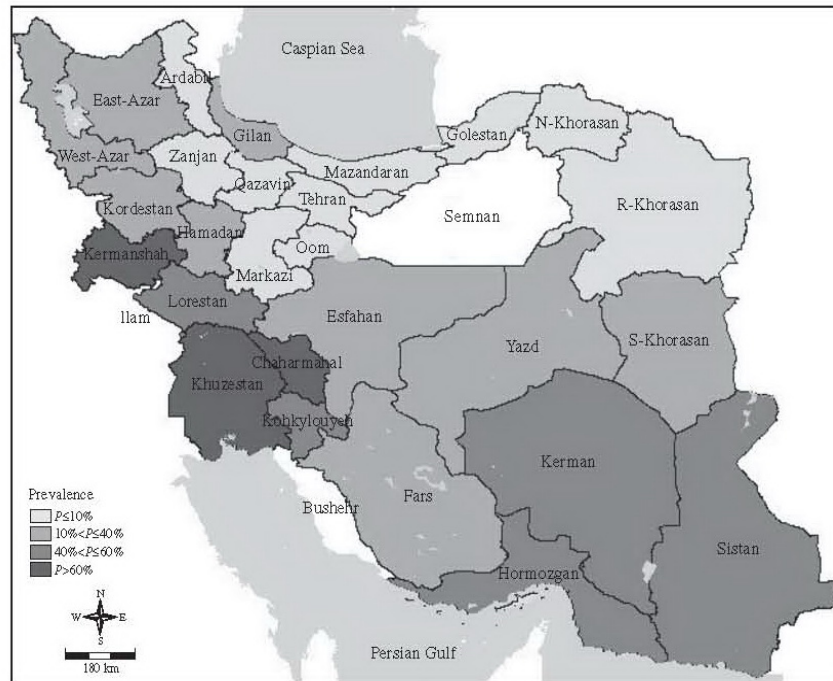


Fig. 2. Anti-WNV seroprevalence determined by plaque reduction neutralization test in equines in 27 out of 30 provinces, Iran, 2008–2009.

an indeterminate result. Nine sera were positive for IgM antibody. The corresponding four provinces had a PRNT seroprevalence rate $> 30\%$.

A logistic regression model showed an association between elevation and seropositivity, with a 60% decrease of seropositivity odds when elevation is increased by 1000 m (Table 1). The Arab breed was a seropositive risk factor, as was being housed in a farm (reference: club). The seropositivity risk also increased with the age of animals, a difference of 5 years corresponding to a change of seropositivity odds by a factor of 1.3.

No significant association was observed between sex and seropositivity, or between group size and seropositivity (Table 1). The ROC analysis resulted in an AUC of 0.887, indicating the model had relatively good discrimination ability. The AUC ranged from 0.884 to 0.889 according to the imputed dataset ($n=20$). Cross-validation showed an individual prediction error of 13.5%: for 86.5% of animals, the predicted PRNT status was thus correct. This prediction error ranged between 12.2% and 14.5% according to the imputed dataset ($n=20$).

DISCUSSION

The results of this study, the first large-scale WNV serosurvey in equines conducted in Iran, show that antibody response to WNV is widespread in this country as seropositive animals were found in 26 out of 27 provinces. The overall serological prevalence rate of WNV antibodies is high (24%) but varies widely according to the geographic location, with southern and western parts of the country being the most affected areas. Nine provinces had a prevalence $\geq 30\%$, five provinces a prevalence $\geq 50\%$, and two provinces a prevalence $\geq 80\%$. We could analyse a double gradient of prevalence between north and south, and between east and west. The greater part of the provinces with high prevalence is located in a dry area (south) rather than a rainy area (north).

Logistic modelling confirmed that geographic location was the main seropositivity risk factor. Furthermore, seropositivity risk decreased with increasing elevation: this association can be explained by variations in the abundance of the vectors. Seroprevalence also varied with the equines' individual

Table 1. Logistic model of anti-WNV seropositivity determined by plaque reduction neutralization test in equines, Iran, 2008–2009

Variable	Value	OR (95% CI)	P value
Northing	$\Delta = 0.5$ degree	0.37 (0.17–0.80)	0.005*, 0.02†
Easting	$\Delta = 0.5$ degree	0.54 (0.32–0.90)	0.008*, 0.001†
Northing–Easting	$\Delta = 0.5$ degree	1.22 (1.06–1.39)	0.02
Elevation	$\Delta = 1000$ m	0.60 (0.43–0.84)	0.04
Sex	Female	Reference	
	Male	0.90 (0.61–1.36)	0.32
Breed	Crossbreed	Reference	
	Arab	3.22 (1.50–6.89)	0.001
	Thoroughbred	1.24 (0.37–4.12)	0.36
	Other	1.50 (0.66–3.38)	0.16
Type of stable	Club	Reference	
	Farm	2.18 (1.28–3.73)	0.002
Age	$\Delta = 5$ years	1.34 (1.04–1.71)	0.01
Group size	$\Delta = 30$ animals	0.80 (0.60–1.06)	0.06

OR, Odds ratio; CI, confidence interval.

* Absolute value.

† Quadratic term.

characteristics. Horses had a higher risk for WNV seropositivity when they were Arab breeds. This may be linked to the geographic location of these horses, found mainly in the southwestern part of the country. The increase in seroprevalence rate with equine age suggests recurrent circulation of WNV, as described in some serosurveys performed in endemic areas [22, 23]. Considering the fact that seropositive animals were more than 1 year old, seropositivity may not be related to maternal antibodies.

Besides individual characteristics, seroprevalence varied according to the conditions in which animals were managed. For example, horses housed in farms appeared to have been more exposed to WNV than those in clubs.

The number of IgM-positive sera observed in the present study ($n=9$) could have been underestimated as only sera positive by competitive ELISA were screened for IgM. The corresponding bias was probably minor as IgG and IgM appear roughly simultaneously after infection [24, 25]. Despite the small number, the positive IgM results revealed a pattern of viral circulation. The corresponding animals were sampled between September 2008 and January 2009 and could thus have been infected from late summer to autumn 2008. Similar observations were made in Europe and the Mediterranean area, with recurring outbreaks in the same seasons that the mosquito population density was highest [3, 16, 26]. The latest IgM-positive serum was obtained at the end of

January 2009, suggesting that the animal was infected during the last days of autumn or during winter. This animal was located in a desert area, Sistan-Baluchestan province. Considering the mild winter in this province, it would not be surprising to detect active, infected mosquitoes at that time of year.

A few sera positive by ELISA were negative for neutralizing antibodies against WNV. The lower analytical sensitivity of the PRNT test can account for this finding. This result was unlikely to be due to assay cross-reactivity as no other related flaviviruses have been reported in Iran.

Circulation and persistence of viruses in the ecosystem depends on environmental, as well as biological, factors. WNV is maintained in nature through a transmission cycle between vector mosquitoes and reservoir birds [27–29]. It appears that Khuzestan province has more favourable conditions for propagation and circulation of the virus than the other provinces. Khuzestan has the most important wetlands which host unique flora and fauna. The importance of migratory birds as virus carriers and transfer hosts between different areas has been previously reported [30, 31]. Iran is located at the crossroads of bird migratory routes, with birds coming from WNV-endemic regions. Although pathways of migratory birds in Iran are not well documented, Iran supports nearly two-fifths of the Middle East's important wetlands. The Iranian wetlands represent wintering areas for millions of migratory waterfowl

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coming from Siberia, Africa, India and South Asia which may introduce different strains of WNV encountered elsewhere.

Since the first reported serological evidence of WNV circulation in the 1950s, the Middle East has been of interest for renewed studies concerning WNV circulation, especially when it was shown that there was a genetic similarity between the New York strain isolated in 1999 and a strain isolated in the Middle East [4]. Co-circulation of different lineages has been previously reported in the Middle East [32]. However, substantiating the circulation of different lineages in Iran needs further study. Efforts are needed to isolate viruses, to identify vectors and vertebrate hosts, to assess the risk of WNV transmission by migratory birds to free ecosystems and also to determine the environmental indicators involved in WNV transmission in Iran.

ACKNOWLEDGEMENTS

We thank all the veterinarians who were involved in field support for this study. We are grateful to J. Maingault, M. Kamyabi, S. Rahimizadeh, S. Jodairy-Eslami, N. Miandehi, A. Moradi-Joshaghan, H. Amiri, H. Najafi, C. Bahuon, M. Chaumien and E. Mazaheri for help with laboratory analyses and their technical assistance. We thank Philippe Pourquier (ID Vet Company) for kindly providing the ID SCREEN[®] competitive ELISA kits. We also thank Francis Geiger and Marc Grandadam for their help in the preliminary study and Mark Zuckerman for editing and improving the manuscript. The first author also thanks Fereshteh Firouzi for the management of the visiting periods in France. This work was supported in part by a grant from the Service de Coopération et d'Action Culturelle (SCAC) of the French Embassy in Tehran, Iran and the Center for International Scientific Studies and Collaboration (CISSC) Ministry of Science, Research and Technology of Iran.

DECLARATION OF INTEREST

None.

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Second Article

Paper to be published

Environmental Risk factors of West Nile Virus Circulation in Population of Equines from Iran

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Abstract

Geographic distribution of West Nile virus (WNV) is heterogeneous in Iran by a high circulation in the southern-western areas. The objective of our study was to determine environmental and climatic factors associated with the risk of WNV equine seropositivity in Iran. Data were obtained from a serosurvey conducted in equine population in 260 districts in the country. The risk factors that were found to be important included annual mean temperature, distance to wetlands, local Normalized Difference Vegetation Index (NDVI) differences and seasonal NDVI differences. The effect of local NDVI differences in spring was particularly notable. This was a normalized differences of average NDVI between two areas: a 5 km radius area centred on the stable and the 5-10 km surrounding area. The model indicated that local NDVI's contrast during spring is a major risk factor of the transmission of West-Nile virus in Iran. This so-called oasis effect consistent with the seasonal production of vegetation in spring, and is associated to the attractiveness of the local NDVI environment for WNV's vectors (mosquitoes and birds).

APPENDIX B: QUESTIONNAIRE FORM

Sample no.	Sampling date
Province	City
Club name	Group size
Geographical coordinates:	
Latitude (Y)	
Longitude (X)	
Elevation (Z)	
Animal name	Owner name
Kind of animal	Age of animal
Sex of animal	Breed of animal
Name of Veterinarian	
Contact address and phone No.	

APPENDIX C: COMPETITION ELISA

- **REAGENTS AND MATERIAL**

- Microplates coated with purified antigen
- Concentrated conjugate 10X
- Positive control
- Negative control
- Dilution buffer
- Concentrated Wash Solution 20X
- Substrate Solution
- Stop Solution (H₂SO₄, 0.5 M)

- **PROTOCOL**

(1) The samples, positive control (PC) and negative control (NC) diluted with Dilution Buffer (1:2) were added to the microwells coated with purified antigen;

(2) After incubating the microplates at 21°C (±5°C), they were washed 3 times with Wash Solution;

(3) Antibody conjugate diluted with Dilution Buffer (1:10) was added to each well;

(4) After incubating the microplates at 21°C (±5°C), they were washed 3 times with Wash Solution;

(5) Substrate Solution was added to each well and let the microplates hold in a dark place at 21°C (±5°C);

(6) After adding Stop Solution (H₂SO₄, 0.5 M) to each well in order to stop the reaction, the plates were read at a wavelength of 450 nm.

APPENDIX D: IGM-CAPTURE ELISA (REAGENTS AND PLATE MAP)

- ***PBS (phosphate-buffered saline)***
 - A PBS pellet (Gibco, ref. 18912014)
 - 500 ml of pure or demineralized water,
 - pH 7. 2-7.6

- ***PBST (PBS-Tween)***
 - 50 µ l Tween 20 (Sigma ref P1379)
 - 100ml PBS

- ***Blocking buffer***
 - 3% (weight / vol) skimmed milk powder (Biorad, ref. 170-6404) to PBS-Tween

- ***Chromogenic substrate***
 - Mix of equal volumes of "TMB peroxidase substrate" and "Peroxidase solution B" (KPL, ref 50-76-00)

- ***Map of plate***

	1	2	3	4	5	6	7	8	9	10	11	12
A	C(+) ₁	C(+) ₁	S ₅	S ₅	S ₁₃	S ₁₃	S ₂₁	S ₂₁	S ₂₉	S ₂₉	S ₃₇	S ₃₇
B	C(+) ₂	C(+) ₂	S ₆	S ₆	S ₁₄	S ₁₄	S ₂₂	S ₂₂	S ₃₀	S ₃₀	S ₃₈	S ₃₈
C	C(-) ₁	C(-) ₁	S ₇	S ₇	S ₁₅	S ₁₅	S ₂₃	S ₂₃	S ₃₁	S ₃₁	S ₃₉	S ₃₉
D	C(-) ₂	C(-) ₂	S ₈	S ₈	S ₁₆	S ₁₆	S ₂₄	S ₂₄	S ₃₂	S ₃₂	S ₄₀	S ₄₀
E	S ₁	S ₁	S ₉	S ₉	S ₁₇	S ₁₇	S ₂₅	S ₂₅	S ₃₃	S ₃₃	S ₄₁	S ₄₁
F	S ₂	S ₂	S ₁₀	S ₁₀	S ₁₈	S ₁₈	S ₂₆	S ₂₆	S ₃₄	S ₃₄	S ₄₂	S ₄₂
G	S ₃	S ₃	S ₁₁	S ₁₁	S ₁₉	S ₁₉	S ₂₇	S ₂₇	S ₃₅	S ₃₅	S ₄₃	S ₄₃
H	S ₄	S ₄	S ₁₂	S ₁₂	S ₂₀	S ₂₀	S ₂₈	S ₂₈	S ₃₆	S ₃₆	S ₄₄	S ₄₄
	Ag(+)	Ag(-)	Ag(+)	Ag(-)	Ag(+)	Ag(-)	Ag(+)	Ag(-)	Ag(+)	Ag(-)	Ag(+)	Ag(-)

S= Sample

APPENDIX E: PLAQUE REDUCTION NEUTRALIZATION TEST
(PRNT) - MEDIA, REAGENTS AND PROTOCOL

- ***Minimum Essential Medium (MEM 2X)***

- Fetal calf serum (10%)
- Sodium Pyruvate (2%)
- Penicillin/streptomycin (2%)

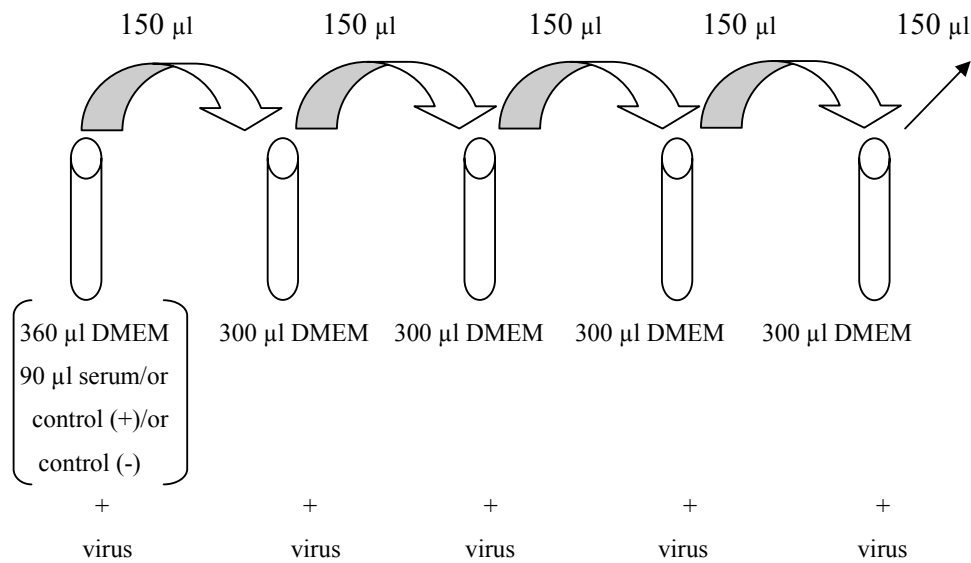
- ***Agarose medium***

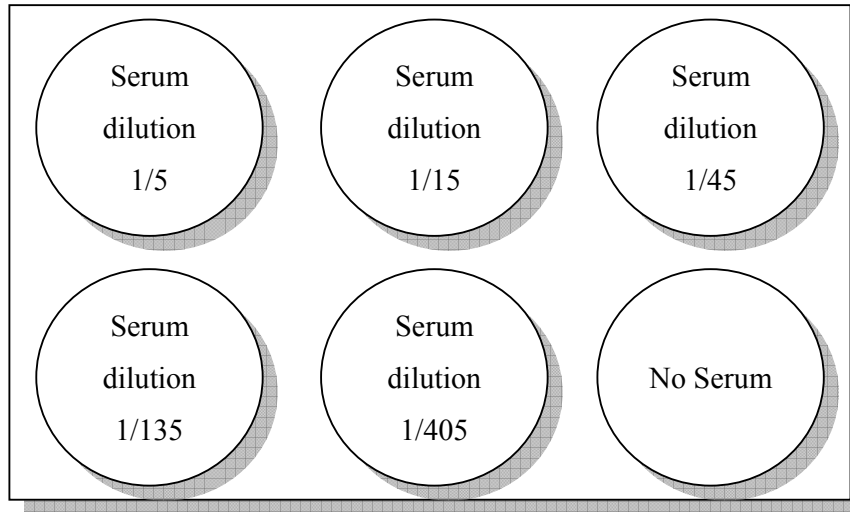
- Seaplate Agarose (2%)
- MEM 2X (InVitrogen)

- ***Cristal violet working solution***

- Purple crystal (0.4%)
- Paraformaldehyde (3.8%)
- Distilled water

- ***Preparing of dilutions***





- **PRNT Protocol**

Briefly, the steps are as follows:

- (1) 7.5×10^5 cells of VeroNK cells distributed in each well of a 6-well plate and incubated for 24h at 37°C in a CO_2 incubator (5% CO_2);
- (2) Heat-inactivated sera were threefold serially diluted in $300\mu\text{L}$ of DMEM, with a starting dilution of 1:5 to 1:405;
- (3) The diluted sera were mixed with an equal volume of a viral suspension (IS-98-ST1 strain, belonging to lineage 1 and amplified in Vero cells) at 4×10^2 pfu/mL;
- (4) The obtained mixtures were incubated for 1h30 at 37°C ;
- (5) Then mixtures were added onto fresh VeroNK cells in 6-well plates and incubated for 1h at 37°C ;
- (6) After a 1h infection, culture medium was aspirated, and replaced with an overlay of 3 ml per well of SeaPlaque Agarose;
- (7) Once the agarose had solidified, the plates were incubated 72h at 37°C in a CO_2 incubator (5% CO_2);
- (8) In order to stain viable cells, 1 ml of crystal violet working solution (see Appendix C) was added into each well and plates were incubated 24h at 37°C in humid chambers;
- (9) After 24 hours, the plate was removed from the incubator, and the agarose layer was discarded and each well was washed 3 times with PBS and viral plaques were counted;
- (10) Titers were expressed as the reciprocal of the highest serum dilution yielding >90% reduction in the number of viral plaques (PRNT₉₀).

Abstract

**West Nile Virus Circulation in Equine Populations from Iran:
Epidemiological: Impact of Environment and Climate**

The outbreak of West Nile in North America in 2002, which affected more than forty states in the United States, has led Public Health Agencies to address the risk of emergence of vectorial zoonosis, outside of the area tropical. This epidemic events associated with climate change, has highlighted the central role of migratory birds in spreading the virus. The biology of birds, especially the migratory phenomenon, ensures a transport of viruses over long distances and across very diverse species.

The Middle East, located at the crossroad between different continents, is extremely prone to the spread of zoonotic diseases, like West-Nile, in the Northern countries. The circulation of WN virus has reported from different countries in the region; such as Egypt, Israel, Lebanon, Iraq, United Arab Emirates and Iran. Saidi et al. (1970) have established the presence of antibodies to West Nile among the population of the Caspian coast (northern Iran), the provinces of Khorasan (Northeast) and Khuzestan (Southwest).

Our study, in the framework of a collaborative programme associating TIMC-IMAG UMR CNRS UJF VetAgroSup, International Network of Institut Pasteur and Centre National d'Etudes Spatiales, aim to: (i) characterizing the circulation of the virus from West Nile in the equine population of Iran, and (ii) and modelling the health impact of the environment and climate on the transmission. The results obtained allow us to assess the risk associated with spatial and temporal spread of the virus by migratory birds. Particular attention is given to the study of environmental and climatic determinants that may increase the potential for transmission.

Mots-clés : Spatial incidence, emergence, environnement, climate, West-Nile.

Résumé

West Nile Virus Circulation in Equine Population from Iran: Epidemiological Impact of Environment and Climate

L'épidémie de West-Nile en Amérique du Nord en 2002, qui a touché plus de quarante états aux Etats-Unis, a conduit les Agences de santé à s'interroger sur le risque d'émergence, à l'extérieur de la zone intertropicale, de zoonoses vectorielles. Cette épidémie associée au changement climatique, a bien mis en évidence le rôle central de l'avifaune migratrice dans la diffusion du virus. La biologie des oiseaux, tout particulièrement le phénomène migratoire, permet un transport des virus sur de longues distances et entre espèces très diversifiées.

Le Moyen-Orient, qui est situé au carrefour de différents continents, est extrêmement propice à la propagation des virus émergents dans les pays du Nord. La circulation du virus West Nile a été rapportée dans différents pays de la région, tels que l'Egypte, Israël, Liban, Irak, Emirats Arabes Unis et Iran. Saidi et al. (1970) ont montré la présence d'anticorps anti-virus du Nil occidental au sein de la population de la côte caspienne (Nord de l'Iran), des provinces du Khorassan (Nord-Est) et du Khuzestan (Sud-Ouest).

Notre étude, conduite dans le cadre d'un programme associant TIMC-IMAG UMR 5525 UJF CNRS VetAgroSup, le Réseau International des Instituts Pasteurs et le Centre National d'Etudes Spatiales, vise: (i) à caractériser la circulation du virus de West-Nile au sein des populations équines d'Iran ; et (ii) et à modéliser l'impact sanitaire de l'environnement et du climat sur la transmission. Les résultats acquis permettent d'apprécier le risque associé à la dissémination spatio-temporelle du virus par les oiseaux migrateurs. Une attention toute particulière est portée à l'étude des déterminants environnementaux et climatiques susceptibles d'accroître le potentiel de transmission du virus.

Mots-clés : Incidence spatiale, émergence, environnement, climat, West-Nile.

